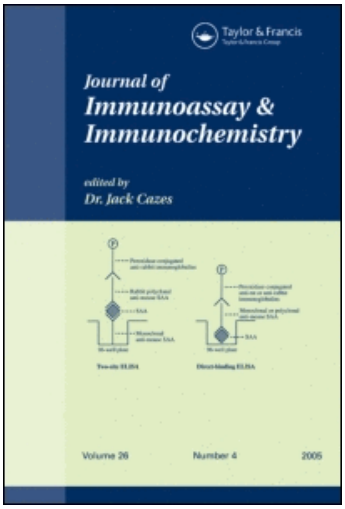

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ESTIMATION OF AIRBORNE RAT-DERIVED ANTIGENS BY ELISA

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ABSTRACT

Double antibody sandwich ELISA procedures have been devised to estimate the concentrations of airborne rat urinary and epidermal antigens. Both assays were capable of detecting at least 3 ng/ml of antigen protein. Unknown concentrations were estimated in terms of the standard, laboratory-prepared material, as calculated from the linear relationship between absorbance and log concentration. The coefficient of variation of a single assay was 12%, the methods have been applied to extracts of the filter material from static and personal samplers in an animal room.

INTRODUCTION

Development of allergy to laboratory animals is an increasingly recognised problem of some magnitude; approximately 1 in 5 of exposed workers become affected (1, 2, 3, 4, 5, 6). It might be expected that a reduction of this incidence would result from decreased exposure to allergen, for example by re-design of animal rooms, adoption of personal protective measures, and reconsideration of working practices. Guidance for the application of such measures must await investigations of those factors which govern the amount of allergen in the air and these, in turn, are dependent on suitable assay procedures. Immunoassay for rat-derived allergen became possible following the demonstration of an allergenic protein in rat urine

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(7, 8). Longbottom (9) estimated the pre-albumin allergenic component of rat urine in dusts and the air of animal rooms by rocket immunoelectrophoresis using a rabbit antiserum specific for this fraction. Edwards (10) has estimated specific rat urinary allergen by the technique of RAST inhibition using sera from allergic individuals containing IgE specific for the allergen. More recently Twiggs et al. (11) have applied a similar technique for the estimation of mouse allergen in air.

ELISA techniques are rapidly gaining wide acceptance, because of their flexibility and lack of complexities associated with the use of isotopes. They are also more sensitive than immunodiffusion and immunoelectrophoretic techniques. The present paper describes the development of an ELISA procedure for the estimation of antigens derived from rat urine and rat epidermis and the application of the technique to the determination of the concentration of these antigens in the air of animal rooms under a variety of conditions.

MATERIALS AND METHODS

Animals

Male rats, Alderley Park Wistar-derived Strain, Specific Pathogen Free, 3 - 4 months old were used as sources of antigens.

Antigens from Rat Urine

Urine was centrifuged and dialysed at 4^{o}C for 48 h against 0.05 M ammonium bicarbonate solution as described by Longbottom (8). The retentate was lyophilized and stored at room temperature in air-tight containers.

Antigens from Rat Epidermis

Epidermis was obtained by a modification (12) of the method of Ashendell and Boutwell (13). Briefly, pieces of shaved flank and dorsal skin (about 8 cm x 10 cm) were frozen by being placed on a glass plate over crushed 'Drikold'. Epidermis was then removed by scraping

with a scalpel blade. Soluble proteins were extracted by sonication in 0.05 M ammonium bicarbonate. The solution was lyophilized and stored at room temperature in air-tight containers.

Rabbit Antisera to Antigens from Rat Urine and Rat Epidermis

Solutions of antigen, emulsified with Freund's Complete Adjuvant were injected intradermally, each animal receiving 250 µg of protein. Injections were continued fortnightly for 6 weeks. Total immunoglobulin (Ig) fractions were prepared by precipitation with ammonium sulphate.

Enzyme Labelling

Aliquots of the Ig fractions were labelled with horse-radish peroxidase (Sigma) by the periodate method of Wilson and Nakame (14) as described by Voller, Bidwell and Bartlett (15).

Line Immunoelectrophoresis

This was carried out as described by Kroll (16).

