

Allergy to rabbits

III. Further identification and characterisation of rabbit allergens

J. A. WARNER & J. L. LONGBOTTOM

Child Health, Southampton University, Southampton, UK

Crossed immunoelectrophoresis and crossed radio-immunoelectrophoresis techniques have been employed for analysis of extracts of various rabbit source materials to identify rabbit allergens in addition to the already described Antigen R1 (AgR1). Urine and dander extracts were found to contain only low levels of AgR1 and its presence in urine was as a contaminant due to mode of collection - it was not present in urine collected directly from the bladder. Other allergens were only recognised by highly rabbit-sensitive individuals, one in particular (Ag2) being present in several source materials. Serum albumin proved to be of minor allergenic importance and except for dander its presence was only in minimal amounts. As both AgR1 and Ag2 are significant components in extracts of fur and dust these extracts are therefore most recommended for use in investigations of individuals sensitive to rabbits.

Key words: crossed immunoelectrophoresis; diagnostic materials; rabbit allergens.

Accepted for publication 27 December 1990

CLINICAL ASPECTS

The favoured diagnostic reagents involved in investigations of allergy to laboratory animals are dander and, more recently, urinary extracts. However, the major rabbit allergen AgR1 is present in minimal amounts in rabbit urine, and dander consists mainly of serum albumin which is apparently not an important allergen. Dust and fur extracts contain large quantities of AgR1 and also the other minor allergen of most importance, Ag2, and should therefore be those recommended for use in diagnostic testing.

Despite rabbits being relatively common domestic pets and widely used as laboratory animals, there have been a limited number of attempts to investigate the specific allergens involved in sensitisation to these animals. There is also little information about the important sources of allergenic components. The major rabbit allergen Antigen R1 (AgR1) (9), has been demonstrated to be an important component of rabbit dust, fur and saliva but not of urine and dander, although these latter have mostly been used for *in vivo* and *in vitro* testing (1, 2, 10, 6). In urine there are many other components present, (with AgR1 being a relatively minor one) and, in

dander, the major component is of serum origin. The aims of this study were to investigate various sources of inhalant materials associated with exposure to rabbits and to identify allergens which may not be present in dust extracts but do, however, show binding of IgE antibodies in rabbit-sensitive individuals.

MATERIAL AND METHODS

Antigens

Dust was collected from surfaces between 3 and 8 feet above the floor in rabbit housing areas

