

Quantitative Immuno-electrophoretic Analysis of Rat Allergen Extracts

II. Fur, Urine and Saliva Studied by Crossed Radio-Immuno-electrophoresis

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Many constituents of rat fur, urine and saliva were identified as being allergenic using crossed radio-immuno-electrophoresis with sera from 14 patients who had asthma and rhinitis on exposure to rats. There was considerable diversity in the spectra of components recognised by individual patients, though the majority reacted with immunoprecipitate 4. In all three extracts this represents a similar component, which was originally characterised as a urinary prealbumin. Most of the allergenic components of urine and saliva have also been detected in the fur extract. Some of the minor allergens are those antigens which appear to be unique to urine, saliva or the skin, suggesting that sensitisation to rats can result from exposure to allergenic material from all three of these sources.

Key words: allergens; crossed radio-immuno-electrophoresis; rat fur; rat urine; rat saliva.

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CLINICAL ASPECTS

An extract of either fur, urine or saliva is likely to prove adequate for use in the diagnosis of allergy to rats, as most of the relevant allergens are represented in each of these source materials. Some individuals react with components which are unique to fur, urine and saliva, in addition to some of the shared allergens, so selection of a suitable preparation for immunotherapy is difficult. Multiple sensitivities to several laboratory animal species are common, though patients do not always associate symptoms with species to which they are skin test positive.

Rat allergens may be derived from many sources. These include rat urine, fur, epithelia, saliva, faeces and serum. The allergenic potency of a number of extracts relevant to exposure to rats has been compared using skin prick tests, bronchial provocation, RAST and RAST inhibition (12, 18). It was concluded that the principal allergens probably originate from urine or saliva, and that these source materials act as contaminants of fur and epithelia (18).

Further evidence for the importance of urine and saliva has emerged from studies using quantitative immuno-electrophoretic (QIE) techniques (19). Many urinary and salivary

components were found on rat fur and in dust collected from a room housing rats, although antigenic similarity was also found between constituents of urine and saliva.

Two major allergens from rat urine have been characterised: an α_2 -euglobulin and a prealbumin (8). These urinary constituents were highly skin test reactive; and in crossed radio-immuno-electrophoresis (CRIE), both exhibited high uptake of IgE from sera of rat-sensitive patients. Molecular weights were found to be 15,000 D for the α_2 -euglobulin and 17,000 D for the prealbumin fraction. These allergens fall within the molecular weight range of a rat

urine fraction for which maximal RAST inhibition potency has been demonstrated (7).

The α_2 -euglobulin and prealbumin allergens shared antigenic determinants, and both showed a reaction of partial identity with a single component (antigen 4) in fur and saliva which had an electrophoretic mobility close to or slightly slower than that of albumin (19). This component may be responsible for a peak of skin test reactivity observed at this mobility on electrophoresis of whole rat pelt (13); though albumin does itself possess some allergenic properties (16).

The aim of this study has been to use CRIE with a group of rat-sensitive patients, to further evaluate the importance as allergens of these and other constituents of fur, urine and saliva; and thus determine more precisely the origins of allergenic material.

MATERIAL AND METHODS

Preparation of extracts

Fur, urine and saliva were obtained from 10- to 14-week-old, male Sprague Dawley/Glaxo rats (Glaxo, Harefield, U.K.) as previously described (18).

Fur was clipped off close to the skin; urine was collected with metabolic cages; and saliva was withdrawn from the mouth following stimulation with pilocarpine nitrate.

Fur was extracted for 3 days in Coca's solution. Urine, saliva and extracted fur were filtered, dialysed and freeze dried.

Antisera

Antisera against rat fur, urine, saliva, serum and other extracts were raised in rabbits as detailed elsewhere (19).

Pooled antisera against fur, urine and saliva each contained about 30 serum bleeds from at least five rabbits, and included antisera raised against other cross-reacting source materials if inclusion resulted in visualisation of additional antigen peaks in crossed immunoelectrophoresis (CIE).

Immunoelectrophoretic techniques

CIE was carried out (10, 19) with 1% agarose in 0.03 M veronal buffer (pH 8.65) containing 0.25 mM thiomersal preservative, which was poured 1.13 mm thick on to 8.4×8.4 cm glass slides.

In the first dimension, 40 μ g fur, 50 μ g urine or 25 μ g saliva in normal saline was electrophoresed for 75 min at 4 V cm^{-1} , and then in the second dimension at 1.25 V cm^{-1} for 20 h. An intermediate gel (5.0×0.8 cm) contained anti-rat serum at $12.5 \mu\text{l cm}^{-2}$, or $62.5 \mu\text{l cm}^{-2}$ where urine was the antigen. Antiserum incorporated in the main gel area (5.0×3.5 cm) was $34 \mu\text{l cm}^{-2}$ anti-fur, $31 \mu\text{l cm}^{-2}$ anti-urine or $37 \mu\text{l cm}^{-2}$ anti-saliva.