Buffers

Coating buffer consisted of Na_2CO_3, 1.59g; $NaHCO_3$, 2.93g; NaN_3, 0.2g in 1 litre of distilled water (final pH 9.6). PBS Tween was NaCl, 40g; KH_2PO_4 1g; $Na_2HPO_4.12H_2O$, 14.45g; Tween 20 (Sigma) 2.5 ml in 5 litres of distilled water (final pH 9.6). Citrate phosphate buffer was citric acid 5g; $Na_2HPO_4.12H_2O$, 17g in 1 litre of distilled water (pH 5.0). For substrate, a stock solution of urea hydrogen peroxide (BDH) 40mg ml^{-1} was made in citrate phosphate buffer and stored at 4^oC. Daily, 40 mg of o-phenylene diamine (Sigma) was dissolved in 100 ml of citrate-phosphate buffer and 0.5 ml of urea-peroxide solution added immediately before use.

ELISA

The double antibody sandwich method (14) was adapted as follows using NUNC micro-titre plates (Gibco): 200 µl volumes of 1:4000 dilution of the Ig fraction of the rabbit antiserum in coating buffer was added to the plates and stored for 2 h at room temperature; after washing,

200 µl of standard antigen dilution or sample for assay both in PBS-Tween containing 0.1% gelatin, was added; after a further 2 h at room temperature the wells were emptied and washed and 200 µl of the appropriate enzyme-labelled Ig fraction added. The plates were stored overnight in a humid chamber at 4°C; after further washing 200 µl of substrate was added. After 30 minutes at room temperature the reaction was stopped by the addition of 50 µl of 0.5 M citric acid and the absorbance measured at 450 nm by means of a Multiskan (Flow Laboratories).

Protein

The method of Lowry et al. (16) was followed.

Sampling of Airborne Antigen

Both personal and background samplers were used, particulate material being collected on glass fibre filters (Whatman GF/A: fuller details of the air sampling procedures will be described in a separate paper). For antigen determination the complete personal filter (22 mm) or for the larger filter (60 mm) 4 x 6 mm punched out circles, were extracted with 1 ml PBS-Tween buffer by sonication.

RESULTS

Characterization of Antigens

The immunoelectrophoretic patterns shown in Figures 1 and 2 indicate that the urinary antigens contained small amounts of serum antigens but very little epidermal antigen, whereas the epidermal antigen was virtually free from contamination with urinary or serum antigens.

Specificity of Assays

Tables 1 and 2 show that, in the concentration range used for assay (3 to 100 ng ml^{-1}), antibody to urinary antigen did not react significantly with serum or epidermal antigens and likewise antibody to epidermal antigen did not react with urinary or serum antigens (Table 1 and 2).

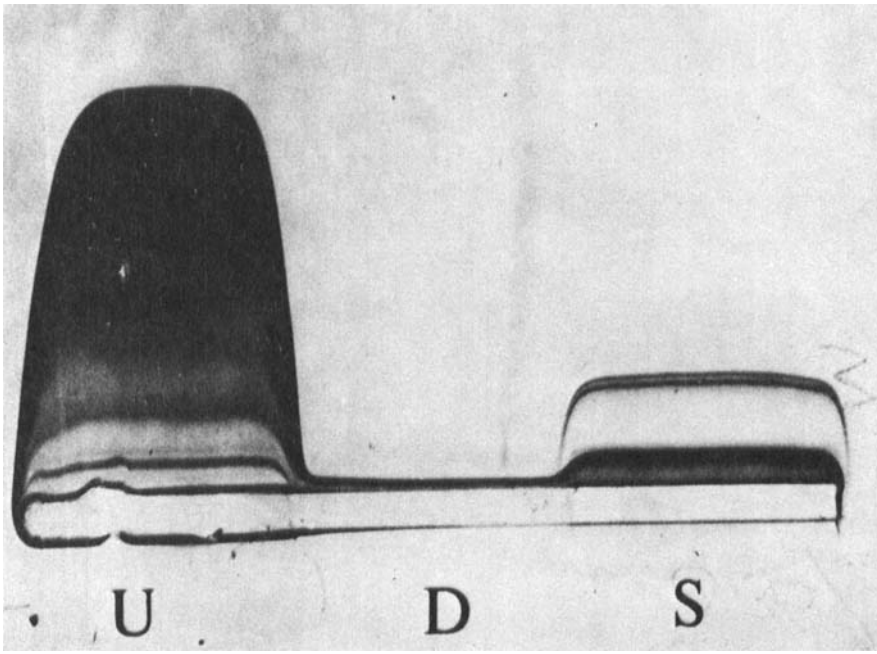

Figure 1. Line immunoelectrophoresis of rat antigens into rabbit anti-rat urine (8% in agarose); U = urine extract (2 mg ml^{-1}); D = epidermal extract (2 mg ml^{-1}); S = serum (1:2)

TABLE 1

Reaction of Rabbit Antibody to Rat Urinary Antigen with Rat Urine, Epidermis and Serum, demonstrating Specificity for Homologous Antigen