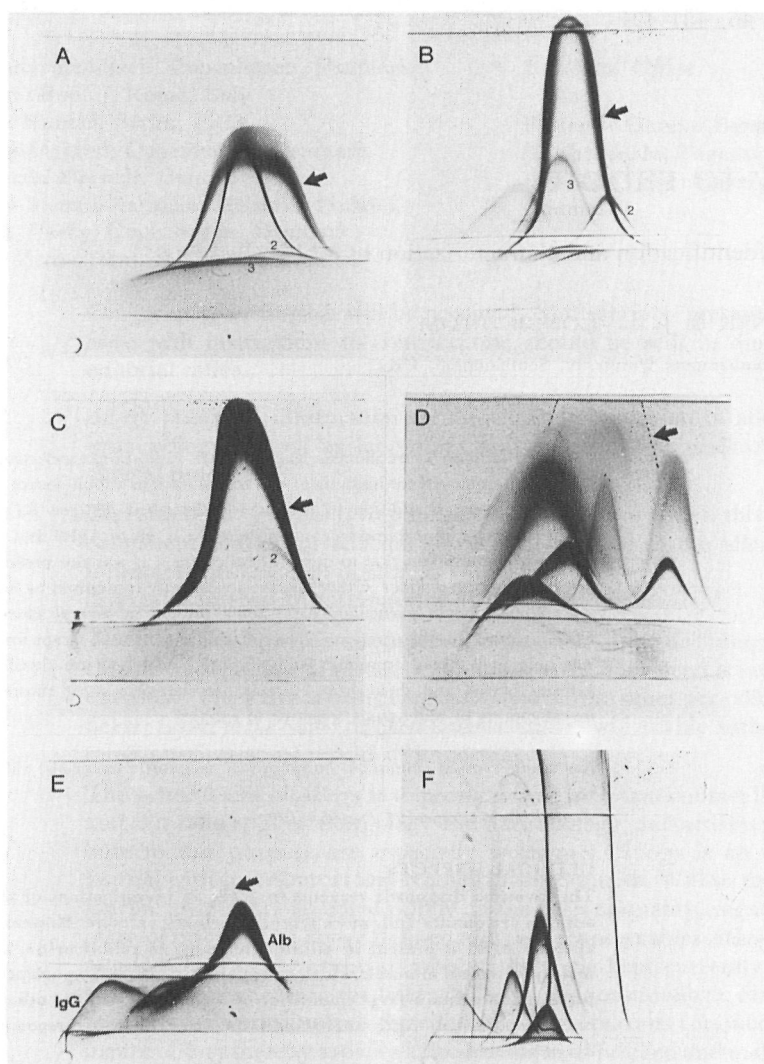


Fig. 1. XIE reference precipitin patterns for (A) dust (30 µg); (B) saliva (20 µg); (C) fur (30 µg); (D) urine (20 µg); (E) dander (34 µg) and (F) serum (5 µg). The intermediate gels in A-E contained anti-rabbit serum (12.5 µl/cm²). The main gels contained (A) anti-rabbit dust (6.5 µl/cm²); (B) anti-rabbit saliva (6.5 µl/cm²); (C) anti-rabbit fur (6.5 µl/cm²); (D) anti-rabbit urine (16.25 µl/cm²); (E) anti-rabbit dander (5 µl/cm²) and (F) anti-rabbit serum (6.5 µl/cm²). AgR1 is arrowed in each extract and similar antigens common between the extracts, i.e. Ag2 and Ag3 numbered.

(New Zealand White and New Zealand White/Dutch Lop Cross).

Urine was collected daily from rabbits of varying ages (3 months to 2 years), placed in metabolic cages where good separation of urine and faeces could be achieved. The pooled urines were centrifuged at 5,000 rpm, filtered through 0.45 µ filters (Oxoid, London) and then dia-

lysed against frequent changes of distilled water before freeze drying. A post-pubertal rabbit urine sample was also kindly supplied by Dr. G.E. Davies and Mrs. A.V. Thompson (ICI Central Toxicology Laboratory, Alderly Park).

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. Normal

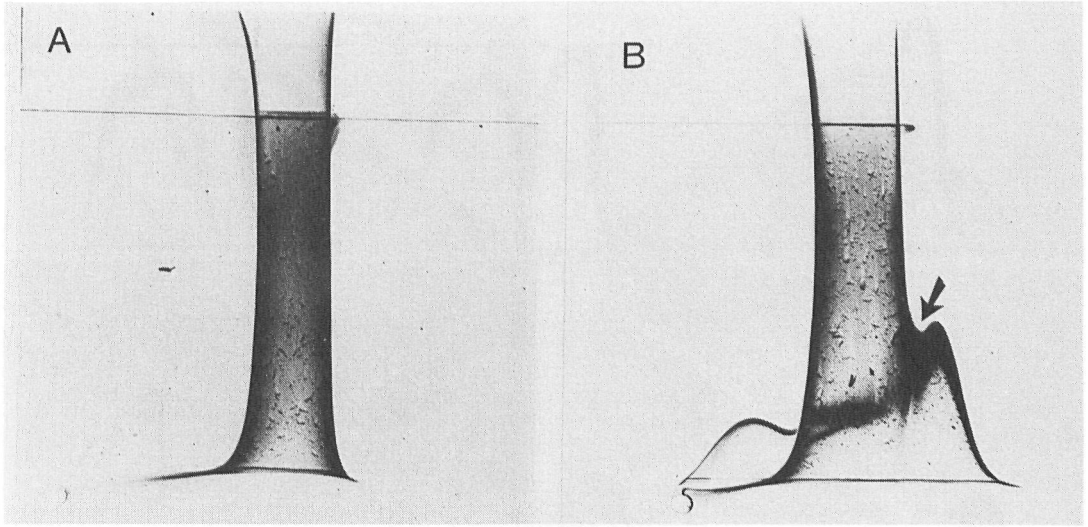


Fig. 2. XIE of (A) rabbit albumin (5 μ g) and (B) tandem XIE of rabbit albumin (5 μ g) and rabbit dander (34 μ g). The main gel areas contained anti-rabbit dander (5 μ l/cm²). The line of identity between the rabbit albumin and the main dander peak is arrowed.

rabbit serum was obtained from 8 to 10-week-old rabbits prior to any immunisation.

Human sera

Sera were obtained from rabbit-allergic individuals who associated symptoms of asthma and/or rhinitis with exposure to rabbits and who were positive in skin-prick tests to rabbit urine and serum and by radioallergosorbent test (RAST) to rabbit dust and urine ($n = 14$). Sera from non-atopic subjects were used as controls ($n = 5$).

