

Allergy to rabbits

II. Identification and characterization of a major rabbit allergen

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Quantitative immunoelectrophoretic techniques have been used to study the antigenic components found in extracts of dust collected from rabbit housing areas. To determine the possible source of these antigens, comparisons have been made to rabbit saliva, urine, fur and dander. Specific antisera for the rabbit extracts were raised in guinea pigs. One major component of the dust (Ag R1) was also found in large amounts in saliva, slightly less in fur and in only minimal amounts in urine and dander. Crossed radioimmunoelectrophoresis (XRIE) of the dust, performed with sera from 14 rabbit allergic individuals who were RAST positive to rabbit saliva, urine and dust identified four IgE-binding constituents. Individual responses varied but all sera reacted with Ag R1, identifying this as a major rabbit allergen. Dust RAST inhibition studies with rabbit dust, saliva and urine indicated saliva to be closely related to the dust. Ag R1 is a glycoprotein which appears to be very heterogeneous in nature. It produced a broad biphasic precipitin peak on immunoelectrophoresis and eluted from Sephacryl S-200 gel filtration over the molecular weight range 30-50 Kd, although a molecular weight of 17 Kd was indicated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gradient gel electrophoresis. The RAST inhibition results and the antigenic similarity of saliva to the dust suggest this to be the most likely source of the major rabbit allergen, Ag R1.

Key words: allergen characterization; crossed radioimmunoelectrophoresis (XRIE); rabbit allergens; radioallergosorbent test (RAST).

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CLINICAL ASPECTS

A major allergen with importance for rabbit allergic patients has been characterized. This allergen constituted the greater proportion of rabbit saliva, fur and dust extracts but was present only in small amounts in extracts of urine and dander. This suggests that the latter, although commonly used, may not be the most appropriate materials for use in diagnostic tests for allergy to rabbits.

In studies to investigate laboratory animal allergy, particularly those concerned with the identification of the major allergens of these animals, much work has been reported for the rat, guinea pig and mouse (5, 11, 12, 14, 15) but little information is available for the rabbit. A study to characterize allergens from the pelts of all four animals (9) reported that, for a group of seven rabbit-sensitive individuals, maximum allergenic skin test reactivity from rabbit pelt

extracts was produced by a protein(s) with a molecular weight between 18 and 38 Kd, and that no reactions were found to rabbit serum.

Probably as a result of such lack of information, studies involving subjects allergic to rabbits have varied as to the source of allergens for both *in vivo* and *in vitro* testing. Some have used urine preparations (1, 2) as had been described for the rat (5), whilst others have used whole pelt (13) or dander extract (8).

The aims of this study have been to investigate the inhalant allergens associated with exposure to rabbits and to determine their sources such that a more consistent preparation can be used for diagnosis of sensitized individuals. Therefore, by studying the antigenic/allergenic content of rabbit dust, which can be considered as more representative of the spectrum of material present in the air than a single source extract, it was hoped that the major rabbit allergen(s) would be identified and their origin(s) determined.

MATERIAL AND METHODS

Dust was collected from surfaces between 3 and 8 feet above the floor in rabbit housing areas (New Zealand White and New Zealand White/Dutch Lop cross). Saliva was obtained by stimulation with pilocarpine nitrate (0.5 ml of a 50 mg/ml solution), injected subcutaneously. Fur was shaved from several rabbits (as above) and used to make a mixed extract. Dander (epithelial scales) was scraped from the shaved pelts with a razor blade after freezing the pelts on a metal surface placed on dry ice. Urine was collected daily and stored at -20°C until sufficient volumes were obtained from rabbits (of varying ages) placed in metabolic cages. Normal rabbit serum was obtained from 8–10-week-old rabbits prior to any immunisation.

All solid materials (including unused food and bedding) were extracted in Coca's solution (pH 7.2) for 48 h. The resulting extracts, and the liquid source materials, were filtered (0.45 μ pore size, Oxoid, London), dialysed extensively against distilled water, and freeze-dried.

Dunkin-Hartley guinea pigs were immunised with whole rabbit dust, saliva, fur, urine and dander extracts. All extracts (25 mg in 1.5 ml distilled water) were emulsified in Freund's Complete Adjuvant and injected (1 ml/guinea pig) into four dorsal, subcutaneous sites. This was repeated twice at 4-week intervals and then the guinea pigs bled by heart puncture. For each extract several bleeds were pooled to provide a reference antisera, which

was then tested by XIE for antibody titre. Swine anti-rabbit serum was obtained commercially (Dakopatts, High Wycombe, UK).

Sera were obtained from rabbit allergic individuals who associated symptoms of asthma and/or rhinitis with exposure to rabbits. Sera from non-atopic subjects were used as controls.