Conc* ng ml^{-1}	Source of Antigen Absorbance at 450 nm ≠ Urine	Epidermis	Serum
100	0.775	0.092	0.15
50	0.663	0.034	0.025
25	0.479		
12.5	0.281		
6.2	0.175		
3.1	0.117		

*Total Protein

≠ Mean value of 3 estimates by ELISA

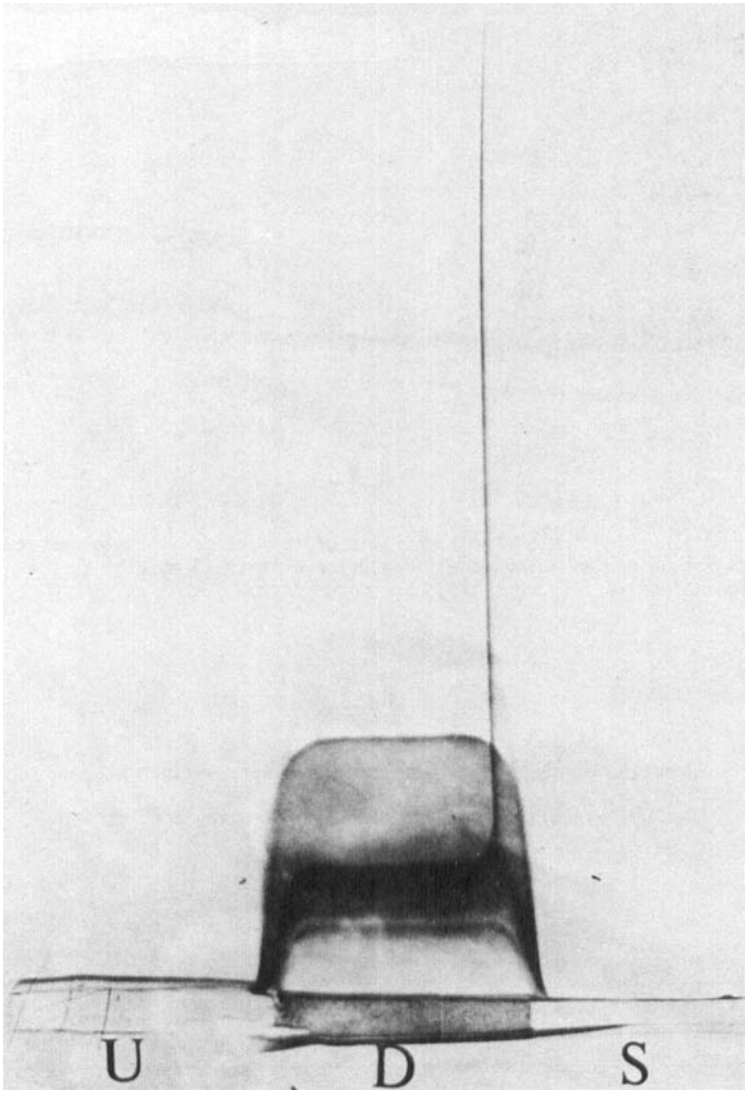

Figure 2. Line immunoelectrophoresis of rat antigens into rabbit anti-rat dander (epidermal protein, 8% in agarose); U = urine extract (2 mg ml^{-1}); D = epidermal extract (2 mg ml^{-1}); S = serum (1:2)

TABLE 2

Reaction of Rabbit Antibody to Rat Epidermal Antigens with Rat Epidermis, Urine and Serum, demonstrating Specificity for Homologous Antigen

Conc* ng ml^{-1}	Source of Antigen Absorbance at 450 nm ≠ Urine	Epidermis	Serum
100	0.035	0.989	0.0
50		0.837	
25		0.730	
12.5		0.551	
6.2		0.346	
3.1		0.210	

* Total Protein

≠ Mean value of 3 estimates by ELISA

Experimental Plan

The micro-titre plates consisted of 8 rows labelled A - H and 12 columns labelled 1 - 12. Row 1 (A to H) was a control row containing no antigen; two columns (wells B to G only) were randomly selected for dilutions of standard antigen; the remaining columns (wells B to G) were used for dilutions of the unknown samples. Calculations of antigen content were made in terms of standard antigen: each plate was treated separately and the value of the unknown derived by reference to the regression line of the samples on its own plate.