Slides were washed three times in physiological saline, pressed and air dried. Storage prior to use in CRIE was at 4°C for up to 1 week.

CRIE was performed essentially as described by Løwenstein (11). In order to economise on patients' serum and ^{125}I -labelled anti-IgE, however, incubation was restricted just to that part of the CIE slide which contained immunoprecipitates (5.0×4.5 cm). Wax crayon was applied to the remainder of the gel to render the surface hydrophobic.

Four ml patients' serum diluted 1 in 10 with phosphate buffered saline (PBS) was added to the central area. Following 24 h incubation at room temperature, serum was aspirated and replaced three times with PBS, for 10 min each wash.

Slides were incubated for a further 20 h with 4 ml ^{125}I -labelled anti-IgE (Pharmacia, Uppsala, Sweden) diluted in PBS to give 400,000 cpm for the total volume added.

After washing with PBS as before, slides were dried, and placed under X-ray film (Kodak NS-2T, Eastman Kodak, Rochester, N.Y., U.S.A.) for autoradiography. For each CRIE slide, two autoradiographs were produced, the first was exposed for 7 days, and the second for 2 days.

Slides were stained with Coomassie Brilliant blue following autoradiography.

Patients

All patients recognised symptoms of asthma and rhinitis as being due to exposure to rats, and were positive in skin prick tests and RAST to rat extracts.

RAST for specific IgE was carried out as described previously (6, 18) with rat fur, urine or saliva extracts covalently coupled to Sepharose 4B (Pharmacia).

PRIST. Total serum IgE was determined using the PRIST technique (4) (Phadebas-PRIST®, Pharmacia).

Skin prick tests

Patients were skin prick tested (18) with urine preparations of rats, mice, rabbits and guinea pigs (9). Extracts were tested initially at 0.01 mg ml⁻¹ dry weight in Coca's/glycerol (1/1), and increased up to 1 mg ml⁻¹ if a positive reaction was not elicited. A wheal of 3 mm or greater was arbitrarily considered as positive. The specificity of reactions was verified by testing these extracts in a control group of 17 subjects who were not sensitive to laboratory animals.

RESULTS

CIE reference patterns for the antigens of rat fur, urine and saliva are shown, with serum proteins absorbed out in an intermediate gel (Figs. 1, 2, 3). These patterns have been described more fully elsewhere (19).

Antigens S1 of fur, urine and saliva have been identified as albumin. Peaks 4 and 13 of urine represent the urinary prealbumin and α_2 -euglobulin allergens which have been characterised (8). Antigens 4 of fur and saliva cross-react with the urinary prealbumin and α_2 -euglobulin allergens (but more completely with the prealbumin). None of the other fur, urinary or salivary antigens have been identified, and the same designation in these different systems does not necessarily imply antigenic similarity.

Antigens X of urine, and X and SX of saliva were visible on autoradiography, but not on the stained CRIE slide.

Radiostaining corresponding to specific immunoprecipitates of rat fur, urine and saliva is shown for sera from 14 rat-sensitive patients (Figs. 4, 5, 6); and some individual autoradiographs are illustrated (Figs. 1, 2, 3). Allergograms have been constructed to show total numbers of patients who reacted with each peak for which radiostaining was observed (Fig. 7); and for each antigen, the presence of cross-reactivity with the other two extracts (found previously using QIE techniques (19)), has been indicated.

CRIE with a control serum pool from 12 non-rat allergic patients caused no ¹²⁵I-anti-IgE uptake which was visible over the period of autoradiography tested.

Diffuse radiostaining over the entire antibody-containing gel area caused by the presence of IgE to rabbit serum proteins in the patient's serum (1), was not observed here (even for those patients who were skin test positive to rabbit urine).

Many of the allergic patients were skin prick test positive to other species of laboratory animal in addition to rats, though in some cases these other animals were not recognised as a cause of symptoms (Fig. 4). None of these patients attributed allergic symptoms to exposure to an animal species to which they did not show a positive skin test reaction.

Positive skin test reactions were not elicited when 17 laboratory workers who were not allergic to laboratory animals (though eight were exposed to them, and seven had an atopic history) were tested with urine from rats, mice, rabbits and guinea pigs.