Crossed immunoelectrophoresis (XIE) was performed as described previously (5). Agarose gels (1%) (BDH, Poole, UK) were poured onto 9×9 cm glass plates (Photoscience, Canvey Island, UK) and electrophoresis of the freeze-dried extract (10 mg/ml in 0.9% NaCl/0.5% NaN_3) was carried out in the first dimension at 4V/cm for 75 min. Electrophoresis in the second dimension was carried out overnight at 1V/cm through an intermediate gel (0.8×5 cm) containing anti-rabbit serum (and where necessary through a second intermediate gel of the same size containing another antiserum) into the main gel area (3×5 cm). These antiserum-containing intermediate gels were incorporated to identify, within the different extracts, components which were antigenically related, e.g. anti-rabbit serum to identify serum-related components.

Crossed radioimmuno-electrophoresis (XRIE) was performed as described previously (16) using a rabbit dust XIE profile as template. Dried plates were incubated overnight with 3 ml of 10% serum from rabbit allergic individuals, diluted in phosphate buffered saline (PBS; pH 7.2) and then, after washing in PBS with 0.05% Tween (BDH, Poole, UK), another overnight incubation with 3 ml Phadebas ^{125}I -rabbit-anti-human IgE (Pharmacia, Uppsala, Sweden) diluted in PBS with 0.3% human serum albumin (HSA) (300,000 cpm per plate). After further washing the dried XIE plates were exposed to X-ray film (DEF-2 Eastman-Kodak Ltd., Hemel Hempstead, Herts, UK) initially for 7 days and then repeated for 2 days to produce two autoradiographs for each serum tested. Radioactive uptake on each antigen peak was graded (by two independent observers) for each serum, as

strong: dense radiostaining after 2 days, moderate: weak radiostaining after 2 days and weak: radiostaining visible only after 7 days exposure.

Rocket immunoelectrophoresis. To detect and quantify the amount of major rabbit dust antigen (Ag R1) in different rabbit extracts the extracts were electrophoresed (1V/cm) overnight directly into a gel containing guinea pig anti-AgR1 and the heights of the rockets compared with those of known concentrations of partially purified Ag R1, obtained by pooling and freeze drying fractions 60–75 from the gel filtration of rabbit dust.

Radioallergosorbent test (RAST) for the determination of IgE levels specific for rabbit dust, saliva and urine was carried out as described elsewhere (15). The extracts were coupled to cyanogen bromide activated Sepharose 4B (10 mg/g) (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The resulting Antigen Particle Complexes (APC) were stored at a stock concentration of 25 % in PBS/0.3 % HSA/0.02 % NaN_3 , at 4°C.

The APC was titrated separately for each extract to determine the optimum concentration at which there was good separation in the uptake of radioactivity between sera from known allergic subjects and sera from non-atopic individuals.

For the assays, with all extracts, 100 μl of 2.5 % APC was incubated with 50 μl of serum (in duplicate) and shaken at room temperature overnight. Each tube was washed with 3 ml of PBS/0.05 % Tween 20 three times with centrifuging (500 g for 10 min). Fifty μl of ^{125}I -rabbit anti-human IgE (Pharmacia) was added to each tube, again shaken overnight, washed (six times) as previously and then counted for 2 min on a gamma counter. Results were expressed as a percentage binding of the ^{125}I -anti-IgE added.

RAST inhibition. Fifty μl aliquots of pooled sera from allergic individuals or from non-atopic control individuals, were added to 100 μl of increasing concentrations of inhibiting

extract in PBS (in duplicate) and incubated, with shaking, at room temperature for 6 h. 100 μl of APC were added to each tube and the RAST procedure performed as above.

The percentage inhibition was calculated as:

$$\frac{(A-C) - (a-c)}{(A-C)} \times 100$$

where A and C were the counts per minute for allergic and control sera respectively without the addition of inhibiting extract, and a and c were the counts per minute for the allergic and control sera with inhibiting extract added.

Gel filtration. A 90 $\times$ 2.6 cm column packed with Sephacryl S-200 (Pharmacia) was equilibrated in 0.5M NH_4HCO_3 pH 8 (eluting buffer) and run at a flow rate of 2 ml/min. Calibration was with thyroglobulin (660 Kd), catalase (232 Kd), bovine serum albumin (67 Kd), chymotrypsinogen A (25 Kd) and vitamin B12 (1.3 Kd).

Rabbit dust and saliva extracts, 50 mg in 5 ml of eluting buffer, were applied to the column, 4 ml fractions were collected and absorbance measured at 280 nm. The molecular weights of the components present in the rabbit extracts were determined using a standard curve of elution coefficient vs. $\log_{10}$ molecular weight of the markers. The separation was monitored by fused rocket immunoelectrophoresis (3) using the guinea pig anti-rabbit dust or anti-rabbit saliva.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis (4) was performed in 20 % acrylamide separating gel in 0.125M Tris (BDH Poole, UK) pH 8.8 containing 0.1 % SDS. Samples were made up in 0.375M Tris/HCl buffer (pH 6.8) and reduced samples were treated with beta-mercaptoethanol (Sigma, Poole, UK) at 100°C for 10 min.

