

Analysis of the non-immunological activity of allergen extracts in cutaneous tests

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Summary

The technique proposed by the National Institute for Biological Standards and Control, London (NIBSC, 1977) for the control of allergenic extracts for non-immunological activity in skin tests has proved to be both sensitive and useful. We have observed 'false positive' results to substances such as merthiolate or glycerine and also to lyophilised preparations with sugar, notably glucose, as the carrier substance.

Other factors studied in 'false positive' reactions to a given extract were pH, osmotic pressure and the presence of traces of endotoxins or histamine. Individually none of these provided satisfactory explanations for the false positive reactions and the use of antagonists or inhibitors of the release of chemical mediators only confirmed the complexity of the non-immunological reaction and the participation of factors other than histamine.

Introduction

A simple though important technique is presented which can be used in the standardisation and control of allergens. This concerns the evaluation of both diagnostic and therapeutic allergen extracts for substances; capable of eliciting reactions via a non-immunological mechanism and not requiring the interaction of antigen with antibody; vaso-active amines and more precisely histamine or pharmacologically related substances.

The NIBSC proposed an experimental protocol with which we have studied products actually in use by clinicians. The technique is to measure the local cutaneous reactions for a test substance when injected intradermally into an experimental animal, normally an unsensitised rat, as shown by the extravasation of a vital stain such as Evans blue.

The frequency and importance of positive non-immunological reactions led us to

attempt to identify the experimental factors or substances capable of causing the increase in capillary permeability.

Material and methods

Choice of the animal model

In the method proposed by the NIBSC assays were performed in the rat. We used both male and female Sprague Dawley rats (Iffa Credo) and in order to assess the importance of any one animal model for the sensitivity of the method, assays were also made in Albino guinea-pigs (Hartley) and California rabbits.

Procedure (rat)

An anaesthetised rat (either trichlorethylene or ether was used as anaesthetic) was shaved on its back and injected with 0.5 ml of 2.5% Evans blue dye into either the lateral tail vein or the penis. After ensuring that there was no spontaneous release of dye into the tissues of the back the test was continued. The rat was injected intradermally using a 1 ml tuberculin syringe (ref. B.D. Plastipak) and a 26 G needle, at six marked sites arranged in two parallel lines of three sites, each 3 cm apart, on either side of the vertebral column. Each was injected with 0.05 ml of one of the following: (a) Four serial dilutions of the test extract (diluted in PBS). (b) Histamine solution—50 µg/ml—positive control. (c) Phosphate buffered saline (PBS)—negative control.

After 20 min the animal was killed and the dorsal skin removed. Fat deposits were cut away from the inner surface so that any areas of blueing around the injection sites could be clearly seen and measured. The skin was then mounted and photographed as a permanent record.

Assuming that there were no abnormalities at the control sites, the results were numerically recorded as the sum of two diameters of the observed blue areas at right angles to each other.

A more precise measurement may be obtained by evaluating the actual quantity of dye released at each specific site (Tarayre & Laouressergues, 1975) we did not use this method because of its semi-quantitative nature.

In order to assess variations in sensitivity caused by the position of the site of injection certain control tests were made using Latin squares (Voorhorst, 1974).

Products studied

A selection of the principal allergens commercially available in France were used. The products were coded for the assay and could be identified as follows:

A = House dust: Glycero-saline extract (50% glycerine + 50% of 0.9% sodium chloride) 1/10 w/v—Batch 70309.

B = House dust: Aqueous extract, prepared by fractionation, concentration and precipitate recovery 1 ampoule of 0.5 ml of a 50% glycerinated extract containing phenol at 0.5% was diluted in 4.5 ml sodium chloride (1%) Batch 052510.

C = House dust: Aqueous diagnostic extract, prepared by fractionation, concentration and precipitate recovery by methods differing from those used in the preparation of B. The extract contained merthiolate, 0.1 gm/litre. The concentration of the extract was given as Noon units. Batch 072029 (C1) and 062029 (C2).

D = Grass pollen mix (cocksfoot, timothy, rye, rye-grass): Diagnostic glycerosaline extract containing merthiolate, 0.1 gm/litre Batch 032021 (D1) and 032031 (D2).

E = Cocksfoot pollen: Glycero-saline extract (50% glycerine + 50% of 0.9% sodium chloride) 1/20 w/v—Batch 40638.

F = Mixed bacterial allergen: 2,000 million organisms/ml—containing merthiolate 0.1 g/litre Batch 0925.

G = Beta haemolytic streptococci: 2,000 million dead organisms/ml containing merthiolate 0.1 g/litre Batch 70307.

H = House dust: 'Delayed absorption' extract, adsorbed on to aluminium hydroxide, containing merthiolate 0.1 g/litre—Batch 33.