CRIE results were analysed statistically by calculation of Spearman's coefficient of rank correlation. When sera were ranked according to the number of antigens radiostained, a significant correlation ($P < 0.025$) was found comparing CRIE for rat fur, urine and saliva. There was also a significant relationship ($P < 0.025$) between the number of antigens radiostained and RAST score (Figs. 4, 5, 6) with the same extract. No significant correlation was

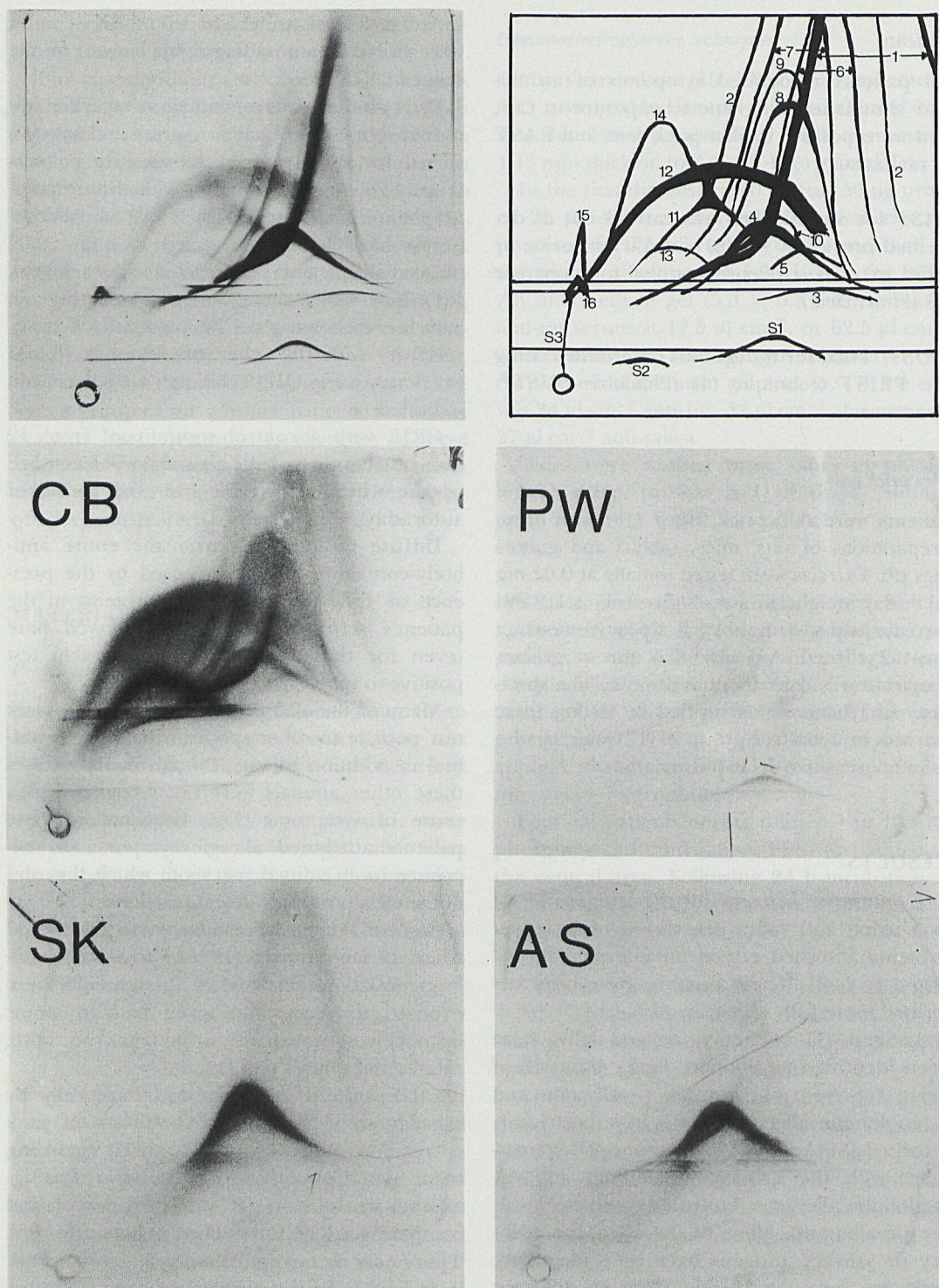


Fig. 1. CIE reference precipitin pattern of rat fur, with line-drawing indicating nomenclature of antigens; and CRIE autoradiographs (after 7 days) for four rat-sensitive patients (C.B., P.W., S.K. and A. B.).