Gels were stained for protein with Coomassie Blue R250 (0.125 % in 95 % ethanol:10 % acetic acid, 1:1), or carbohydrate using periodic acid/Schiff's (PAS) reagent (BDH, Poole, UK). Standard low molecular

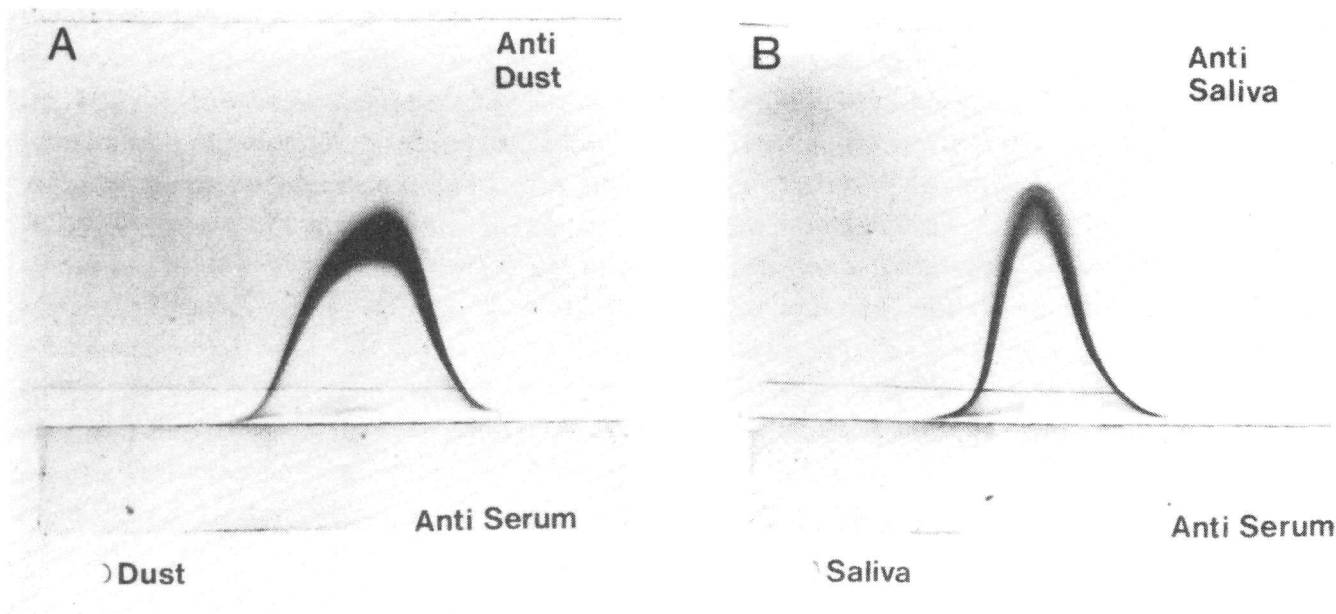


Fig. 1. A) XIE of rabbit dust (30 μ g) against anti-rabbit dust (6.5 μ l/cm²) with an intermediate gel of anti-rabbit serum (12.5 μ l/cm²). B) XIE of rabbit saliva (20 μ g) against anti-rabbit saliva (6.5 μ l/cm²) with an intermediate gel as in Fig. 1A. Ag R1 is the visible peak in both profiles.

weight markers (LMW, Pharmacia) were used for calibration.

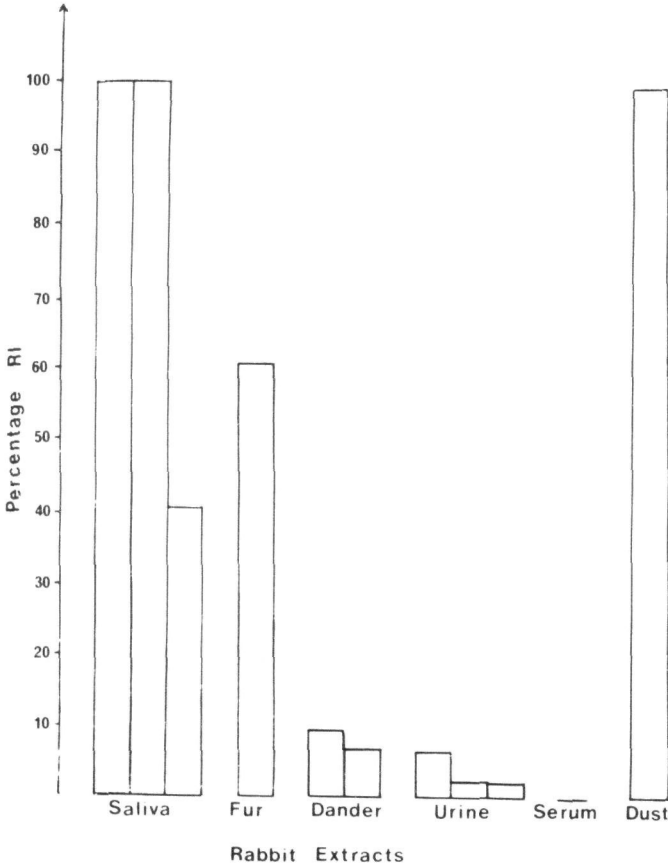


Fig. 2. Percentage of Ag R1 present in rabbit extracts as determined by rocket immunoelectrophoresis.