The following solutions were also tested as serial dilutions prepared in distilled water:

(a) Preserving or carrier substances used in the preparation of allergens: Sodium ethylmercurithiosalicylate (sodium merthiolate); Glycerol; Phenol.

(b) Impurities or possible contaminants of an allergen extract: Endotoxins (ref. Lipopolysaccharide derived from *Shigella flexneri* n° L 3255); Histamine.

Antihistamines and inhibitors of histamine release were also tested for their effects on reactions: Clemastine fumarate; Theophylline.

Results

Selection of the principal allergens commercially available in France

The results, summarised in Table 1, showed:

(i) positive reactions either equal to or greater than the histamine control, obtained for: concentrated glycero-saline extracts—A and E; extracts containing merthiolate at 0.1 gm/litre—C1, D1, F and G; house dust aqueous extract—1/200 dilution, containing phenol 0.5%—B; merthiolate solution 0.1 g/litre; glycero-saline solution containing 50% glycerine; isotonic glucose solution—Codex 5%.

(ii) parallel sensitivity in the rat and guinea-pig. The rabbit appears less sensitive.

(iii) a clear role was played by merthiolate in the non-immunological epidermal reaction. All of the products containing merthiolate at 0.1 g/litre were positive irrespective of the allergen concentration e.g. C1 and D1. However when these extracts were further diluted by 1 in 10 in PBS they lost all epidermal activity and in the case of the bacterial allergens F and G a 1/10 dilution of the pure merthiolated solution with PBS was sufficient for the total loss of epidermal activity.

This action of merthiolate is further shown when all of the allergen dilutions are prepared in PBS. Table 2 shows the results obtained with commercially available allergen extract dilutions containing merthiolate, compared with identical dilutions prepared from a concentrated commercial extract using PBS. However it must be noted that even with the commercial preparations the order of the reaction is reduced with allergen concentration though the solutions supposedly contain the same concentration of merthiolate.

Study of the preservatives and adjuvants used in the preparation of allergens

The female rat seemed more sensitive in these tests than the male, and therefore before continuing with the other assays we tried to determine what effect the choice of either male rats or female rats would have on the results.

We found that the female rat is significantly more sensitive than the male rat with aqueous house dust extract A ($P < 0.01$), aqueous house dust extract C2 (1,000 Noon units— $P < 0.001$), low concentrations of grass pollen mix D2 (1/100,000— $P < 0.05$)

Table 1. Comparison of extracts in different solutions

Product		Concentration	Guinea-*		
			Rat*	Pig	Rabbit*
A	House dust	{ Pure	22	23	22
	aqueous extract	{ 10%	0	0	0
B	House dust	{ 0.5%	12	12	10
	aqueous extract	{ 0.2%	0	0	0
C ₁	House dust	{ 1,000 Noon units	15	17	10
	aqueous extract	{ 100 Noon units	10	10	8
		{ 10 Noon units	8	6	0
		{ 1 Noon unit (diluent = PBS)	0	0	0
D ₁	Grass pollen mix diagnostic extract	{ 0.1%	20	20	15
		{ 0.01%	13	12	8
		{ 0.001%	11	9	0
		{ 0.0001% (diluent = PBS)	0	0	0
E	Cocksfoot pollen aqueous extract	{ Pure	25	24	20
		{ 10%	5	6	6
		{ 5%	0	0	0
F	Bacterial aqueous extract	{ Pure	12	10	8
		{ 10%	0	0	0
G	Bacterial allergen aqueous extract	{ Pure	10	10	8
		{ 10% (diluent = PBS)	0	0	0
	Merthiolate solution	{ 0.1 g/l	16	15	12
		{ 0.01 g/l	0	0	0
	Glycero-saline solution batch F.250	{ Pure	23	20	12
		{ 10% (diluent = PBS)	0	0	0
	Isotonic glucose solution codex 5%	Pure	18	16	10
	Histamine (positive control)	50 µg/ml	22	20	15
	PBS (negative control)		0	0	0

* Mean results obtained from three animals.

and glucose ($P < 0.01$). It is therefore important to use only one sex throughout all the assays. For all of the products we chose the male rat despite its lesser sensitivity because we found it to be easier to inject.

The results obtained when using sodium merthiolate, phenol, glycerol and glucose in skin tests suggest that all of these products are capable of eliciting a response and moreover at concentrations approaching those in use commercially e.g. merthiolate which is the most widely used product for allergenic extracts in France (Table 3).

In order to eliminate the chance of such artefacts it is necessary to set the following new limits of concentration: Sodium merthiolate 0.01 g/litre; Glycerol 3%; Phenol 0.4%; Glucose 1%.