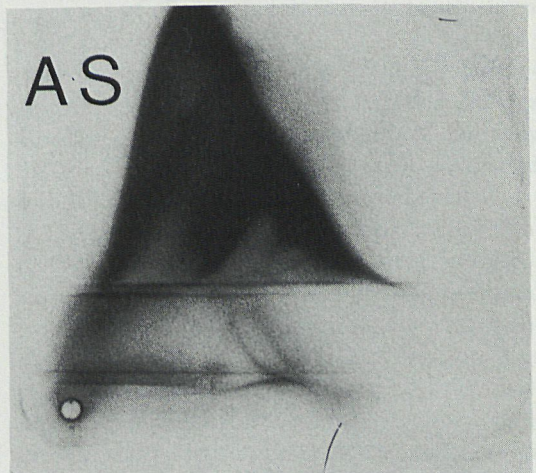
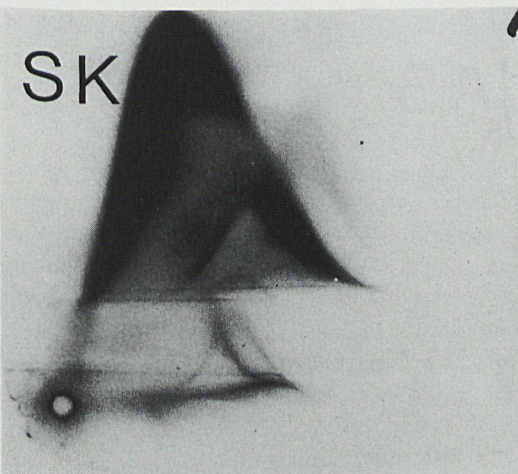
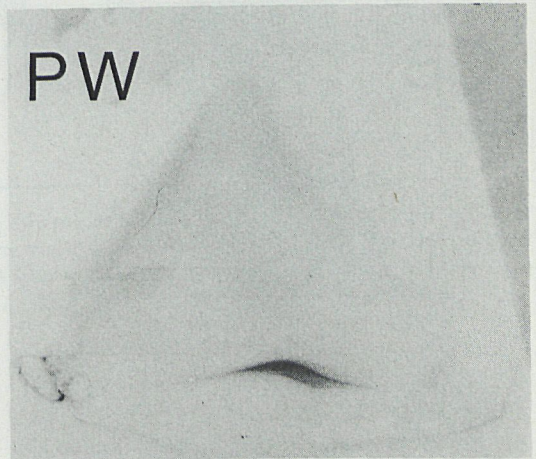
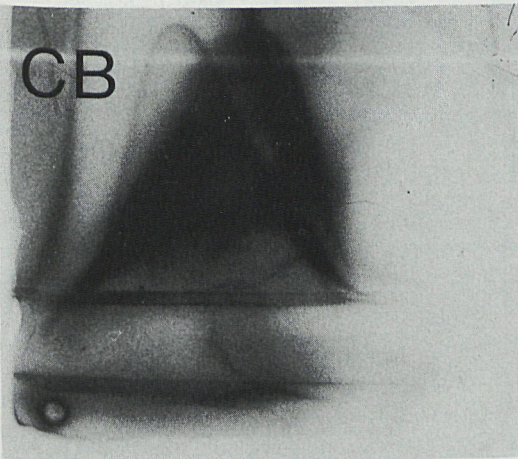
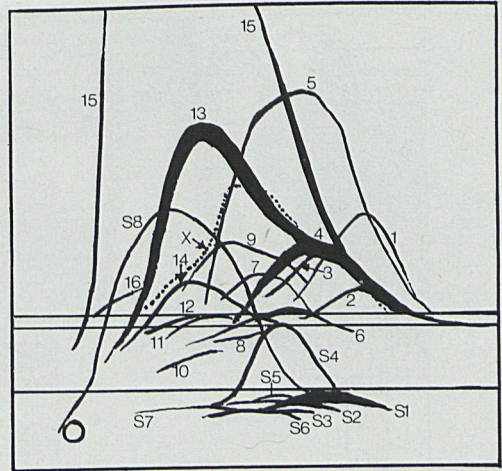
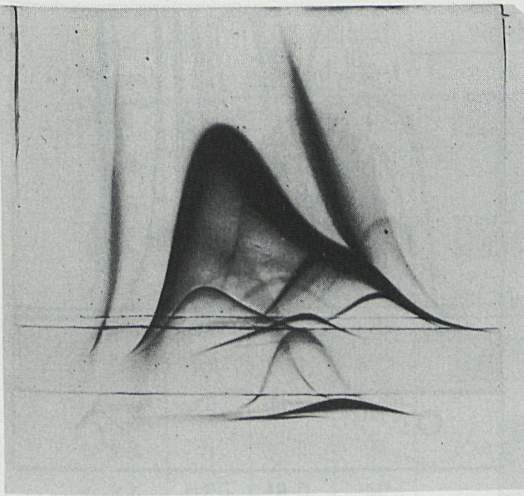


Fig. 2. CIE reference pattern of rat urine, with line-drawing and CRIE autoradiographs for four rat-sensitive patients.