RESULTS

XIE

On XIE of three different extracts of rabbit dust against guinea pig anti-rabbit dust, one major component Ag R1, which produced a broad, biphasic precipitin peak, and several minor components were detected (Fig. 1a). A noticeable similarity was observed between the XIE profiles of dust and saliva (Fig. 1b) with dense protein staining of the antigen R1 peak, although with saliva it appeared as a more symmetrical narrow peak without the biphasic nature as occurred with the dust. The presence of Ag R1 in saliva, fur, dander and urine was demonstrated by the partial absorption of this precipitin peak by antisera raised to these four extracts (not shown). In tandem XIE against anti-dust a reaction of identity was observed between the saliva and dust components. No reactions were obtained with any of the antisera to the rabbit-derived mate-

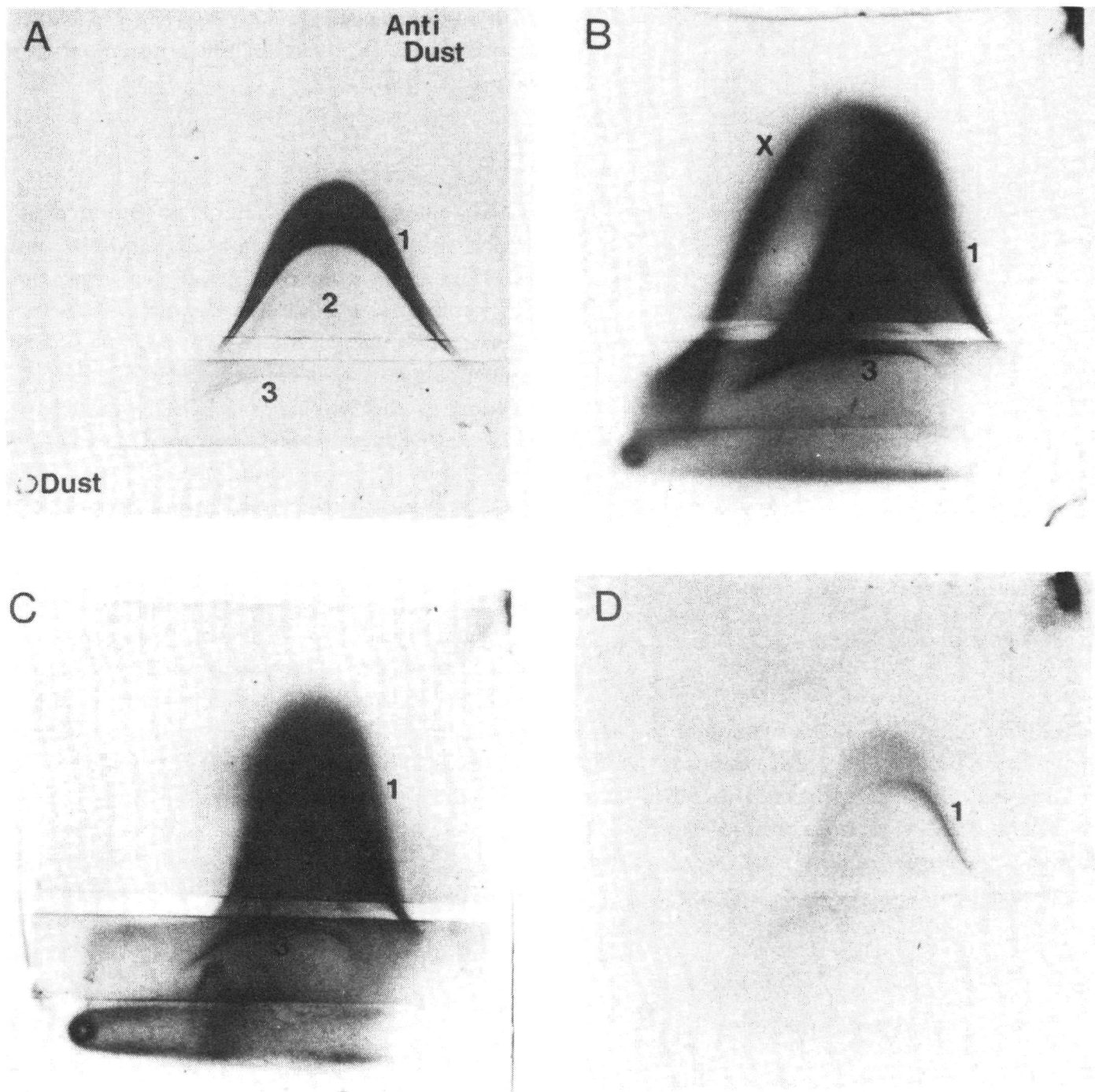


Fig. 3. A) XIE precipitin pattern for rabbit dust/anti-rabbit dust with an intermediate gel containing anti-rabbit serum (antigen peaks numbered arbitrarily from the anode).