Antisera

Sheep antisera to rabbit urine was produced and kindly donated by Dr. Davies and Mrs. Thompson (ICI). Antisera (swine) to rabbit serum was purchased from Dakopatts (A/S Dako Immunoglobulins, High Wycombe, UK). Duncan-Hartley guinea pigs were immunised with whole rabbit dust, 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 anti-serum which was then tested by crossed immunoelectrophoresis (XIE) for antibody response.

Crossed immunoelectrophoresis (XIE)

This was performed as described previously (4). Agarose gels (1%) (BDH, Poole, UK) were poured onto 9 $\times$ 9 cm glass plates (Photo-science, Canvey Island, UK) and electrophoresis of the freeze-dried extract (10 mg/ml in 0.9% NaCl/0.5% NaN₃) was carried out in the first dimension at 4 volts/cm for 75 min. Electrophoresis in the second dimension was carried out overnight at 1 volt/cm through an intermediate gel (0.8 $\times$ 5 cm) containing anti-rabbit serum (and, where necessary, through a second and third intermediate gel of the same size containing other antisera) into the main gel area (3 $\times$ 5 cm).

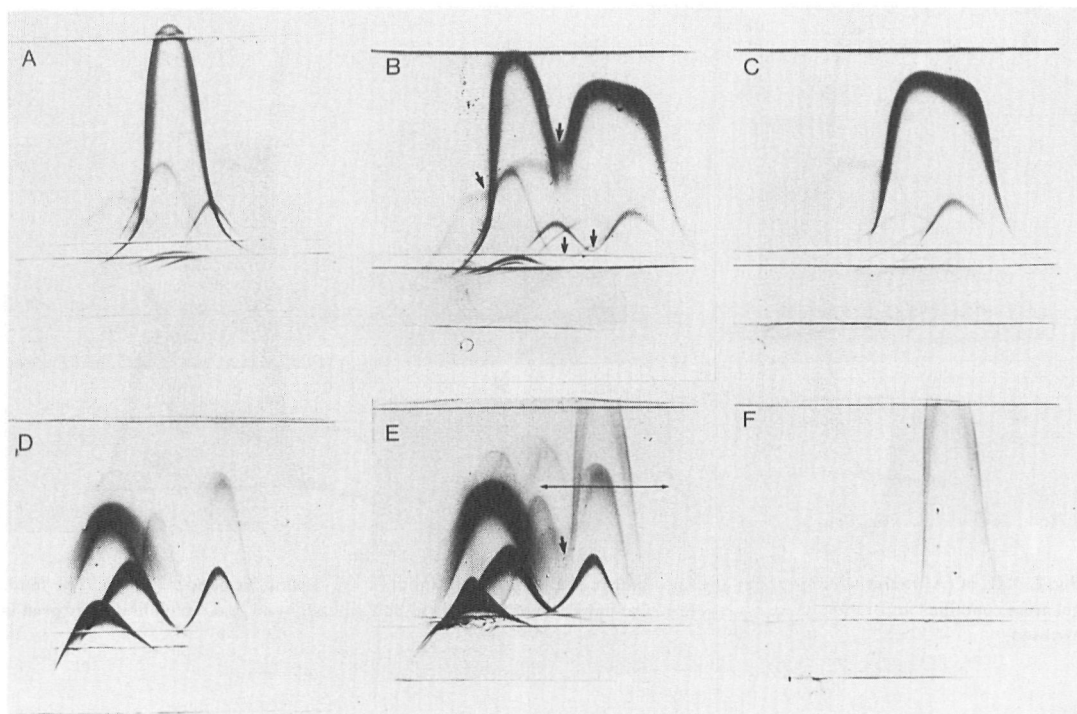


Fig. 3. XIE of (A) rabbit saliva (20 µg); (C) rabbit dust (20 µg) and (B) TXIE of the two extracts. The intermediate gels contained anti-rabbit serum (12.5 µl/cm²) and the main gels, anti-rabbit saliva (6.5 µl/cm²) XIE of (D) rabbit urine (20 µg); (F) rabbit dust (10 µg) and (E) TXIE of the two extracts. The intermediate gels contained anti-rabbit serum (12.5 µl/cm²) and the main gel areas, anti-rabbit urine (16.25 µl/cm²). The lines of identity between similar components are arrowed or an increase in peak width indicated.

These antisera 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.