The reactions to the 'pure solutions' are less than those obtained with commercial extracts containing the respective solutions. This suggests that, either other components are intervening in the reaction as for pollens and house dust (impurities, contaminants), or that the concentration of the preserving solutions in the allergen extracts is not accurate.

Table 2. Comparison of effects of dilution with PBS or sodium merthiolate solution

Product	Commercial preparations		Laboratory preparations (PBS dilutions)	
	Concentration	Results*	Concentration	Results*
C ₁ House dust aqueous extract	1,000 Noon units	15	1,000 Noon units	15
	100 Noon units	10	100 Noon units	0
	10 Noon units	8	10 Noon units	0
			1 Noon unit	0
D ₁ Grass pollen mix diagnostic extract	0.1%	20	0.1%	20
	0.01%	13	0.01%	0
	0.001%	11	0.001%	0
F Bacterial allergen extract	Pure	12	10%	0
G Bacterial allergen extract	Pure	10	10%	0
H House dust adsorbed extract	1,000 Noon units	13	1,000 Noon units	13
	100 Noon units	8	100 Noon units	0
	10 Noon units	6	10 Noon units	0
			1 Noon unit	0
Histamine (positive control)	50 µg/ml	22		
PBS (Negative control)		0		

* Mean results obtained from three animals.

Table 3. Action of sodium merthiolate

Product injected		m*	s
Sodium merthiolate diluted in PBS	0.1 g/l	17.5	1.37
"	0.05 g/l	11.7	1.96
"	0.025 g/l	6.66	1.15
"	0.01 g/l	0	—
Histamine—50 µg/ml (positive control)		21.5	1.38
PBS (negative control)		0	—

* Mean results obtained for 6 rats.

The figures represent the size of the papule given as the sum (in mm) of two diameters measured at right angles to each other.

Role of impurities and contaminants—physico-chemical factors

Presence of histamine in allergenic extracts. Tests were performed to see whether those extracts giving a very strong epidermal reaction contained small quantities of histamine.

Using the technique of Magnus (1904) on the guinea-pig ileum, we examined several batches of allergen, notably the different concentrations of adsorbed house dust extract (H) which showed the presence of small quantities of histamine at approximately 0.1 µg/ml for the 1,000 Noon unit extract.

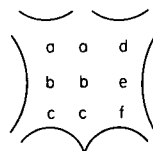
Unfortunately, without having large quantities of extract, it was not possible to repeat the assays several times in order to see if the histamine was derived from the allergen or from a contaminant.

Endotoxins. Different authors (Bergman & Munoz, 1977; BITO, 1977; FDA, 1977b; Snella & Rylander, 1977) have written of the possible contamination of biological proteins with bacterial endotoxins, capable, even in minute quantities, of provoking inflammatory responses.

We therefore determined the sensitivity of the rat skin to serial dilutions of endotoxin, prepared in PBS, by prick test and by intradermal injection.

The following injections were made in each of three rats after their backs had been shaved and each had been injected intravenously with 0.4 ml of 2.5% Evans blue dye:

- (a) i.d. 0.05 ml–5 mg/ml.
- (b) i.d. 0.05 ml–1 mg/ml.
- (c) i.d. 0.05 ml–100 µg/ml.
- (d) Prick test 5 mg/ml.
- (e) Prick test 1 mg/ml.
- (f) Prick test 100 µg/ml.



Non-immunological histamine release was only obtained with a relatively high concentration of endotoxin (5 mg/ml) giving a 15 mm papule when injected intradermally. It therefore seems unlikely that the results observed previously when using commercial allergen extracts are influenced by endotoxin only. However it is advisable to determine the level of endotoxin in allergenic extracts since there could be a possibility of a synergistic relationship between trace levels of histamine and endotoxin resulting in the non-immunological release of mediators (Bergman & Munoz, 1977).

pH. No reaction was observed when using a range of pH between 4.0 and 8.0 prepared using PBS to which either 30% hydrochloric acid or 0.2 M sodium hydroxide had been added according to the pH required.

Variation in pH alone is not responsible for the non-immunological histamine release.

Ionic strength. Six products were selected which had previously given either definite positive or negative responses and had a range of osmotic pressures.

It can be seen in Table 4 that ionic strength is insignificant for non-immunological histamine release.

Action of antagonist and inhibitors of histamine release

The role of histamine as the mediator of the response in this cutaneous test was studied, using clemastine fumarate which has been shown to be effective in man (Burns & Shelanski, 1977) and theophylline (Sullivan & Parker, 1976) (Tables 5 and 6).

