

Quantitative Immunoelectrophoretic Analysis of Rat Allergen Extracts

I. Antigenic Characterisation of Fur, Urine, Saliva and Other Rat-Derived Materials

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Crossed immunoelectrophoresis reference patterns were established for the antigenic constituents of rat fur, urine and saliva. Using quantitative immunoelectrophoretic techniques, extensive cross-reactivity was observed between these extracts, and with epithelia, shaved pelt, serum, faeces and dust. No major qualitative sex differences were observed, though levels of certain components differed. Constituents of many source materials were found to show a reaction of partial identity with previously isolated rat urinary allergens; and relative skin test reactivity of a range of these extracts was associated with the amount of urinary allergen present. Material from two different rat strains had similar constituents. Rat source materials did not cross-react with the corresponding extracts from guinea pigs.

Key words: allergens; crossed immunoelectrophoresis; rat fur; rat saliva; rat urine; species; cross-reactivity.

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CLINICAL ASPECTS

Allergen avoidance for the rat-sensitive patient who wishes to continue working with rats is not easy, because many source materials share the same antigenic components. Similar constituents were shown to be present in extracts derived from both sexes of rat, and also from two different strains. Dual sensitivity to rats and guinea pigs is unlikely to be due to antigenic cross-reactivity between these species.

The rat sensitive subject may encounter potentially allergenic material from many sources in the environment of rats. Inhaled dust is likely to contain particles derived from fur and dander, and also bedding and diet, all of which could be contaminated with urine, faeces and saliva. Moreover, the latter could be introduced into the skin through bites and scratches.

Allergenicity has been demonstrated for rat fur (4, 5, 17, 28), whole pelt (15, 28), serum (2, 13, 15, 28), urine (2, 3, 8, 13, 28), faeces and saliva (28). Two major rat urinary allergens have been identified as an α_2 -euglobulin and a prealbumin (8), while rat serum albumin can also act as an allergen (26).

In a previous study (28) the allergenic potencies for a wide range of extracts relevant to allergy to rats were compared using skin prick tests (SPT), RAST and RAST inhibition for dust taken from a rat room. Most potent were fur, urine, saliva, epithelial scrapings and dust, although results of RAST and RAST inhibition suggested that important allergens were shared by rat extracts derived from different sources.

It is possible to collect rat urine and saliva which is not contaminated with other material. In the case of fur or dander, however, this is difficult, as urine, saliva and other substances are likely to be present in the natural state.

The aim of this study was to use quantitative

immuno-electrophoretic (QIE) techniques to investigate the extent of antigenic cross-reactivity between different allergenic extracts in order that the sources of rat allergen may more accurately be determined.

MATERIAL

Extracts

A range of extracts was prepared from 10- to 14-week-old Sprague-Dawley/Glaxo (SD/G) rats (Glaxo, Harefield, UK). For purposes of comparison, material was collected also from Lister Hooded/Olac (LH/O) rats of the same age (Olac, Bicester, UK) and from 10- to 14-week-old Dunkin Hartley guinea pigs (Redfern, Frant, Sussex, UK).

Material was collected as previously described (28) and included clipped fur, epithelial scrapings, shaved pelt, urine, saliva, faeces, serum, dust from the ventilation grill of a rat room, sawdust and diet (PRD diet, Labsure, Poole, UK). Animal material was derived from at least four animals.

Metabolic cages (Techniplast, Varese, Italy) were used in collection of urine, though a small volume was collected directly by bladder massage.

Saliva was stimulated using pilocarpine nitrate (McFarlan Smith, Edinburgh, UK) with ketamine hydrochloride anaesthesia (Ketalar, Parke-Davis, Pontypool, UK).

Source materials were defatted in ether, extracted in Coca's solution (except urine, saliva and serum), filtered ($0.45\ \mu$), dialysed against 0.05 M ammonium hydrogen carbonate and freeze dried (28).

Rabbit antisera

NZW and NZW-Dutch Lop cross-bred rabbit (2–3 kg) were immunised with 10 mg dry weight of extract in 4.6 ml of an emulsion of 50% saline and 50% Freund's complete adjuvant. Injection was in multiple sites with six of 0.1 ml intradermally on the back, two of 1 ml subcutaneously on each flank, and two of 1 ml intramuscularly in each thigh.

On day 21, the procedure was repeated with 10 mg extract in Freund's incomplete adjuvant. Rabbits were bled on day 56 and thereafter at 2 weekly intervals. After periods of 6 weeks, animals were boosted by subcutaneous and intramuscular injections of 1 mg of extract in saline.

Optimum antiserum pools were prepared for male fur, urine and saliva which contained a total of 25 or 35 bleeds, and from five or six rabbits, which had been immunized with one of these or a related extract. Bleeds were tested individually and in combination by crossed immuno-electrophoresis (CIE). Antisera produced against cross-reacting extracts were included in the pools if their presence allowed the detection or better resolution of particular components. For example, in addition to antisera against rat fur from both sexes, and two strains of rat, the "anti-rat fur" pool contained antisera against rat epithelial, urinary and salivary extracts.

Antiserum against the major prealbumin allergen of rat urine was raised against a purified fraction of this component (8). This antiserum was characterised by its incorporation in CIE and in intermediate gel CIE, which demonstrated that it was specific for the urinary prealbumin allergen and cross-reacting α_2 -globulin.