Tandem-crossed immunoelectrophoresis (TXIE)

TXIE was performed as described for XIE but with a second extract added in a well cut in line and a short distance (0.5–1 cm) from the first well. The two extracts were electrophoresed simultaneously in the first dimension (3).

Crossed radioimmuno-electrophoresis (XRIE)

This was performed as described previously (12) using rabbit urine and dander as antigens and

antisera to rabbit urine and dander in the main gel areas (5 × 3 cm). Intermediate gels (5 × 0.8 cm) incorporating anti-rabbit serum, anti-rabbit IgG and anti-rabbit dust were used to determine their absorbing effects on the urinary and dander XIE profiles. The plates were washed and dried (cold air) and then 3 ml of a 1:10 dilution of patient's serum was overlaid and allowed to react overnight (18–20 h).

After 4–5 washes there was a further overnight incubation with 3 ml of ¹²⁵I rabbit anti-human IgE (Phadebas RAST isotope, Pharmacia Diagnostics, Milton Keynes, UK) diluted to approximately 10,000 cpm/100 µl (300,000 cpm/plate). The washed and dried plates were then exposed to X-ray film (DEF/2 Eastman-Kodak Limited, Hemel Hempstead, Herts, UK) for 5–7 days, after which the films were developed, according to manufacturer's instructions and the glass slides stained for protein in

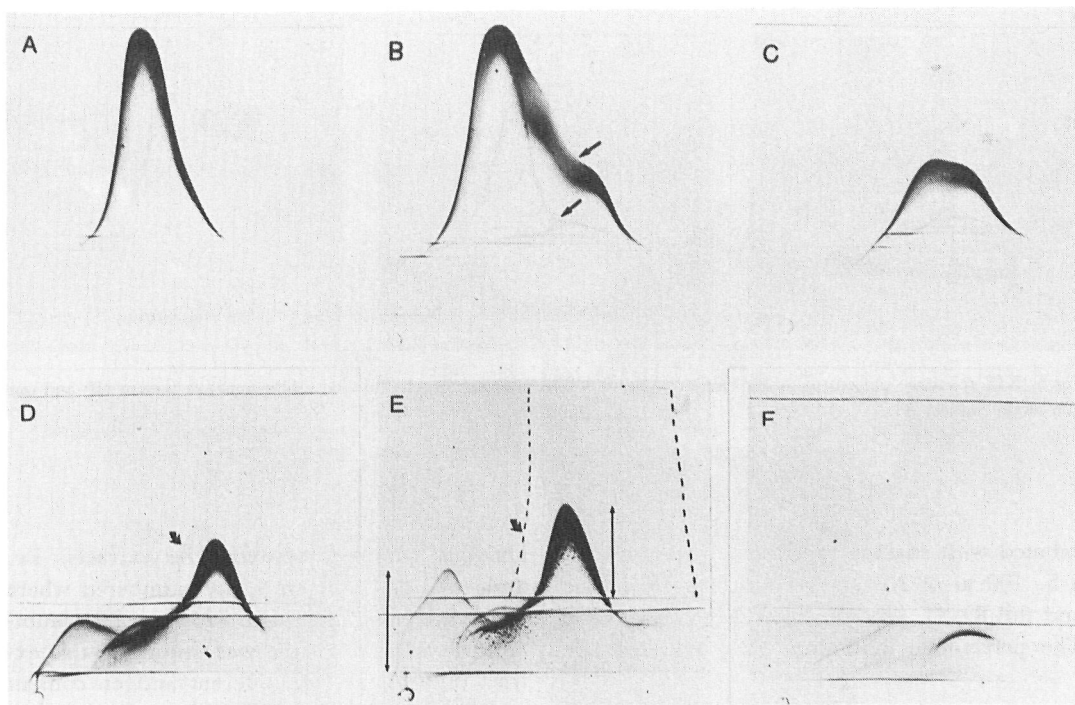


Fig. 4. XIE of (A) rabbit fur (30 µg); (C) rabbit dust (30 µg) and (B) TXIE of the two extracts. The intermediate gels contained anti-rabbit serum (12.5 µl/cm²) and the main gels, anti-rabbit fur (6.5 µl/cm²).

XIE of (D) rabbit dander (34 µg); (F) rabbit dust (30 µg) and (E) TXIE of the two extracts. The intermediate gel contained anti-rabbit serum (12.5 µl/cm²).

The lines of identity between similar components are arrowed, or where components have completely united the increase in peak height is indicated.

0.5% Coomassie brilliant blue R250 (Sigma Chemical Company, Poole, UK).