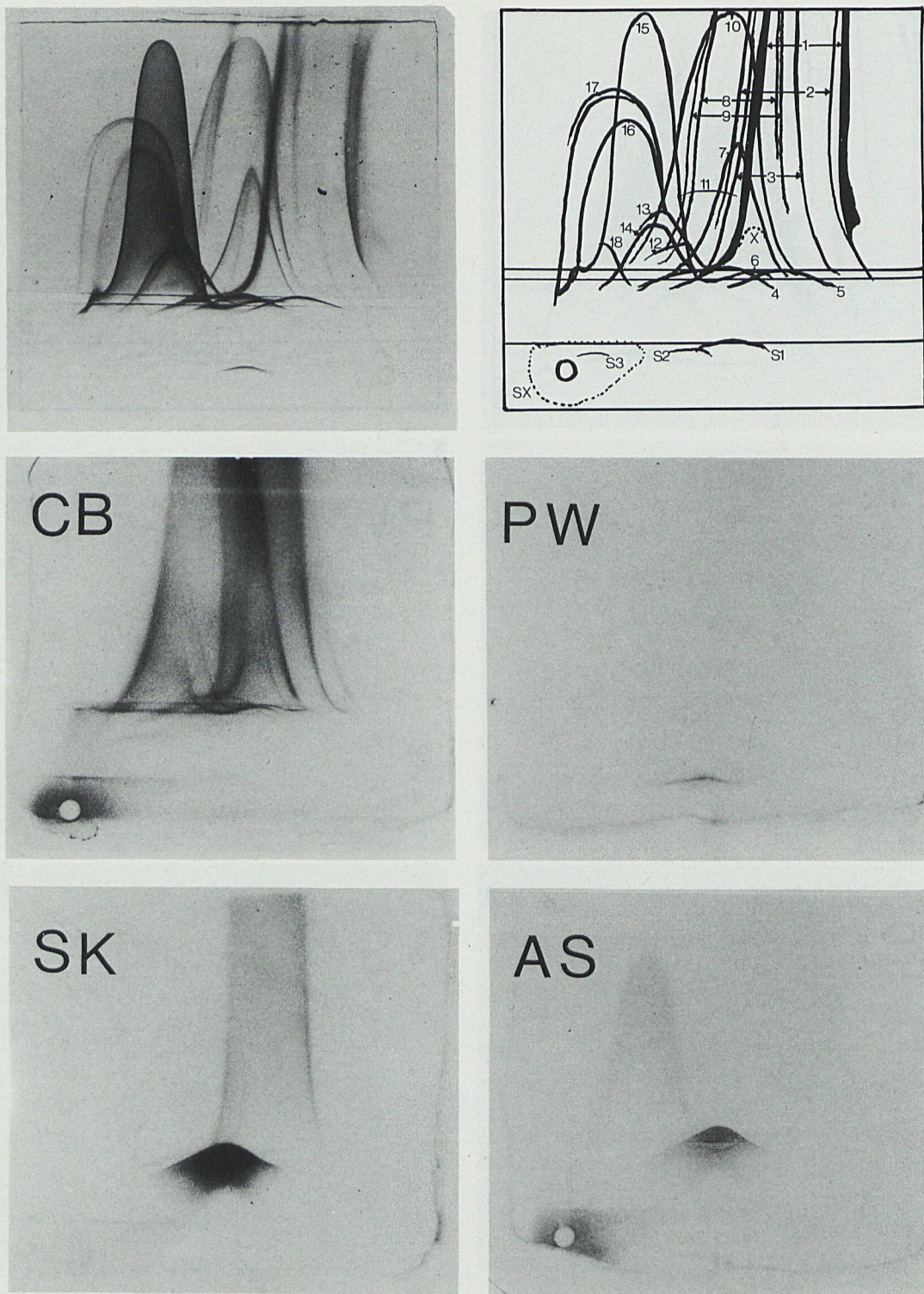


Fig. 3. CIE reference pattern of rat saliva, with line-drawing and CRIE autoradiographs for four rat-sensitive patients.

Patient	Allergy to	RAST % binding	Total IgE μml^{-1}	Radiostaining of fur antigen number														
				1	2	4	5	6	7	8	11	12	13	15	16	S1	S3	
R.B.	M G	35.0	530															
C.B.	M R G	34.9	41															
P.F.	M(R)G	32.2	208															
S.L.	n.t.	27.3	106															
A.C.	M R G	23.8	104															
G.H.	M G	22.7	70															
C.A.	(G)	22.2	570															
P.W.	M(G)	21.2	19.2															
S.K.	M	20.9	32															
P.K.	M R G	19.8	50															
A.S.	M(R)G	19.7	104															
D.A.	(M)(R)(G)	14.3	42															
G.S.	M R G	10.5	70															
J.L.		2.4	52															
Control pool		1.0	200															

Fig. 4. Total IgE, specific IgE to rat fur and CRIE radiostaining corresponding to immunoprecipitates of fur due to individual sera. Hatched squares represent peaks visible by autoradiography after 2 days and dotted squares after 7 days. Skin prick test positivity to mice (M), rabbits (R) and guinea pigs (G) is shown. A letter in parenthesis indicates that the patient did not associate symptoms with that species.

n.t. = not tested.

found, however, between numbers of peaks visible on autoradiography and total IgE levels (Fig. 4).

DISCUSSION

Many antigens of rat-derived extracts are allergenic as determined by radiostaining in CRIE. The allergenic properties shown previously for the rat urinary α_2 -euglobulin and prealbumin allergens (8) and for rat albumin (16), are confirmed here.