B-D) XRIE autoradiographs for three rabbit allergic patients. Antigen X is indicated in Fig. 3B.

rials with the extracts of unused food or bedding.

Rocket immunoelectrophoresis of rabbit extracts were compared with doubling dilutions of the partially purified preparation of Ag R1 as standard. The extract of dust and two extracts of saliva contained almost 100 % Ag R1 (Fig. 2) whilst fur contained 60 %, dander 9 %, and urine 6 %. There were no detectable levels of Ag R1 in serum.

XRIE

In XRIE tests for 14 rabbit allergic sera four allergenic dust components were recognised, i.e. precipitin peaks 1,2,3 and X (X not visualised by Coomassie blue staining of the XIE template) (Fig. 3). Autoradiographs from three rabbit allergic subjects are illustrated (Fig. 3b-d). From the XRIE results (as determined from the degree

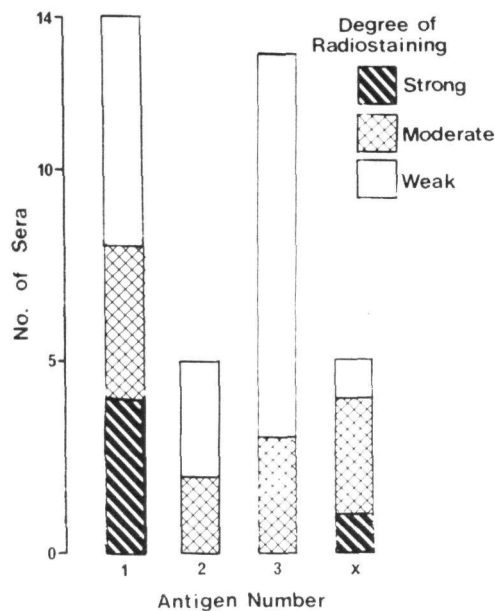


Fig. 4. XRIE radiostaining of rabbit dust antigens with sera from 14 rabbit-allergic individuals. Radioactive uptake for each antigen was graded as strong, moderate, and weak, as described in Methods.

of radiostaining seen on each antigen for each serum after both 2 and 7 days exposure of the autoradiographs – see Material and Methods) an allergogram has been compiled (Fig. 4) showing the total numbers of sera reacting with each antigen. All sera reacted strongly

with antigen peak 1, i.e. Ag R1. No radio-staining was observed on the autoradiographs of control sera.

RAST

RAST values for specific IgE to rabbit dust, saliva, urine, serum and serum albumin, are given in Table 1 for the 14 rabbit allergic and five control sera. Although direct comparisons of the different APC are not possible, these results suggest a decrease in % binding in relation to the amount of Ag R1 determined for each extract. There was good correlation ($P < 0.001$) between the RAST values for dust, saliva and urine. Also using Spearman's Rank Correlation these RAST values correlated ($P < 0.01$) with the number of antigens and the degree of radiostaining seen on those antigens in XRIE. (The scoring system for each antigen being: strong staining = 3; moderate = 2; weak = 1 and the total score for each serum was correlated with the single extract RAST value.)

Specific IgE to serum albumin and components of whole serum was only detected in those sera with RAST values $> 10\%$ to the other extracts.

Table 1
Results of RAST to rabbit extracts in a group of rabbit-sensitive individuals

Patient	RAST % binding				
	Dust	Saliva	Urine	Serum	Serum albumin
RS	30.3	43.2	34.6	4.8	5.6
AB	26.5	31.4	25.0	12.8	12.2
ZN	26.3	22.8	23.6	4.6	6.5
LP	23.5	38.6	31.6	3.0	15.9
NZ	18.9	22.9	29.2	4.5	6.0
IL	18.0	16.4	20.8	3.8	6.0
GS	14.6	12.4	14.8	0.7	0.4
AC	10.1	9.6	10.6	1.2	0.9
RA	4.0	1.0	7.7	0.7	0.5
AW	3.5	1.0	6.0	0.9	0.5
BC	3.3	2.6	8.4	0.8	0.4
TB	2.7	1.4	5.8	0.9	0.7
AF	2.6	1.3	6.3	2.8	2.0
RF	0.7	1.6	3.0	0.5	0.3
*	0.508	0.344	0.668	0.404	0.313