METHODS

Immuno-electrophoretic techniques

Crossed immuno-electrophoresis (CIE) was carried out as described previously (10). 8.4×8.4 cm glass slides were precoated with a thin layer of 0.1% agarose and dried before addition of 8 ml 1% agarose in 0.03 M veronal buffer (pH 8.65) with 0.0025% thiomersal.

The extract at 10 mg ml⁻¹ in saline (4 μ l fur, 5 μ l urine or 2 μ l saliva) was electrophoresed in the first dimension for 75 min at 4 Vcm⁻¹. "Windows" of 3.5×5 cm (main gel area) and 0.8×5 cm (intermediate gel) were cut out and filled with agarose, containing appropriate rabbit antisera before electrophoresis in the second dimension at a potential difference of 1.25 Vcm⁻¹ for 20 h. The intermediate gel, which contained anti-rat serum, was placed between

the electrophoresed extract and the main gel containing pooled antiserum against fur ($34 \mu\text{l cm}^{-2}$), urine ($31 \mu\text{l cm}^{-2}$) or saliva ($37 \mu\text{l cm}^{-2}$).

Plates were washed in saline, dried, and stained with Coomassie Brilliant Blue stain.

Tandem CIE (6) was performed by adding a second extract to a well cut in line with and 0.5 cm away from the well in which the appropriate reference extract was applied.

In *crossed line immunoelectrophoresis* (CLIE) (7), 500 μg of extract was incorporated in a further intermediate gel ($0.8 \times 5 \text{ cm}$), situated below that containing anti-rat serum. In addition, smaller amounts of extract were added for purposes of comparison and confirmation.

Relative amounts of rat serum albumin, and of the urinary prealbumin allergen in a range of extracts were estimated by *rocket immunoelectrophoresis* (RIE) (31). Various volumes and concentrations of extracts were added to a series of wells 0.5 cm apart, and were electrophoresed directly into antiserum-containing gel ($15.4 \mu\text{l cm}^{-2}$) for 20 h at 1.25 V cm^{-1} .

Skin prick tests (SPT)

SPT were carried out (28) at a concentration of 0.01 mg/ml^{-1} in Coca's solution/glycerol (1/1). Mean wheal areas were calculated for 13 rat-sensitive patients, all of whom associated symptoms of asthma and rhinitis with exposure to rats. Specificity was verified by a lack of SPT responses to these extracts in asymptomatic control subjects (28).

RESULTS

Characterisation of extracts

Reference CIE patterns for male rat fur, urine and saliva are shown in Fig. 1. No cathodal migrating components were detected when antiserum-containing gels were placed in such a way that this could be tested. No components were found to migrate faster than those in the reference pattern, with first dimension electrophoresis of 15 min. Improved resolution of antigen peaks was achieved by incorporation of the inter-

mediate gel containing anti-rat serum. By this means, three of the components of fur, eight of urine and three of saliva were recognised as being present in rat serum.

Total numbers of antigenic components observed for these three extracts were 19 for fur, 24 for urine and 21 for saliva. Precipitates have been numbered arbitrarily for each extract.