In addition to these recognised rat allergens, many other components of fur, urine and saliva appear to bind patients' IgE in CRIE. A few peaks are visible by CRIE but not by Coomassie Brilliant blue protein staining in CIE. Their presence may be ascribed to the greater sensitivity of the radiostaining technique (3).

Though many allergens appear to be involved, there is considerable diversity in the spectrum of allergenic components to which an individual patient reacts.

Those sera with high RAST values for specific IgE, also show radiostaining to a greater number of antigenic components. A tendency for the degree of IgE uptake to be associated with RAST score should be expected (11).

The absence of any relationship between total IgE levels and uptake of ^{125}I -anti-IgE in CRIE, and the finding that no radiostaining occurred with pooled control sera, suggests that non-specific binding of IgE was insignificant. The possibility that results were affected by the binding of human serum IgE specific for rabbit immunoglobulins rather than the rat allergen, seems unlikely. Some dominant immunoprecipitates did not exhibit radiostaining in any of the

	RAST % binding	Radiostaining of urine antigen number																		
Patient		1	2	3	4	5	6	7	9	13	15	16	X	S1	S3	S7	S5	S6	S7	S8
R.B.	40.2																			
C.B.	37.6																			
P.F.	32.3																			
S.L.	30.1																			
A.C.	28.0																			
G.H.	29.0																			
C.A.	23.8																			
P.W.	21.2																			
S.K.	27.1																			
P.K.	18.0																			
A.S.	27.5																			
D.A.	24.3																			
G.S.	12.8																			
J. L.	8.1																			
Control pool	1.9																			

Fig. 5. RAST for IgE to rat urine, and radiostaining of rat urinary antigens. Hatched squares represent peaks visible after 2 days autoradiography, and dotted squares, after 7 days.

CRIE autoradiographs. Moreover, diffuse radiostaining over the whole gel area reported to be typical of sensitivity to rabbit serum (1) was not seen in this study.

Several of the patients were skin test positive to rabbit urine (Fig. 4) and this extract has been shown by QIE to contain trace amounts of rabbit serum proteins (unpublished observation). Although sensitive to rabbits, it is improbable that relevant allergens for these patients included rabbit serum, or at least rabbit IgG. In an investigation by Ohman and colleagues, positive skin test reactions to rabbit serum were not elicited in seven patients with a history of sensitivity to rabbits, though all responded with an extract of pelt (13).

There was no clear association between radiostaining of rat antigens and allergy to other laboratory animals. Binding of patients' IgE to specific peaks is unlikely to be related to their allergy to a certain other species of experimental animal, in view of the apparent lack of

cross-reactivity between extracts of these species. In previous studies with these rat fur, urine and saliva extracts, antigenic similarities with the corresponding materials from guinea pigs were not observed using QIE procedures (19), nor was the IgE RAST for rat room dust inhibited by preincubation with dust from a guinea pig room (18). Similar findings have emerged using intermediate gel CIE and RAST inhibition with urines from rats, guinea pigs and mice (9), and the RAST for IgE specific for rat urine has not been found to be inhibited with rabbit urine (unpublished observation).

Possible reasons for multiple sensitivities to laboratory animals have been discussed elsewhere (19). It was found in this, and in another study (5), that certain allergic patients gave positive skin test reactions to species which they did not implicate as a cause of their symptoms. It is improbable that this is due to an artifact in skin testing in view of the lack of positive

	RAST % binding	Radiostaining of saliva antigen number												
Patient		1	2	3	4	10	11	13	16	X	S1	S3	SX	
R.B.	39.3		■	■	■	■			■	■	■			
C.B.	32.2		■	■	■	■				■			■	
P.F.	28.9	■		■	■									
S.L.	22.2	■	■		■			■		■				
A.C.	24.4												■	
G.H.	18.4	■	■	■	■						■			
C.A.	24.7	■			■	■	■	■		■	■	■		
P.W.	18.1										■			
S.K.	21.0			■	■	■								
P.K.	16.8			■	■	■	■		■		■			
A.S.	20.7			■	■	■			■				■	
D.A.	17.5	■		■	■	■			■					
G.S.	11.1		■	■	■	■	■		■					
J.L.	3.8				■									
Control pool	1.2													

Fig. 6. RAST for IgE to rat saliva, and radiostaining of rat salivary antigens. Hatched squares represent peaks visible after 2 days autoradiography and dotted squares, after 7 days.

reactions elicited in asymptomatic subjects. Moreover, RAST and RAST inhibition with sera from such patients has indicated that they possess specific IgE for the animal extract to which they are skin test positive (unpublished observation). Working in an animal house is likely to entail exposure (even indirectly) to several species of laboratory animals, and in such conditions, one should not expect a precise identification of those animals responsible for symptoms. There may also be a time lag between first elicitation of positive skin tests, and recognition by the patient that allergic symptoms are resulting from a particular animal species.