*5 control sera. Mean + 2 SD

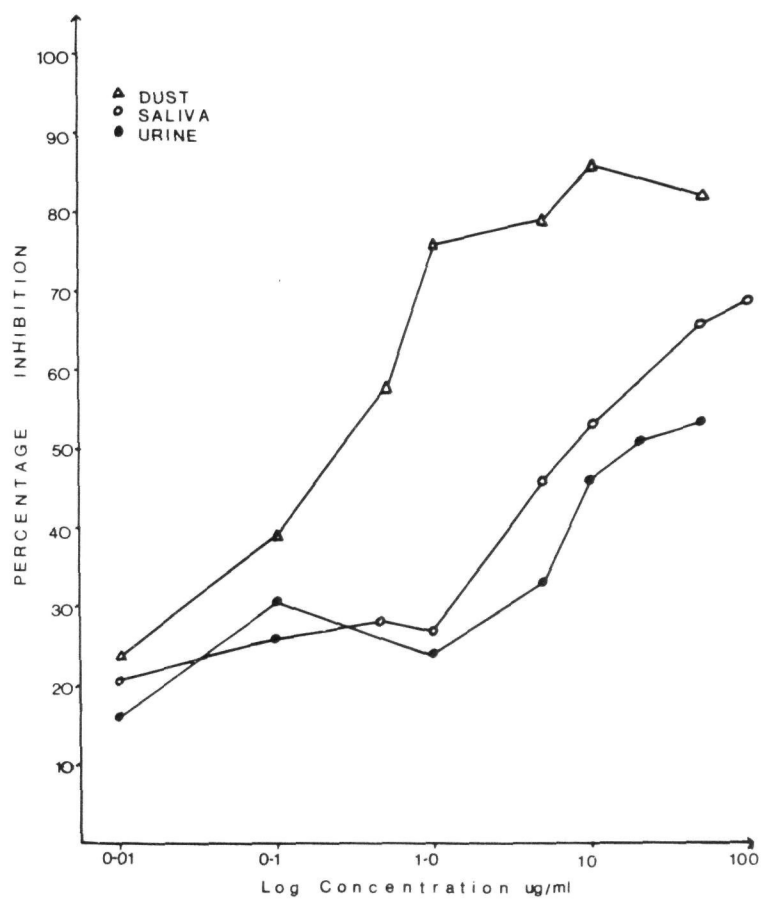


Fig. 5. Inhibition of IgE RAST for rabbit dust with the homologous dust extract and the heterologous saliva and urine extracts.

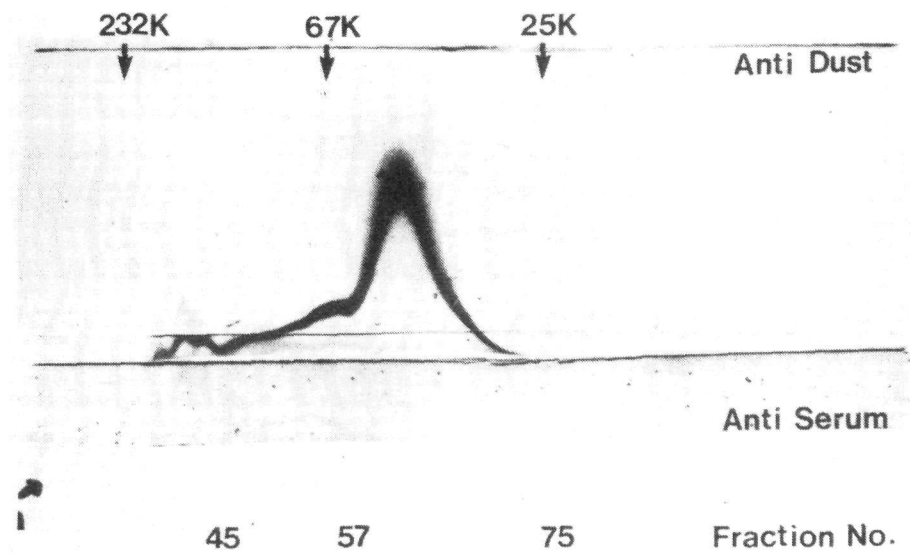


Fig. 6. FRIE of rabbit dust fractionation on Sephacryl S-200. The elution on standard molecular weight markers is indicated.