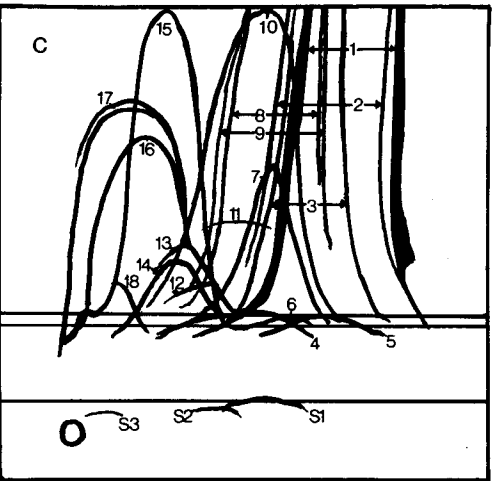
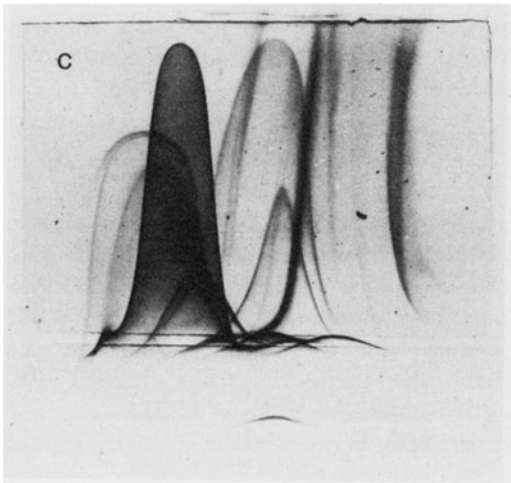
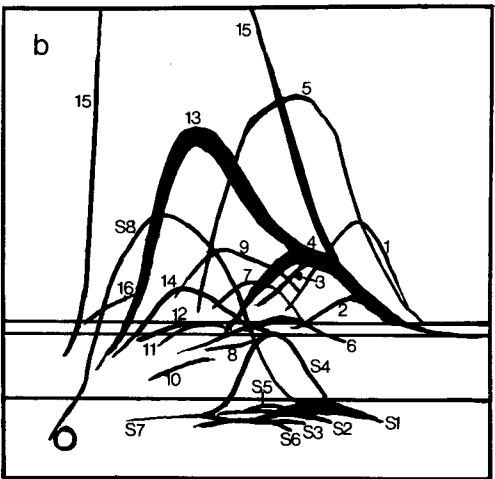
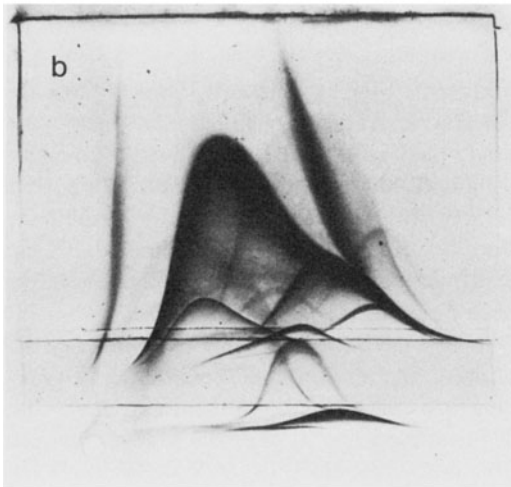
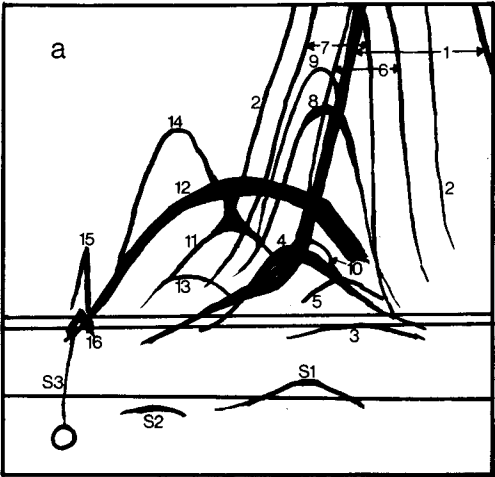
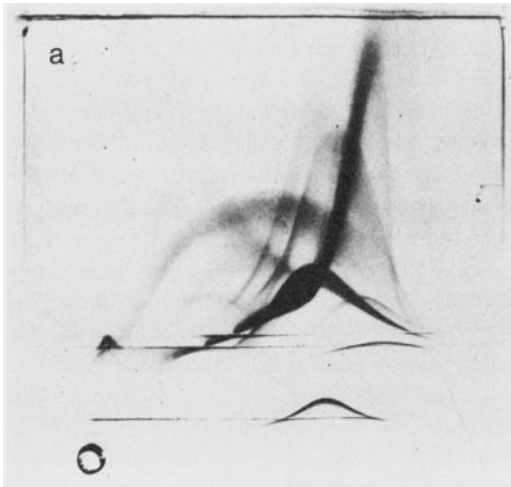
Peaks S1 of fur, urine and saliva were identified as albumin using rat serum albumin (Sigma, Poole, UK) in CLIE. QIE characterisation of the commercial preparation showed that immunochemical identity was with albumin and not with a minor contaminant.

Antisera raised against fractions of the two major rat urinary allergens (8) were added to intermediate gels in order to enable identification of these proteins in the reference precipitin patterns. The prealbumin allergen was represented in rat urine by peak 4 and the partially identical α_2 -euglobulin by peak 13. A single peak in both fur and saliva cross-reacted with both these components, but exhibited a greater antigenic identity with the prealbumin. Both the fur and salivary antigenic equivalents of urinary prealbumin allergen have therefore been designated peak 4.

It is interesting to note that the electrophoretic mobility of this "prealbumin" component was not the same in these extracts. While in urine the antigen peak did indeed possess a prealbumin mobility, in saliva the mobility was approximately equal to, and in fur was less than that of serum albumin.

Sex of source animals

CLIE, tandem CIE, intermediate gel CIE and RIE were carried out to compare extracts of fur, urine and saliva derived from male and female rats. Antisera raised against extracts from both sexes of animal were used. Marked differences in levels of many components were found between sexes (Figs. 1, 2, Table 4). Comparison of rat fur, urine and saliva extracts which were prepared on two different occasions from similar animals, indicated a degree of variability between levels of certain components. Such batch to batch differences were,



