

The Rat Mast Cell Degranulation Assay as an *In Vitro* Allergy Test

31863

J. D. Wetherall*, Roslyn Hackshaw† and K. J. Turner‡

Clinical Immunology Unit, Princess Margaret Hospital for Children, Perth, W.A.

Summary: Rat mast cell degranulation has been utilised to detect human reagins to the drugs penicillin, insulin, rifampicin, cycloserine and lignacaine and to rheo-macroderm. The specificity of the method for human IgE was established by the induction of degranulation with anti-IgE. The technique appears to have limitations as a potential tool for routine clinical diagnosis.

Type I hypersensitivity reactions are mediated predominantly by antibodies of the immunoglobulin E class¹. They may be detected *in vivo* by direct skin tests in the allergic patient or by indirect skin testing (P-K reactions) in normal human subjects or in primates. Since skin testing, while effective, is difficult to quantitate, attempts have been made to develop satisfactory *in vitro* assays for the quantitation of IgE antibodies.

In vitro allergic histamine release from sensitised basophils² has been used to quantitatively detect Type I hypersensitivity towards a wide range of allergens. The method suffers from the disadvantage that it requires fresh leukocytes from the allergic subject since attempts to use passively sensitised leukocytes from normal subjects have met with limited success³. Cell suspensions or slices of monkey lung tissue may, however, be passively sensitised with serum from allergic subjects and induced to quantitatively release histamine upon subse-

quent exposure to specific allergens⁴. Apparently individual variations of donor serum or recipient cells do not affect this assay since there is no evidence to suggest that a given suspension of monkey lung mast cells cannot be adequately sensitised by human serum from any donor. Rabbit basophils⁵ and rat peritoneal mast cells^{6, 7, 8} have also been passively sensitised with human serum and induced to degranulate upon subsequent exposure to pollen, drug and food allergens. Gillman and Haddad⁹ have recently developed the rat mast cell degranulation technique to allow for quantitation of the amount of histamine released during the reaction. The explanation for the success reported in the use of heterologous cell systems, monkey lung tissue, rabbit basophils and rat mast cells, as recipients for sensitisation by human reagins lies in the fact that such cells share common receptor sites with human mast cells for human IgE. It follows therefore that rat, monkey and rabbit reagins should serologically cross-react with human IgE, a deduction which has since been confirmed by experimentation^{10, 11, 12}.

This paper reports experiments designed to test the feasibility of using rat mast cell degranulation as a routine diagnostic procedure for the confirmation *in vitro* of clinically suspected Type I hypersensitivities in humans.

Materials and Method

Human serum

Serum obtained from clotted blood taken by venepuncture was filtered through a millipore filter (HAWP type, 0.45) and stored at -20°C until ready for use. Blood was collected from patients who presented at one of the three teaching hospitals in Perth and who subsequently developed serious clinical symptoms consistent with immediate hypersensitivity reactions.

*Research Fellow, Princess Margaret Children's Medical Research Foundation (C.M.R.F.)

†Graduate Research Assistant (C.M.R.F.)

‡Senior Research Fellow, N.H. & M.R.C.

Correspondence: Dr. K. J. Turner

Accepted for publication: 8 November, 1973

TABLE 1

Effect of specific antisera to human immunoglobulins on rat mast cell degranulation

Serum	Maximum Specific Degranulation %
Anti γ^*	≤ 5
Anti μ^*	
Anti α^*	
Anti $\epsilon^{*†}$	42

*Behringwerke sera

†W.H.O. sheep anti-IgE

Rat mast cells sensitised with human allergic sera.

Rat mast cell degranulation technique

The method used was adapted from that described by Perelmutter and Millard¹³. Mast cells from the peritoneal cavity of Wistar rats were harvested in 10 ml of sterile collecting medium containing disodium ethylenediamine tetracarboxylic acid (Na₂EDTA)—(Sodium chloride 0.14M, potassium chloride 0.005M, glucose 0.007M, Na₂EDTA 0.5 mg/ml, BSA 10 mg/ml buffered to pH 6.80 with HEPES* and bicarbonate). These cells were centrifuged at 750 g and resuspended in 1 ml sterile suspending medium containing foetal calf serum (FCS)—(Sodium chloride 0.14M, potassium chloride 0.005M, glucose 0.007M, BSA 10 mg/ml, FCS 40% buffered to pH 6.85 with HEPES and bicarbonate). The rat mast cells (RMC) were stored at 4°C until used and were not kept for more than 1 hour.