Discussion

The collation of all of these results confirms the value of the test proposed by the NIBSC before making immunological studies in animals. It is essential to determine for each product the concentration which gives no non-immunological histamine release before proceeding with immunological studies. The responses to sodium merthiolate, phenol, glycerol and glucose are particularly interesting. They suggest that similar studies should be made in man, particularly with sodium merthiolate, given that its effect has already been noted by other workers (FDA, 1977) (Gilbert, Bastien & Pelletier, 1977) (Magnusson & Moller, 1977). However, it must be noted that the

Table 4. Ionic strength

Product injected		Osmotic pressure mmole/Kg	m*	s
H	House dust adsorbed extract 1,000 Noon units	282	14.2	1.17
C ₁	House dust aqueous diagnostic extract 1,000 Noon units	236	15.7	1.03
A	House dust aqueous extract Dilution 10% in PBS	1,080	0	—
E	Cocksfoot pollen aqueous extract Dilution 10% in PBS	1,289	0	—
	Histamine 50 µg/ml PBS	229 396	21.2 0	1.5 —

* Mean results obtained for six rats.

The figures represent the size of the papule given as the sum (in mm) of two diameters measured at right angles to each other.

Table 5. Action of clemastine fumarate on the non-immunological release of histamine

Product injected	Control rats		Tests rats**		t	P
	m*	s	m*	s		
House dust 10% aqueous extract (A)	26	3	22.6	1.6	2.37	<0.05
House dust 0.5% aqueous extract (Batch 1125)	13.3	1.03	11.7	0.8	3.1	<0.05
Cocksfoot pollen 5% glycero-saline extract (batch 60153)	23.1	2.1	16.7	1.36	6.77	<0.001
Isotonic glucose solution 5% codex	18.7	1.21	12	1.6	7.9	<0.001
Sodium merthiolate 0.1 mg/ml solution	17.3	1.5	14.6	1	3.57	<0.01
Histamine 50 µm/ml PBS	21.8 0	1.17 0	0 0	0 0	0 0	<0.001 —

* Mean results obtained for six rats. The figures represent the size of the papule given as the sum (in mm) of two diameters measured at right angles to each other.

** Oral route—Dose: 2 capsules/rat equivalent to 2.68 mg/rat (weight: 200 gm).

Table 6. Inhibition of immunological and non-immunological histamine release by theophylline. Intravenous route—Dose = 15 mg/rat (weight = 200 g)

Site of injection	Product injected	Control rats†		Test rats§		t	P
		m	s	m	s		
a*	Cocksfoot pollen extract E diluted 5%	18.5	1.5	4.33	4.8	6.88	<0.01
b	Cocksfoot pollen extract E diluted 10%	0	—	0	—	—	—
c	Cocksfoot pollen extract E	22	—	18.16	—	—	—
d	Histamine 50 µm/ml	20.17	1.17	17.8	0.75	4.11	<0.01
e	PBS	0	—	0	—	—	—

* Site sensitised with rat anti-cocksfoot serum 24 hr before intradermal allergen challenge.

† Control rats received 1 ml PBS 30 min before allergen challenge.

§ Test rats received 1 ml of theophylline—15 mg/ml—intravenously 30 min before intradermal allergen challenge.

response to a solution of sodium merthiolate alone is generally markedly less than to the allergen extracts containing merthiolate, particularly in the female rat. The responses for bacterial allergens containing merthiolate is less however (Tables 1 and 2).

It seems therefore that the total response is not due to merthiolate alone but seemingly also depends on factors governed by the quality of the raw material and the method of preparation of the extract.

This hypothesis is confirmed by the significant difference in response observed when using various concentrations of aqueous house dust extract C2 and grass pollen extract D2, though all supposedly contained the same concentration of sodium merthiolate (0.1 g/l).

The possible role of endotoxins and other contaminants must be considered; pH and ionic strength of extracts appear to be of no significance.

The local reaction seen by the NIBSC technique does not depend on a unique process for the extravasation of the dye following the action of chemical mediators on capillary permeability. It is however clear that histamine is one, following upon the results obtained with clemastine fumarate which significantly blocked the non-immunological reaction of the extract (Table 5) whilst neutralising completely the positive histamine control. Moreover the blocking action of theophylline on both immunological and non-immunological release (Table 6) further confirms the role of histamine. It is possible that the mechanisms involved in increasing vascular permeability following challenge with conserving solutions or 'charged substances' are different.

The role of the mercury ion in the displacement of mediators and in the liberation of histamine in the absence of degranulation has already been recorded (Parrot, 1976). Parrot has warned of a similar action by glucose but it remains to be seen whether the difference in concentration of glucose at the capillaries following local intradermal challenge results in immediate exosmosis which would explain the rapidity and importance of the reaction when using glucose in the skin test.

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