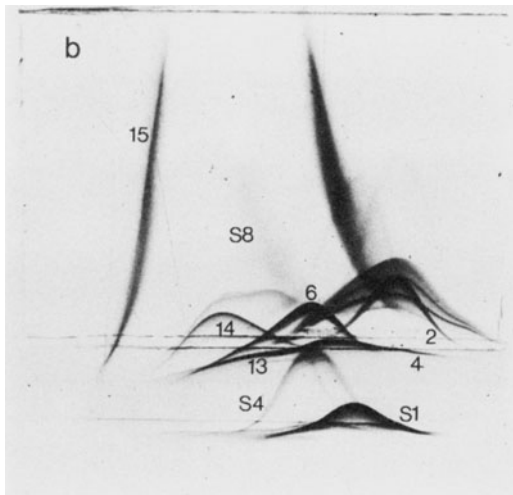
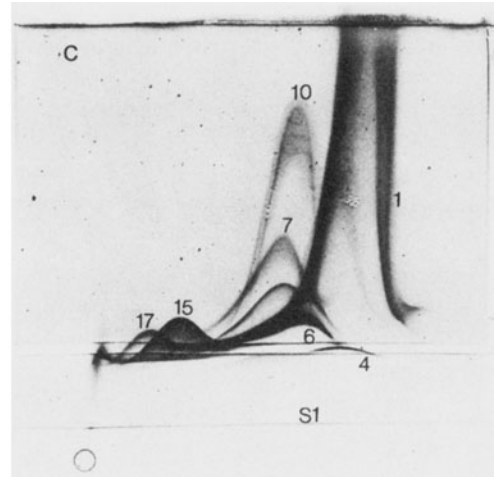
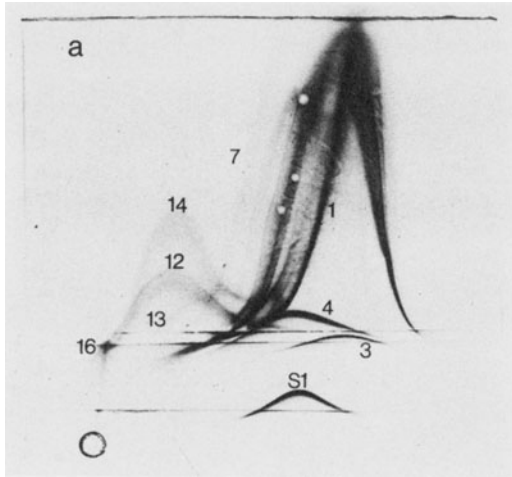


Fig. 2. CIE for (a) fur; (b) urine; and (c) saliva from female rats. The antiserum pools, concentrations and conditions were the same as those for CIE of the corresponding extracts from male rats in Fig. 1.

however, much less than those observed between extracts from male and female animals.

Despite quantitative sex differences considerable similarities were found in the antigens which were present in male and female extracts. All antigens of male fur and saliva were found also in the female extract, and *vice versa*. In female urine, all of the male components except number five were present; while no dis-

tinct female urinary components were observed.

Cross-reaction between sources of allergen

Many antigenic similarities between various materials derived from male rats were found using CLIE. Comparison with extracts from female rats was not carried out because of the

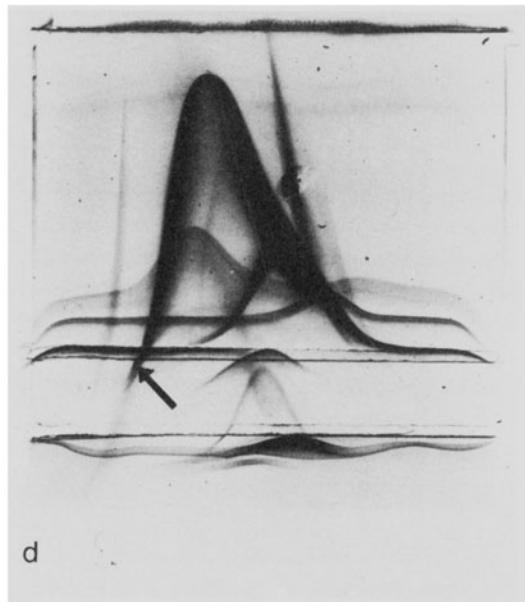
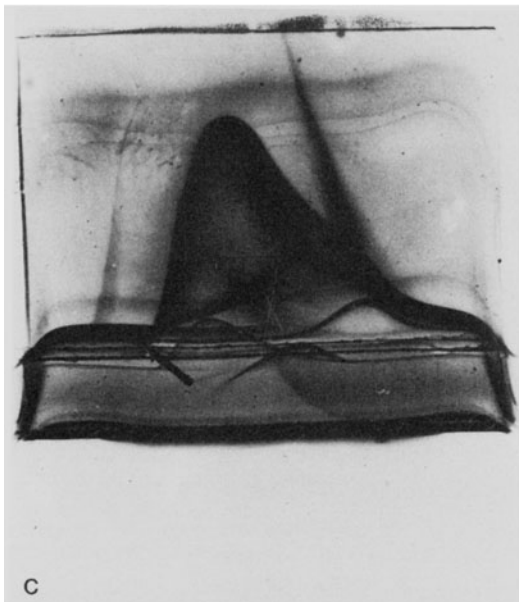
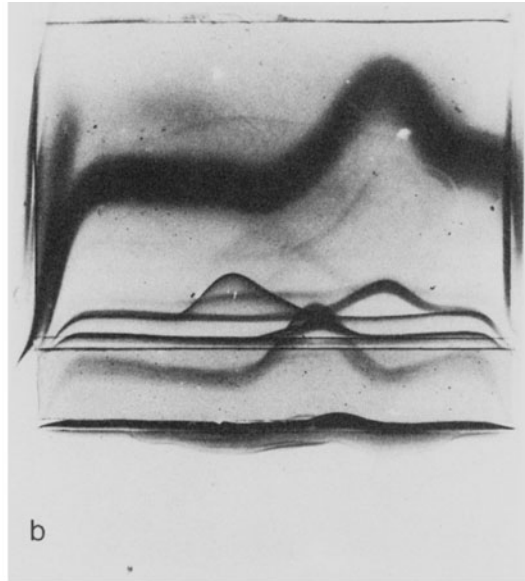
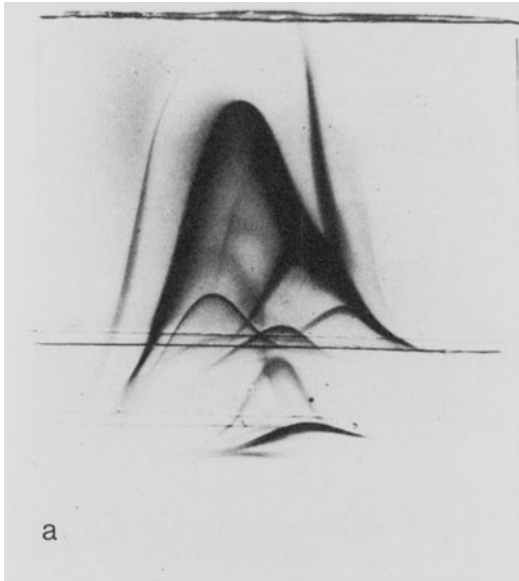
Fig. 1. CIE reference precipitin patterns with line drawings for (a) fur (40 μg); (b) urine (50 μg); and (c) saliva (20 μg) derived from male rats. The intermediate gel contained anti-rat serum (12.5 $\mu\text{l cm}^{-2}$ for (a) and (c), 62.5 $\mu\text{l cm}^{-2}$ for (b)); and the main gel: pooled antiserum against fur (34 $\mu\text{l cm}^{-2}$), urine (31 $\mu\text{l cm}^{-2}$) and saliva (37 $\mu\text{l cm}^{-2}$) respectively.