Equal volumes of 0.05 ml of allergic serum, RMC suspension and allergen solution at various concentrations were mixed and incubated at 37°C for three minutes. An aliquot of the reaction mixture was then removed and examined immediately by placing it on a siliconised slide and covering it with a cover slip coated with 0.18% neutral red stain in ethanol. The percentage of RMC's undergoing degranulation was estimated microscopically. Meanwhile, the remainder of the reaction mixture was fixed for 1 hour at 4°C with a 2% solution of glutaraldehyde in 0.2M sodium phosphate buffer pH 7.3. The fixed cells were then concentrated by centrifugation, smeared on a slide, and stained with Wright's stain and mounted. These slides could then be examined at leisure and provided a check for the neutral red method. Control cell suspensions which contained either allergic serum or allergen solution were done with every test. The percentage of degranulating RMC's in both preparations was estimated on a count of not less than 200 RMC's per slide. Degranulating mast cells manifested at least one of the following morphological alterations: enlargement in size, expulsion of granules, or movement of granules to the periphery of the cell resulting in a "mulberry" appearance.

*HEPES: N-2 hydroxyethylpiperazine-N-2-ethanesulphonic acid.

Results

The specificity of the technique for detecting IgE class antibodies was confirmed by challenging RMC's, sensitised with human allergic serum, with rabbit antisera specific for human γ , μ , α and ϵ immunoglobulin polypeptide chains. Only anti- ϵ chain sera induced degranulation, hence the technique specifically detects IgE antibodies. These results have been summarised in Table 1.

Positive reactions were obtained with sera from patients with allergic symptoms to a variety of substances. These are listed in Table 2. RMC degranulation was not demonstrable with serum from patients clinically hypersensitive to bacteria. Moreover, in our hands only approximately 15% of patients with suspected hypersensitivity to penicillin were positive when assessed by RMC degranulation. It is not clear whether this reflects inadequacy of either the clinical diagnosis or the assay system. Compound 48/80, a non-specific irritant of mast cells, elicited, at a level of 10 μ g/ml, approximately 95% degranulation of normal mast cells indicating that the assay system was sufficiently reproducible under ideal circumstances to detect degranulation.

From Table 2 it can be seen that the levels of non-specific degranulation resulting from the allergic serum and allergen controls were often high and that it is essential therefore that these be done with each test carried out. It was found that some human sera contain a toxic substance which causes high levels of non-specific RMC degranulation. This substance can be removed by adsorption with a HAWP type millipore filter (0.45 μ). Human sera therefore were routinely filtered through a double thickness millipore filter before use. The extent of specific degranulation also depends on the concentration of the allergen used to challenge the sensitised mast cells. A range of allergen concentrations must be tried with every test attempted. It is interesting to note that of the two patients tested for insulin hypersensitivity only patient RD was positive by direct skin testing for immediate hypersensitivity. The other patient, RM, was negative by skin testing for immediate hypersensitivity and gave a negative mast cell degranulation result also. Patient RM, however,

TABLE 2
Rat mast cell degranulation assays*†

Patient	Allergen & Concentration	Percentage Degranulation			Percentage Specific Degranulation
		Serum Control	Allergen Control	Test	
R.B.	Rheomacrodex 2% soln.	3	4	15	8
	1 "	3	3	24	18
	0.2 "	3	2	35	30
	0.1 "	3	2	24	20
R.D.*	Insulin 2 units/ml	6	33	73	34
	1	6	25	79	48
	0.5	6	15	57	26
	0.1	6	14	45	25
R.M.*	Insulin 2 units/ml	5	25	31	1
	1	5	39	42	2
N.T.	Rifampicin 2.8 mg/ml	11	5	40	24
	0.3	11	3	19	5
D.T.	Cycloserine 5 mg/ml	15	3	32	14
	1	15	3	32	14
	0.1	15	2	23	6
L.J.*	Lignacaine 2% soln.	5	10	50	35
	.2 "	5	6	5	16
	.02 "	5	4	9	0
D.T.	Penicillin G 1000 μ g/ml	14	5	31	12
	100	14	4	24	6
	10	14	2	17	1
—	Compound 48/80 10 μ g/ml	—	—	92	92

*Hypersensitivity confirmed by direct skin testing. †Mean of duplicates.

showed a weak positive delayed hypersensitivity skin reaction when assessed at 48 hours.