The diversity of CRIE patterns is likely to result from genetic differences between patients. The degree, nature and time course of exposure to rat antigens is also likely to be of relevance, but these parameters are difficult to measure in an objective way retrospectively.

For CRIE, as with RAST (18), there was a

clear relationship between rat fur, urine and saliva in uptake of ^{125}I -anti-IgE. Patients recognising many allergens in one rat source material, were also likely to do so in another. This must be on account of the antigenic similarities between these extracts (19).

CRIE should be considered as a qualitative or semiquantitative technique (11). It is not possible, therefore, to compare directly these three series of CRIE experiments, and one should expect discrepancies in total numbers of patients reacting with a similar antigen in each CRIE series.

Some of the difficulties involved in comparison of CRIE systems are illustrated by the radiostaining of rat albumin (antigen S1) where the disparity in numbers of sera which show binding is particularly evident. For, whereas all 14 allergic sera caused some degree of radiostaining of the albumin of fur and urine, for salivary albumin, only five of the sera did so.

QIE analysis of cross-reactivity between ex-

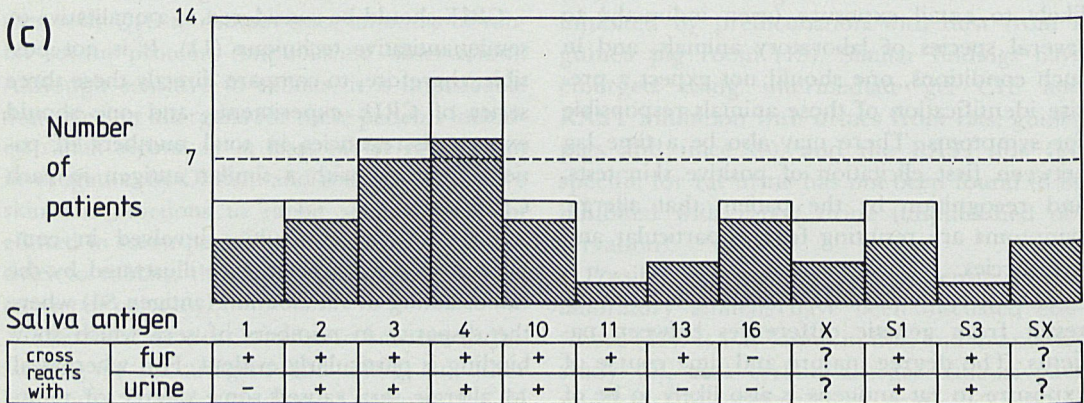
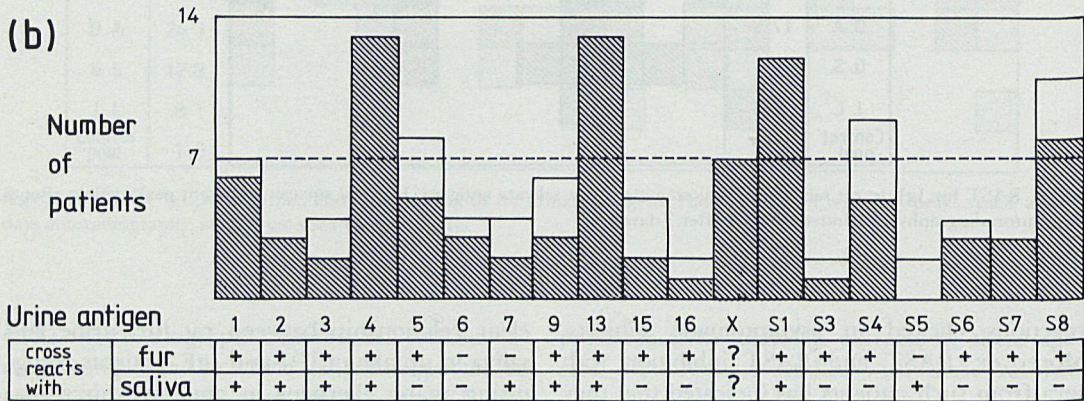
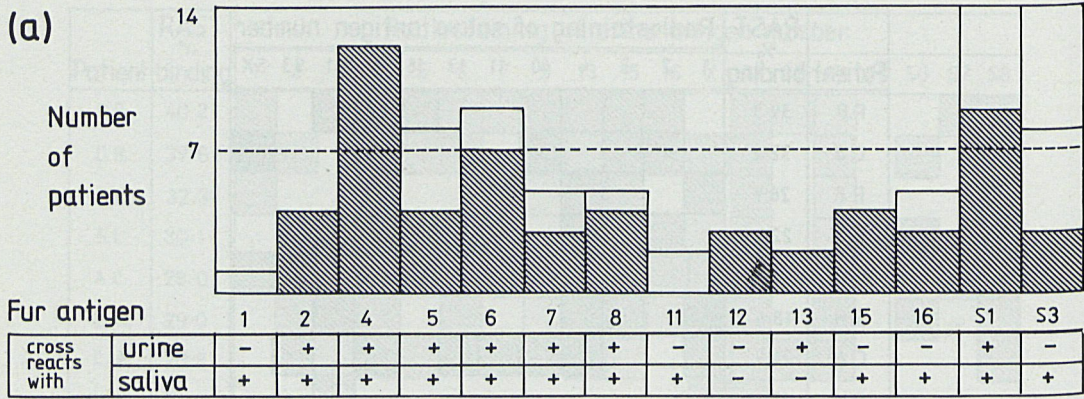