With the exception of peaks 4 and S1, similar antigens in the different extracts are not necessarily designated by the same number.

lack of qualitative sex differences. With the maximum of 500 μ g dry weight incorporated in an intermediate gel, antigenic components observed in the CIE reference patterns for fur, urine and saliva, were seen to cross-react with constituents of other rat-derived extracts (Tables 1, 2, 3). These findings were confirmed

using CIE with intermediate gels containing specific antisera raised against fur, urine, saliva, faeces, serum and dust.

For all peaks where cross-reactivity was observed, the reaction was seen as one of complete identity in the CLIE system (1); the only exception was with the α_2 -euglobulin allergen (peak



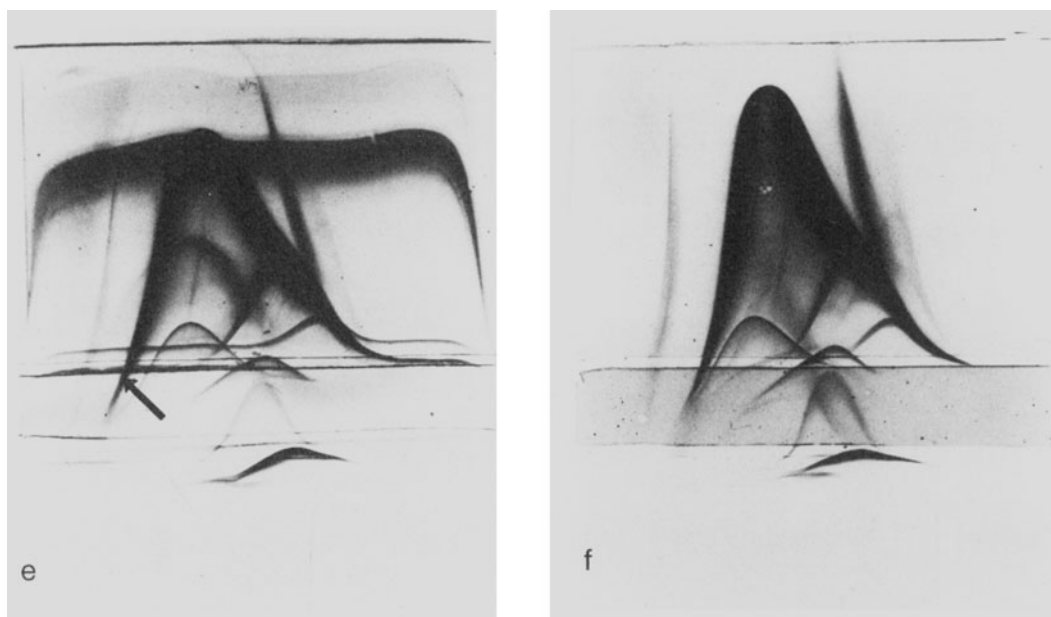


Fig. 3. CLIE showing effects on the CIE reference pattern for male rat urine (50 μ g) of incorporating in the first intermediate gel: (a) no extract; (b) 300 μ g male urine; (c) 500 μ g male fur; (d) 100 μ g male saliva; (e) 500 μ g male faeces; (f) 500 μ g male guinea pig urine. The second intermediate gel contained anti-rat serum (62.5 μ l cm^{-2}) and the main gel: anti-rat urine (31 μ l cm^{-2}).

The peak for urinary α_2 -euglobulin (antigen 13) begins below the lines corresponding to the urinary prealbumin allergen in fur, saliva and faeces (arrowed (c), (d) and (e)).