Typical Assays

Examples of assays of the amounts of rat urinary and epidermal antigens in the air of animal rooms are shown in Tables 3 and 4, with the corresponding calibration curves illustrated in Figure 3. An

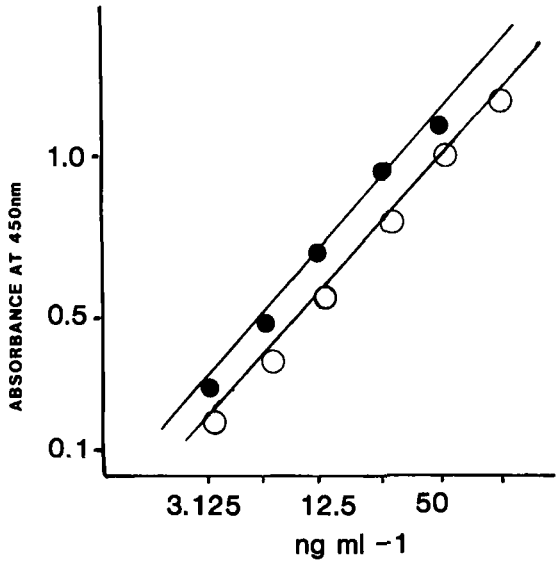

Figure 3.

Calibration curves for assay of rat epidermal and urinary antigens.
For urine (●) Log_e Concentration = (3.3 x Absorbance) + 0.175
r = 0.976
For epidermis (○) Log_e Concentration = (3.35 x Absorbance) + 0.56
r = 0.997

TABLE 3

Assay of Urinary Antigen in Air

Dilution of Extract	Absorbance at 450 nm	Estimated Concentration in Sample ng ml^{-1}	Mean ng ml^{-1}
1:16	0.634	14.7 x 16 = 234	
1:32	0.459	8.2 x 32 = 262	248
1:64	0.241	3.9 x 64 = 249	
1:2	0.555	11.2 x 2 = 22	
1:4	0.226	3.7 x 4 = 15	203
1:8	0.133	2.7 x 8 = 22	

Log_e concentration = (3.35 x Absorbance) + 0.56

r = 0.997

TABLE 4

Assay of Epidermal Antigen in Air

Dilution of Extract	Absorbance at 450 nm	Estimated Concentration in Sample ng ml^{-1}	Mean ng ml^{-1}
1:8	1.03	35.5 x 8 = 284	289
1:16	0.833	18.7 x 16 = 299	
1:32	0.61	8.9 x 32 = 285	
1:2	0.787	16.0 x 2 = 32	26
1:4	0.460	5.4 x 4 = 22	
1:8	0.238	2.6 x 8 = 21	
1:16	0.139	1.9 x 16 = 30	

Log_e Concentration = (3.3 x absorbance) + 0.175

r = 0.996

TABLE 5

Calculation of Antigen Concentration of Air

Total area of filter = 1809 mm^2

Area of 4 x 6 mm discs = 113 mm^2

Conc. of Antigen in Assay Sample (Table 3) = 248 ng ml^{-1}

Volume of Air Sampled = 53 m^3

$\therefore$ Conc. of Antigen in Air

$$= \text{Conc. in Sample} \times \frac{\text{Area of Filter}}{\text{Area of 4 Discs}} \times \frac{1}{\text{Vol of Air}}$$

$$= 74.9 \text{ ng } m^{-3}$$

example of the method of calculation of airborne antigen concentration is shown in Table 5.

Statistical Considerations

The variability of the standard response curve slope was investigated. About half the variability was identified with the day of the experiment and a quarter with each of the plate-to-plate variability on a given day and the column-to-column variability on a given plate. As all of our estimates of the unknown levels were made by reference to the standard result on the same plate on the same day, only the column-to-column variability applies to these estimates. The variance of the standard response curve slope within a given plate on a given day (ie the column-to-column variance) was 0.25. The relevant coefficient of variation was 12%.

Airborne Antigen Levels in an Animal Room

A control animal room was selected in order that the assay procedure could be tested under actual conditions. During the period of investigation the room was not used for any other purpose. It was stocked initially with 800 male rats and after a settling period of 10 days static samples were taken in the centre of the room each night (for 16 hours) and day (for 8 hours) over a 10 day period: 400 animals were then removed and the day and night sampling continued for a further 10 days. Figure 4 illustrates the relationship between antigen levels and stocking density and also demonstrates nocturnal activity of the animals. In the next phase of the investigation 10 to 12 experienced animal technicians were requested to carry out some standard procedures each on 100 animals (weighing, dosing by gavage, cleaning, and merely holding up an animal to examine it). Figure 5 shows the range of antigen levels estimated and indicates the individual variability between operators and also the very high levels experienced during examination, presumably during which the animals urinated.