Allergic serum heated at 56°C for 10 minutes lost its capacity to specifically sensitise rat mast cells. It was found that serum which had been abused by successive freezing and thawing or by bacterial contamination also lost its capacity to sensitise rat mast cells.

Two other parameters were found to influence the RMC degranulation assay. These were the pH of the media and the age and health of the rats. The pH of the media was always checked therefore to ensure it did not vary by more than ± 0.05 pH units from the stipulated pH. Variation by more than this amount resulted in fragile mast cells which tended to spontaneously degranulate. Old rats and rats in poor health gave mast cell suspensions in which

there were very high levels of non-specific degranulation, although morphological differences between non-degranulating mast cells from old and young rats were not observed. Young rats of about 140 gm were found to be the best donors of mast cells. It was occasionally necessary to prepare several RMC suspensions before a suitable one with a low level of non-specific degranulation was obtained.

Discussion

The data presented in this report shows that human IgE antibodies to a wide variety of antigens can be detected using the RMC degranulation technique. The technique measures human IgE antibodies since, following sensitisation with human serum, rat mast cells degranulate in the presence of antiserum specific

for IgE but not for any other immunoglobulin class. Moreover, fluorescent antibody studies have shown the presence of human IgE on the surface of sensitised rat mast cells¹⁴. The assay described in this report is dependent on the subjective assessment of the morphological characteristics of degranulating and normal mast cells and is subject to several technical limitations.

Firstly, the age and health of the animals can be critical. Old rats or rats in poor condition gave mast cell suspensions with high levels (~60%) of non-specifically degranulating mast cells. Such suspensions were unsuitable, although apart from the high level of non-specific degranulation there were no obvious morphological differences between the mast cells from old and young rats. Serum subjected to multiple (3X) freezing and thawing or contaminated with bacteria, loses its ability to specifically sensitise mast cells. Non-specific degranulation of the mast cells with the serum alone (i.e. serum control) can also be a serious problem. This can usually be minimised by diluting the serum 1 + 1, by filtering through a double thickness 0.45 μ HAWP millipore filter, or by the addition of bovine serum albumin to the suspending medium. These latter two procedures have been routinely adopted in our assay methods. In a few cases the non-specific degranulation of the serum control could not be reduced to workable proportions and consequently the assay could not be carried out.

It is also important to perform the assay using a range of allergen concentrations. The data presented suggest that there is an optimal concentration at which degranulation is maximal. In this respect RMC degranulation is similar to the allergen induced histamine release from sensitised human basophils². It has been postulated that the optimal allergen concentration for histamine release from sensitised human basophils reflects a stereochemical requirement for the bridging of two adjacent IgE molecules by a multivalent allergen molecule¹⁵. A similar mechanism could be operative in the case of rat mast cell degranulation. Although a maximum of 48% specific degranulation was observed with antigen antibody induced degranulation, compound 48/80 resulted in greater than 90% de-

granulation. Similar values for antigen antibody induced degranulation have been reported by Perelmutter and Khera⁷.