Table 1
Cross-reaction of rat fur antigens with other extracts

Fur antigen	Epithelia	Shaved pelt	Saliva	Urine	Faeces	Serum	Dust
1	+	+	+				+
2	+		+	+			+
3	+		+	+	+		+
4	+	+	+	+	+		+
5	+			+			+
6	+		+	+			+
7	+		+	+	+		+
8	+		+	+			+
9	+						
10	+		+				+
11	+		+				+
12	+	+					+
13	+			+			+
14	+	+	+	+			+
15	+		+				+
16	+		+				+
S1	+	+	+	+	+	+	+
S2	+	+	+	+	+	+	+
S3	+	+	+			+	+
Total 19	19	7	15	11	5	3	18

Table 2
Cross-reaction of rat urinary antigens with other extracts

Urine antigen	Fur	Epithelia	Shaved pelt	Saliva	Faeces	Serum	Dust
1	+	+		+	+		+
2	+			+	+		+
3	+			+	+		+
4	+	+	+	+	+		+
5	+	+	+				+
6							+
7	+	+	+	+	+		+
8	+	+	+		+		+
9	+	+	+	+			+
10	+			+			
11	+	+	+	+			
12	+	+		+	+		+
13	+	+	+	+	+		+
14	+	+		+			
15	+	+					+
16	+	+					+
S1	+	+	+	+	+	+	+
S2	+	+	+	+		+	
S3	+	+	+			+	
S4	+	+	+		+	+	+
S5		+	+	+		+	
S6	+	+	+			+	
S7	+	+	+			+	
S8	+	+	+		+	+	+
Total 24	22	20	15	14	11	8	16

13 of urine). The α_2 -euglobulin of male rat urine shows a reaction of partial identity with the prealbumin allergen in CIE, and the appearance of the peaks seems to indicate that it is lacking in some major antigenic determinants of prealbumin. Nevertheless, CLIE demonstrates that α_2 -euglobulin does itself possess unique antigenic components. Where fur, saliva or faeces were added in an intermediate gel, the peak for urinary α_2 -euglobulin began below the line representing the fur, salivary and faecal antigenic equivalents of urinary prealbumin (Fig. 3). This was not the case where urine, dust and epithelia were added in: CLIE of urine showing that these extracts possess components with a reaction of identity to urinary α_2 -euglobulin.

No antigens of rat diet or unused sawdust bedding were shared with any rat-derived extracts.

Tandem CIE showed the similarity between urine collected with metabolic cages, and that obtained by bladder massage from animals of the same age, strain and sex (Fig. 4). Only peak 14 of metabolic cage urine was lacking from urine collected directly.

Comparison of two rat strains

SD/G urinary components were all found by CLIE to be present in LH/O urine, except for S5 and S6. All fur and salivary antigenic constituents were present in the corresponding extracts of LH/O rats.

Table 3
Cross-reaction of salivary antigens with other extracts

Saliva antigen	Fur	Epithelia	Shaved pelt	Urine	Faeces	Serum	Dust
1	+	+	+				+
2	+	+		+			+
3	+	+	+	+			+
4	+	+	+	+	+		+
5	+	+		+	+		+
6				+			+
7	+	+					+
8	+						
9	+						
10	+	+		+	+		+
11	+						
12				+			+
13	+						+
14							
15				+			
16	+	+		+			
17							
18							
S1	+	+	+	+	+	+	+
S2		+	+			+	+
S3	+	+	+	+	+	+	+
Total 21	14	11	6	11	5	3	13

Table 4

Levels of urinary prealbumin allergen and rat serum albumin (antigens 4 and S1 respectively of fur, urine and saliva) determined by RIE, and mean SPT wheal areas (for 13 patients) for rat-derived extracts

Rat-derived extracts	Relative amounts of		Relative SPT reactivity
	Urinary prealbumin allergen	Rat serum albumin	
♂ fur	151	226	71.4
♀ fur	18.0	79.0	84.0
♂ epithelia	16.8	2,766	65.5
♀ epithelia	51.0	4,840	72.3
♂ shaved pelt	11.7	5,130	22.7
♂ whole pelt	12.0	5,380	29.4
♂ serum	0	660	10.1
♂ saliva	48.0	80.0	55.5
♀ saliva	39.0	226	70.6
♂ urine	910	371	89.1
♀ urine	85.0	460	57.1
♂ faeces	6.60	88.7	16.0
Rat-room dust	100	100	100

Results are expressed relative to the values for rat-room dust (100%).

Antisera raised against LH/O extracts failed to reveal antigens which were not found in SD/G fur, urine or saliva.

Comparison with guinea pig extracts

Incorporation of fur, urine or saliva derived from male guinea pigs into an intermediate gel in CLIE, failed to reveal cross-reactivity with corresponding male rat antigens.

Relative levels of albumin and prealbumin allergens in rat extracts

Relative amounts of serum albumin and urinary prealbumin allergen as determined by RIE are tabulated for a range of rat extracts (Table 4).