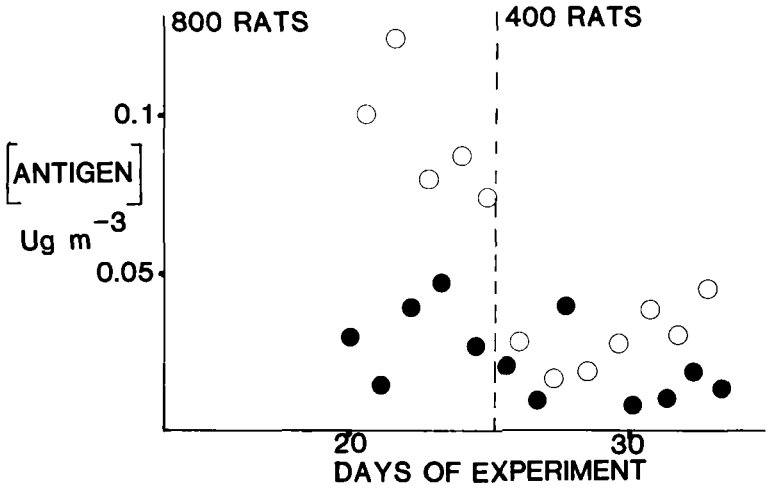

Figure 4. Levels of rat urinary antigen in an animal room under resting conditions. ◯= night samples; ● = day samples.

DISCUSSION

The purpose of this communication is to describe the immunoassay procedure which we are now using for a detailed study of the factors governing the amount of animal-derived allergen in the air. As described the system estimates antigen rather than specifically, allergen. There are two reasons for this: first, specific estimation of allergen would require a large and constant supply of human serum containing the appropriate antibodies and as we envisage the necessity for several thousand assays, this would be inconvenient, and second, it seems reasonable to assume that antigen concentration is related to allergen concentration, at least with the antigens we are using. Finally it must be emphasized that all concentrations are quoted in terms of the standard preparations and are not absolute values. The results of studies in the controlled animal room are included in this paper merely

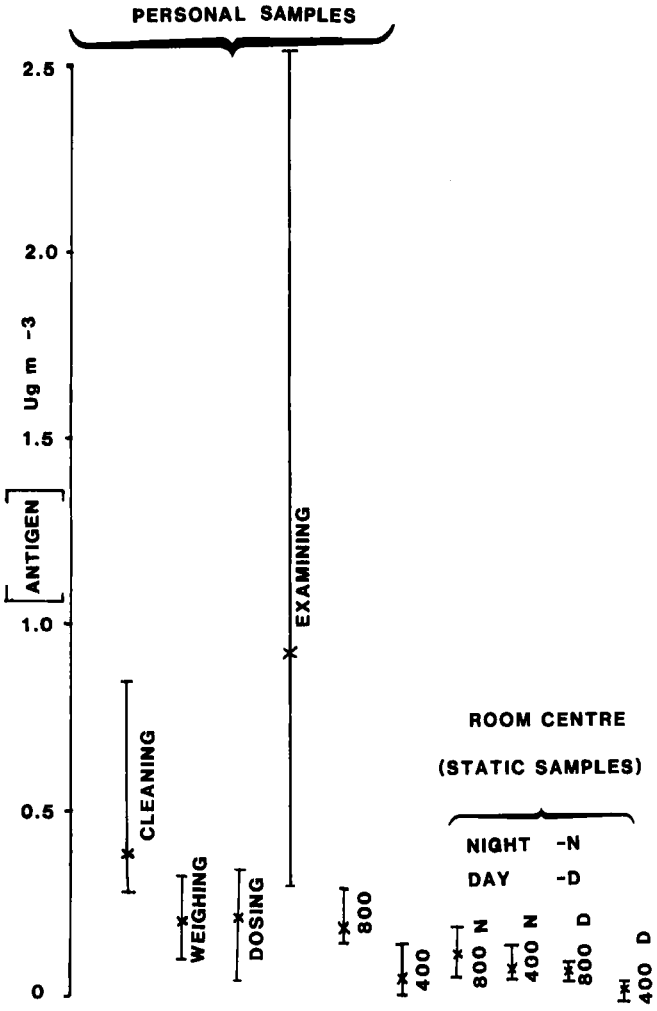

Figure 5. Levels of rat urinary antigen in an animal during various types of activity

to illustrate the assay procedure. The number of observations and the range of conditions under which they were made are far too small for any firm conclusions to be made. Similar but much more extensive investigations are currently in progress and will be reported separately.

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