A recent paper by MacLaren *et al*¹⁶ reports good correlation between rat mast cell degranulation and direct skin testing with a variety of allergens. This finding justifies the use of the technique to confirm and even establish the presence of a state of clinical hypersensitivity but the method is not sufficiently refined to monitor the severity of the hypersensitive state. This is of particular relevance in the case of drug hypersensitivity. Our lack of correlation between suspected penicillin hypersensitivity and RMC degranulation may be explained on the basis that the assay system is not sensitive enough to detect low levels of hypersensitivity. Moreover, the technique records only the presence of serum reagins which have bound to rat mast cells in sufficient amount to trigger off subsequent degranulation and gives no indication of the potential degree of sensitisation of the patient's own target cells. On this basis a negative result might indicate that the hypersensitisation is not acute but it still might be of sufficient magnitude to suggest that continued use of the drug is contraindicated. This limitation is also shared by other *in vitro* techniques currently in use to detect clinical hypersensitivity. However, we feel that, notwithstanding the positive results obtained in this paper, the difficulties encountered in obtaining reproducible results, which may in part be attributed to the limitations reported above, limit the use of the technique for a rapid *in vitro* diagnosis of clinical hypersensitivity.

Acknowledgements

This project was supported by grants from the Princess Margaret Children's Medical Research Foundation and the National Health and Medical Research Council of Australia. We wish to acknowledge the co-operation of the clinical staff of the Princess Margaret Hospital.

References

1. ISHIZAKA, K. and ISHIZAKA, T. (1970): The significance of immunoglobulin E in reaginic hypersensitivity. *Ann. Allergy*, **28**, 189.
2. LICHTENSTEIN, L. M. and OSLER, A. G. (1964): Histamine release from human leukocytes by ragweed pollen antigen. *J. exp. Med.* **120**, 507.
3. LEVY, D. A. and OSLER, A. G. (1966): Studies on the mechanisms of hypersensitivity. XIV. Passive sensitization *in vitro* of human leukocytes to ragweed pollen antigens. *J. Immunol.* **97**, 203.
4. MALLIY, A. and HARRIS, R. L., Jr. (1968): Passive sensitization of monkey lung fragments with serum of timothy sensitive patients. *J. Immunol.* **100**, 915.
5. SHELLEY, W. B. (1962): New serological test for allergy in man. *Nature (Lond)*, **195**, 1181.
6. SCHWARTZ, J., KLOPFLOCK, A., ZIKERT-DUVDEVANI, P. and HONIG, S. (1965): Detection of hypersensitivity by indirect rat mast cell degranulation. *Int. Arch. Allergy* **26**, 333.
7. PERELMUTTER, L. and KHERA, K. (1970): A study on the detection of human reagins with rat peritoneal mast cells. *Int. Arch. Allergy* **39**, 27.
8. KOROTZER, J. L., HADDAD, Z. H. and LOPAPA, A. (1971): Detection of human IgE antibody by a modified rat mast cell degranulation technique. *Immunology* **20**, 545.
9. GILLMAN, S. A. and HADDAD, Z. H. (1972): Histamine release from rat peritoneal mast cells after passive sensitization with human reaginic serum or E myeloma protein. *J. Allergy & Clin. Immunol.* **50**, 3, 131.
10. KANYEREZI, B., JATON, J. C. and BLOCH, K. J. (1971): Human and rat γ E: serological evidence of homology. *J. Immunol.* **106**, 1411.
11. ISHIZAKA, K. and ISHIZAKA, T. (1971): Immunoglobulin E and hemocytotropic properties. *Progress in Immunology*, Academic Press, New York, page 859.
12. ZVAIFLER, N. J. and ROBINSON, J. O. (1969): Rabbit hemocytotropic antibody. A unique rabbit immunoglobulin analogous to human IgE. *J. exp. Med.* **130**, 907.
13. PERELMUTTER, L. and MILLARD, E. (1971): A modified rat mast cell technique for the detection of human IgE type reagins. *Int. Arch. Allergy* **40**, 675.
14. HADDAD, Z. H., LOPAPA, A. and KOROTZER, J. (1971): Direct demonstration of human immunoglobulin E (IgE) on rat mast cell after passive sensitization *in vitro*. *J. Allergy* **47**, 88.
15. DE WECK, A. L. and SCHNEIDER, C. H. (1969): Molecular and stereochemical properties required of antigens for the elicitation of allergic reactions. *Bayer Symposium 1*, pages 32-46.
16. MACLAREN, W. R., HO, F. C. and LOPAPA, A. (1972): Rat mast cell degranulation and routine skin tests in allergic patients. *Ann. of Allergy* **30**, 579.