Statistical comparison of levels of these allergenic components with relative values of mean SPT wheal area for 13 rat-sensitive patients, was performed by calculating Spearman's coefficient of rank correlation. No significant rela-

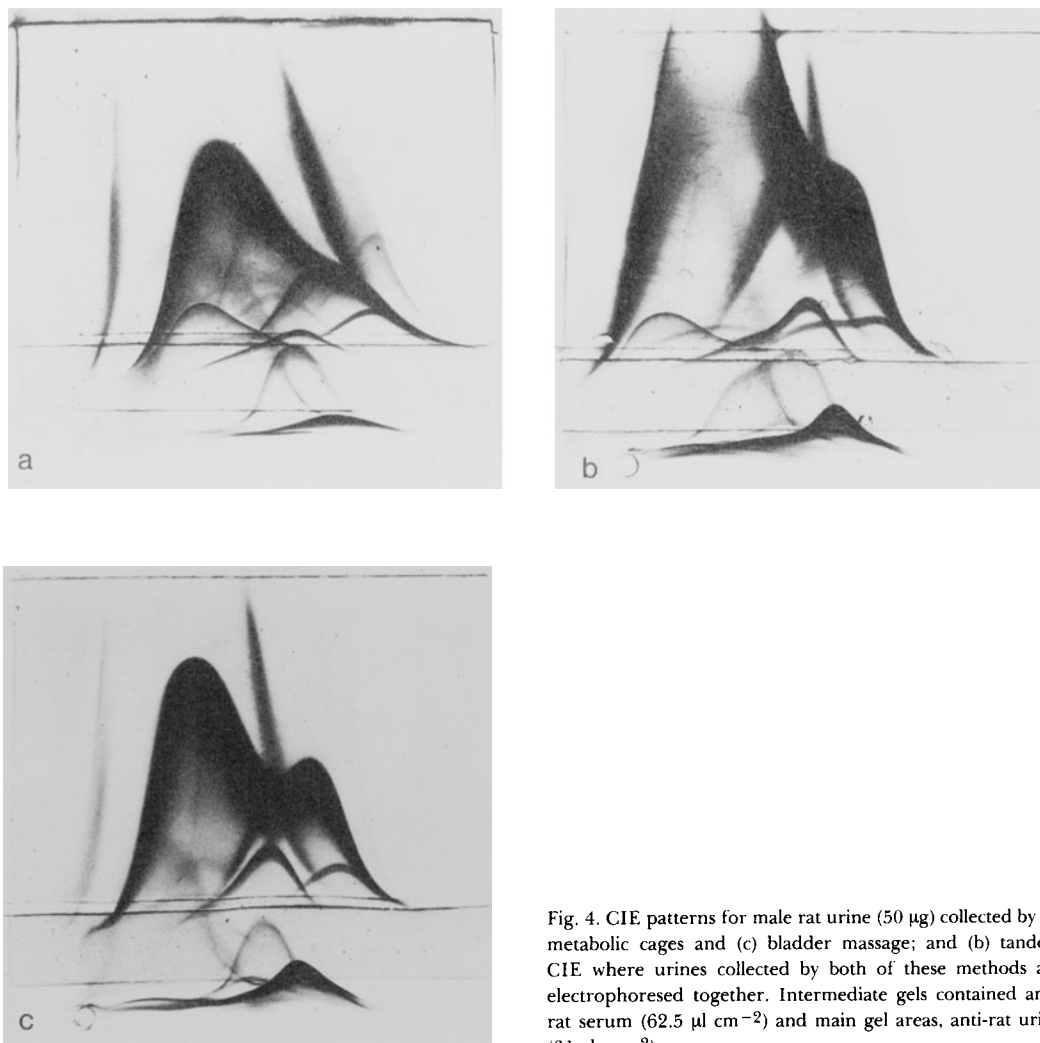


Fig. 4. CIE patterns for male rat urine (50 μg) collected by (a) metabolic cages and (c) bladder massage; and (b) tandem CIE where urines collected by both of these methods are electrophoresed together. Intermediate gels contained anti-rat serum (62.5 $\mu\text{l cm}^{-2}$) and main gel areas, anti-rat urine (31 $\mu\text{l cm}^{-2}$).

tionship was found between albumin levels and SPT reactivity, but the correlation between ranked values of amounts of prealbumin allergen and SPT reactivity was significant ($P < 0.01$).

DISCUSSION

The considerable antigenic similarities found between different rat source materials confirm the findings of RAST and RAST inhibition (28), which have demonstrated cross-allergenicity.

The presence of rat serum proteins in all of rat-derived extracts explains why many rat-sensitive individuals react to rat serum (13, 15, 28), while their exposure to rat serum itself has probably been minimal.

The rat urine studied here contained eight serum components. With older rats, the number is likely to be much higher on account of the spontaneous nephropathy with proteinuria which develops with age (22, 23, 30). In a previous study, at least 14 constituents of serum were demonstrated in a rat urine extract (8).

Allergenic properties have been described for

rat serum albumin in a proportion of rat-sensitive patients (26). The lack of correlation of albumin levels in rat extracts with relative SPT reactivity for a group of patients, would indicate that the role of this allergen is a minor one. Furthermore, the low allergenic potency of serum in comparison with extracts of urine, fur and saliva (13, 28), indicates that the non-serum components of these extracts are more important in allergy to rats.

When the rat urine extract obtained using metabolic cages was compared with urine collected by bladder massage, it was found that these non-serum components (with the possible exception of peak 14) do not represent contamination during collection. There is evidence that some non-serum urinary constituents are derived from the kidney or associated genital glands (16). Others may be present in very low amounts in serum, but are actively concentrated by the kidney (19, 20).