Fig. 7. Numbers of patients who reacted with each antigenic component of (a) fur, (b) urine and (c) saliva. Hatched bars represent patients for whom CRIE binding was present after 2 days autoradiography, and open bars, after 7 days. The presence of cross-reactivity with the other two extracts is shown for each antigen.

tracts has not revealed antigenic dissimilarity between rat albumin from these three sources (19), so such differences are unlikely to cause different levels of IgE binding. Another factor which should be considered is a possible difference in the ^{125}I -anti-IgE between each CRIE series. Had the period of autoradiography been increased, more sera may have shown radio-staining of rat salivary albumin.

Much of the radiostaining of rat albumin, however, may be due to the trapping of other allergenic molecules in the precipitate, a process described in another allergen system (2). The position of albumin in the intermediate gel renders it particularly susceptible to this process of co-precipitation (especially with antigen 4). Different degrees of co-precipitation are likely to occur with these three rat extracts.

The CRIE series for fur and urine almost certainly exaggerate the relevance of albumin as an allergen. Wahn and colleagues (16) found rat serum albumin to cause histamine release from basophils of only eight out of 33 rat-allergic patients. Moreover, the low skin test reactivity of an albumin-rich fraction of rat urine (8), and of certain other extracts with high albumin levels (19), must mean that this component is of minor significance only. Nevertheless, it may play an important role in the allergic response of certain rat-sensitive individuals: one patient (P.W.) reacted strongly to albumin, but to little else.

Antigen 4, the urinary prealbumin allergen, or its analogue in fur or saliva, emerges clearly as a major allergen. In CRIE, for all three source materials, the majority of patients reacted with this antigen, and the position of the immunoprecipitates makes them less susceptible to the problem of co-precipitation.

These CRIE studies are in accord with previous work which showed that the purified urinary prealbumin allergen had high potency in skin tests (8), and that relative skin reactivity of a wide range of rat-derived extracts correlated with the amount of this allergen present (19). All patients reacting with the urinary prealbumin allergen also reacted with the partially identical α_2 -euglobulin (urine antigen 13) thus indicating that the major allergenic sites of antigen 4 are shared by antigen 13.

The fur extract contained antigens which were otherwise specific to saliva or urine, suggesting contamination with both of these source materials, but the absence of certain salivary and urinary components from fur has been taken as evidence for chemical modification or degradation (19). Nonetheless, most allergenic components of saliva and urine detected by CRIE were antigens which were also present in fur. This probably reflects the stability of these antigens on fur (and possibly on other materials as well) and hence their abundance in the environment.

Though of less significance in provocation of allergic reactions, it is the allergens unique to a particular extract which can provide an indication of the most relevant sources of allergenic material. Caution should be exercised in interpretation of the results of QIE analysis for the rat-derived extracts, for reasons discussed previously (19), but assuming their validity, then CRIE demonstrates that there are allergens which are present in urine (e.g. urinary antigen 6) and in saliva (salivary antigen 11) but which are not represented in fur. This suggests that these antigens may have become airborne without prior adherence to fur, or at least very soon afterwards; or perhaps sensitisation can result from bites and scratches.

There are also allergenic components which are present in both urine and fur, but not in saliva, and which are in saliva and fur, but not urine. In addition there is a fur allergen (antigen 12) which is not found in urine or saliva, but which seems to originate from the skin (19).

The allergens responsible for sensitisation to rats therefore appear to originate from urine, saliva and the skin; though most fur allergens probably represent contamination with saliva and urine.

Those allergenic constituents unique to either fur, urine or saliva are all of minor significance, and all patients studied have reacted to components of all three major source materials. This accords with RAST and skin test results which have shown the same trend (18). Thus, for diagnostic purposes, any of these materials in a sufficiently high concentration is likely to prove adequate.

With immunotherapy the situation is more complex, as any preparation containing all known rat allergens would have to contain a mixture of fur, urine and saliva. Nevertheless, some success has been reported using a crude rat pelt preparation in hyposensitisation (17).

Preparing a purified rat allergenic extract for hyposensitisation presents difficulties on account of the diversity in allergic responses. Ideally, the preparation should be tailored to the individual patient (14, 15), but such an approach is unlikely to be feasible on economic grounds. An extract for general use, however, clearly ought to contain rat antigen 4.

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