RAST inhibition

The inhibition by dust, saliva and urine of a dust RAST is shown in Fig. 5. On a

dry weight basis the inhibition curve produced by saliva more closely paralleled that of dust self-inhibition than did that of urine, a further indication for higher levels of the

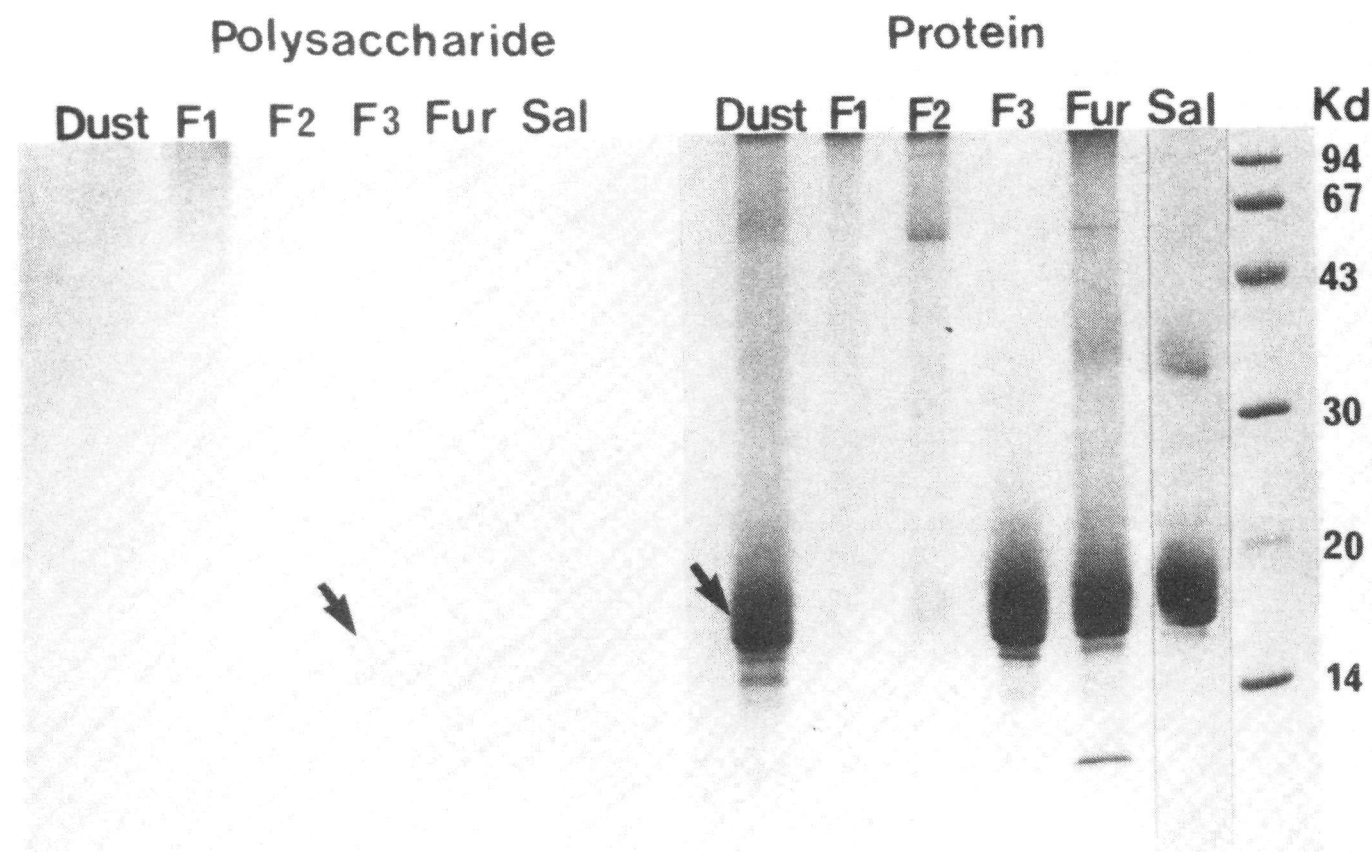


Fig. 7. SDS-PAGE of rabbit extracts and Sephacryl S-200 column fractions of dust stained for polysaccharide and protein. Weak PAS staining of Ag R1 is indicated with its molecular weight of 17 kD compared with standard molecular weight markers.

major allergen Ag R1 in saliva compared to urine.

Gel filtration

The molecular weight of the main Ag R1 peak estimated from its K_{av} value (calculated using the fused rocket immunoelectrophoresis profile of the eluted fractions of dust, Fig. 6; or saliva, not shown) and calibration curve, ranged between 30–50 Kd. However, as the antigenic peak also began eluting in earlier fractions it may exist in higher molecular weight forms. Fractions 60–75 were combined and freeze dried for use as a partially purified preparation of Ag R1.

SDS-PAGE

The electrophoretic profiles of various rabbit extracts and Sephacryl S200 column fractions of rabbit dust (F1 = fraction 39–45, F2 = 46–58 and F3 = 60–75) compared with the molecular weight markers indicated a molecular weight of 17 Kd for

Ag R1 (Fig. 7). Although not shown here, results obtained in the absence of SDS on gradient gels (PAA 4/30 Pharmacia) agreed with this value.

Staining of a similar gel with Schiff's (PAS) stain (Fig. 7) showed weak staining in the area associated with Ag R1, indicating its glycoprotein nature.