The occurrence of sex differences in levels of certain rat urinary proteins has been the subject of several studies (18, 20, 21). This work confirms such findings and extends them to saliva and fur. Noteworthy is the observation that male rat fur, urine and saliva contain higher levels of the prealbumin allergen and the components with which it cross-reacts.

In rat-sensitive patients, larger skin test reactions to male rat urine may be attributed to higher levels of prealbumin and α_2 -euglobulin allergens. The lack of such a trend in the SPT reactivities of fur and saliva, suggests that the concentration differences involved were not of a sufficient order to affect SPT reactions, and that other allergenic components compensate for this deficiency. Nevertheless, the significant relationship between levels of urinary prealbumin allergen and relative SPT reactivity for the 13 rat-derived extracts is evidence for the importance of this allergen.

The qualitative similarities demonstrated here between male and female allergenic extracts would appear to indicate that the sex of animal is not of major importance. However, female rats are smaller than males of the same age, and excrete much less urinary protein even when compared on the basis of size (22,

23). Therefore, it is possible that male rats contribute more than females to the allergenic load of animal houses.

The presence of many shared components for both urine and saliva makes it difficult to gauge the relative importance of these source materials in allergy. It is also difficult to assess to what extent fur would become contaminated with urine or saliva, though some antigens are clearly derived from the skin (e.g. fur antigen 12).

There are some discrepancies in total numbers of antigens which were found by QIE to be shared by different source materials. For instance, 11 urinary constituents cross-reacted with the extract of saliva, yet there were 14 salivary components which cross-reacted with urine.

Such disparities could be explained in part by antigens exhibiting antigenic identity with more than one peak in the cross-reacting extract. They are likely also to be a reflection of the sensitivity of the QIE techniques used, whereby owing to differing concentrations of antibody and antigen, certain peaks not seen in a CIE reference pattern could be visualised indirectly in CLIE, when they cross-reacted with another extract. These considerations of sensitivity apply in particular to components which are at the limits of detection in CIE such as some of the serum proteins in this study.

Despite the large number of salivary and urinary antigens detected on fur, there were some important omissions. Some components which were represented by dominant peaks in CIE reference patterns for saliva and urine were not found in the CIE reference patterns for fur (even using an anti-fur pool which contained antiserum raised against both urine and saliva). Even for the same source material, variation between extracts in amounts of certain components present was observed; and some antigens visualised in one source material could be in such low levels in another that they escape detection. Nevertheless, close similarities observed between different batches of the same material and between certain other extracts tend to discount the importance of sample variation.

Saliva stimulated by pilocarpine may not be the same as that which the rat would deposit on its coat during grooming. Salivation does not occur without some stimulus (25), however, so finding an appropriate control is not easy.

The absence in fur of the α_2 -euglobulin allergen, a major antigen of male rat urine, is surprising, though this component was detected in epithelial scrapings. Fur peak 4 does show antigenic identity with the partially identical pre-albumin component of urine, but the electrophoretic mobility differs from the corresponding antigenic constituents of both urine and saliva. It seems likely that many antigens derived from urine and saliva undergo chemical modification or degradation following deposition on fur.

The significance in allergy to rats of specific fur, urine and saliva antigens is being studied further using crossed radioimmuno-electrophoresis, with a view to evaluating more precisely the origin of rat allergens (29).

The close similarity in composition of extracts from two very different outbred rat strains, which has been reported here, seems to indicate that strain differences are not important in allergy to rats. It is possible, however, that comparison of materials from various inbred strains would reveal greater differences.

When rat fur, urine and saliva were compared with guinea pig extracts, no cross-reactivity was observed. This accords with the finding that IgE RAST scores against rat-room dust were not inhibited by pre-incubation of rat allergic sera with dust from a room housing guinea pigs (28); and with some more extensive work with animal urines (9). Moreover, in P-K neutralisation tests with fur (5), and in the IgG response of patients undergoing immunotherapy with pelt extracts, there was no cross-reactivity found between laboratory animal species (27).

Antigenic similarities have been demonstrated between extracts of dog, horse, cow and some other mammalian extracts using QIE (11). It is possible that under other conditions cross-reactivity would be found between these two species of laboratory animal. By using a maximum of 500 μ g of extract in CLIE (ap-

proximately 10 times the amount of extract electrophoresed in the first dimension), a quantitative element was introduced into this qualitative technique.

Nevertheless, any species cross-reactivity must be at a very low level. If one assumes that human IgE is not directed against antigenic determinants which are unrecognised by rabbit-precipitating immunoglobulins, then the observation that rat sensitive patients are frequently allergic to other species in the animal house as well (12, 27, 29), cannot readily be explained by cross-reactivity of allergens. A non-specific mechanism seems more likely.

Multiple sensitivities to several animal species may be a reflection of genetic predisposition to allergy. There is some association between symptoms of asthma to laboratory animals and an atopic history, though a clear relationship has not been shown for other allergic symptoms (2, 13, 23). Or perhaps the tissue damage resulting from the inflammatory reactions of asthma, rhinitis and conjunctivitis provoked by one animal species, permits greater entry of potential allergens from other species at this time, leading to sensitisation.

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