DISCUSSION

Extracts of dust obtained from rabbit housing areas were used as sources of rabbit-derived inhalant antigens. On XIE one dust component, Ag R1, which proved to be the main constituent of the dust, was also recognised as being the main component of saliva, present to some extent in the fur extract and in small quantities in extracts of urine and dander, but not detectable in serum.

All the rabbit dust antigens investigated appeared to be derived from the rabbits themselves as extracts of food and bedding did not contain components recognised by the guinea

pig anti-rabbit dust. Although components of these materials are likely to be present in any sample of dust, none were recognised as being antigenic by the guinea pigs.

By XRIE four dust antigens were recognised as being allergenic, but only the antigen R1 was identified as a major allergen as defined by this test method (6, 7). One of the four allergens (antigen X) was visualised only by autoradiography. A variety of patient responses were obtained to these antigens and the absence of radiostaining on the control autoradiographs indicated the specificity of these results.

There was good correlation between the RASTs for dust, saliva and urine and therefore all these RAST values correlated with the number of antigens recognised by specific IgE taking into account the degree of radiostaining seen on those antigens in the dust XRIE. However, the dust RAST inhibition studies showed that although saliva more closely paralleled the dust self-inhibition than did urine on a weight basis, it was 10–30 times less potent. This may be explained both by the slight difference in the immunological nature of the saliva and dust components as noted in their XIE profiles, and also by the additional components visualized in the dust extract on XRIE. Fur extracts also contained high levels of Ag R1. These results suggest that saliva, deposited on the fur during grooming, becomes airborne on drying, and is therefore the probable source of Ag R1 in dust.

As urine and dander contained much lower amounts of Ag R1 than dust and saliva it might be expected that they would also be less allergenic. However, the urine RAST produced a range of values which correlated significantly with those of dust. Edwards et al. (2) evaluated the use of RAST with the urines of all four laboratory animals and found that although the rabbit RAST produced significant results there were problems of non-specificity with some sera which contained IgG antibodies to rabbit IgG. This was caused by direct cross-linking of the ^{125}I -labelled rabbit IgG (anti-human IgE) to the rabbit light chains present in the rabbit urine that was bound to the solid phase (2). Further studies have demonstrated that the presence of rabbit light chains also caused similar non-

specific binding to sheep antibodies in XRIE (10), although addition of normal rabbit serum to the radioactive tracer in both RAST and XRIE eliminated this non-specific effect. As there are only minimal amounts of this light chain component in dust and it was not detected in saliva, the use of these extracts may reduce the possibility of false positive results in diagnostic tests for rabbit-sensitive individuals since this component was demonstrated to be of relatively minor allergenic importance (10). However, the positive urine RAST results indicate that other allergens may be present in the urine, although they were not detected in the dust.

No Ag R1 was detected in rabbit serum, and results of RAST to rabbit serum and serum albumin were very much lower than those of the other three extracts.

In immunotherapy studies (13) for the treatment of laboratory animal allergy, washed whole pelt extract was used and significant increases in the levels of blocking antibody and a decrease in symptoms over the treatment period were reported. Whether this was the case for all the animals tested or only for the rabbit appears unclear. Although the major allergens may be expected to be present in an extract of the pelt, significant levels of the major rabbit allergen would only be found if all the salivary constituents had not been removed from the fur by the washing. In the present investigation the dander scraped from the pelt after removal of the fur contained only small amounts of Ag R1. Instead, the component present in largest amounts was serum albumin (data to be published) and this component was found to be positive by RAST only for those individuals giving the highest values in RAST to the other rabbit extracts. The study of allergens from washed rabbit pelt extracts fractionated by gel filtration on Sephadex G-100 (9) demonstrated one fraction with the electrophoretic mobility of serum albumin (67 Kd) and a second fraction with a component(s) of molecular weight 18–38 Kd. These fractions when compared to the other separated fractions both showed greater allergenic activity as measured by skin prick test in rabbit-sensitive individuals.

The findings of the present study are mostly

in agreement with these earlier observations. The 18–38 Kd M.W. fraction probably contained Ag R1, although very little is likely to remain in a washed pelt extract. However, Ag R1 was in fact found in several different molecular weight forms, from 17 Kd on SDS-PAGE to between 30 and 50 Kd on Sephacryl S-200 gel filtration, indicating the possibility of a dimeric form, and its presence in still higher M.W. fractions, suggests that Ag R1 may also exist in higher polymerised forms. The glycoprotein nature of Ag R1 was revealed by staining for polysaccharide.

These studies suggest that since saliva and dust contain high levels of a component demonstrated to be a major rabbit allergen, Ag R1, extracts of either source material may be more relevant than dander or urine for use in investigations of individuals with sensitivity to rabbits, i.e. in skin test and RAST, and also possibly for desensitisation.

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