Radioallergosorbent test (RAST)

IgE levels specific for rabbit dust, urine, dander, and albumin were carried out as described elsewhere (11). The extracts were coupled to cyanogen bromide activated Sepharose 4B (10 mg/g) (Pharmacia) according to the manufacturer's instructions. The resulting antigen particle complexes (APC) were stored at stock concentrations of 25% in PBS/0.3% HSA/0.02% NaN₃ at 4°C. The APC were titrated separately for each extract to determine the optimum concentration at which there was a 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 neat 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 (5000 rpm for 10 min). Fifty µl of ¹²⁵I rabbit anti-human IgE 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 ¹²⁵I-anti-IgE added.

RAST inhibition

50 µl aliquots of pooled sera from allergic individuals or from non-atopic control individuals were added to a 100 µl of increasing concentrations of inhibiting extract (0.001 to 100 mg/ml) in PBS (in duplicate) and in-

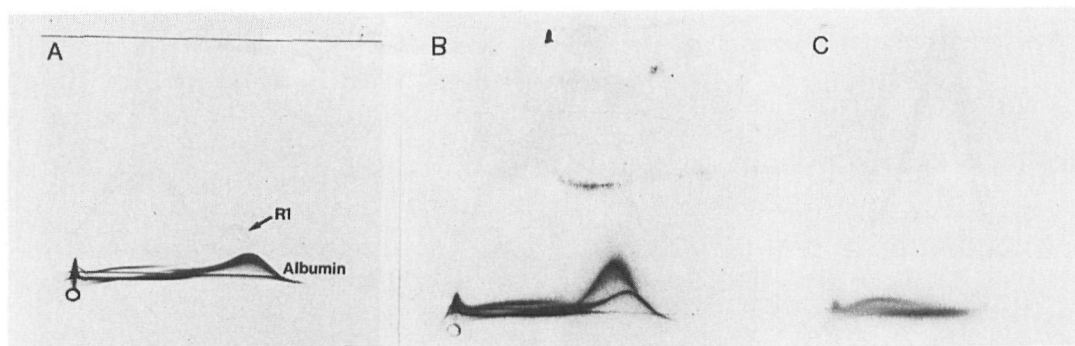


Fig. 5. XIE reference pattern of rabbit dander (A) and XRIE autoradiographs for one rabbit-sensitive patient (B) and one non-atopic subject (C).

cubated with shaking at room temperature for 6 h. 100 μ l of APC were added to each tube and the RAST procedure performed as above. The percentage inhibition was calculated as:

$$\text{percentage inhibition} = \frac{(A-C) - (a-c) \times 100}{(A-C)} \%$$

Where "A" and "C" were the cpm for allergic and control sera, respectively, without the addition of inhibiting extract, and "a" and "c" were the cpm for the allergic and control sera with inhibiting extracts added.

RESULTS

XIE

Reference XIE patterns for rabbit dust, saliva, fur, urine, dander and serum were established (Fig. 1). In each pattern (except Fig. 1F) an intermediate gel containing anti-rabbit serum was included and small quantities of serum components were found in dust and urine (Fig. 1A & D), with the major component of dander (Fig. 1E) being serum albumin. This was confirmed by tandem XIE of rabbit serum albumin with dander (Fig. 2). The major component of dander showed a reaction of complete identity with the major albumin peak (Fig. 2B). Similar

antigens common between the extracts, i.e. antigen 2 and antigen 3, are numbered where they have been identified. However, the numbering system for urine was unique to this extract due to the very different antigen content and does not necessarily relate to the same numbers in the other extracts (the word "urinary" always precedes the antigen number for antigens of this extract).

Tandem XIE of dust with saliva and urine using the homologous antisera (Fig. 3) identified components in these extracts which were also present in the dust. Four saliva components showed identity with dust components (Fig. 3 A-C). It was interesting to note that between these extracts there was only partial identity of the AgR1 peak (Fig. 3B), indicating some difference in the nature of the component depending on its source material.

Although urine contained more antigenic constituents than the other extracts, only two of those were seen in the dust: one of which was a small amount of AgR1, identified by the increase in width of its peak in Fig. 3E (compared with Fig. 3F), its associated peak being barely detectable in Fig. 3D. This was, however, shown to be a contaminant in the urine extract due to the method of collection since comparison of the XIE profile of a urine extract collected in a metabolic cage with the profile of urine collected directly from the bladder showed AgR1 to be absent from the latter (not illustrat-

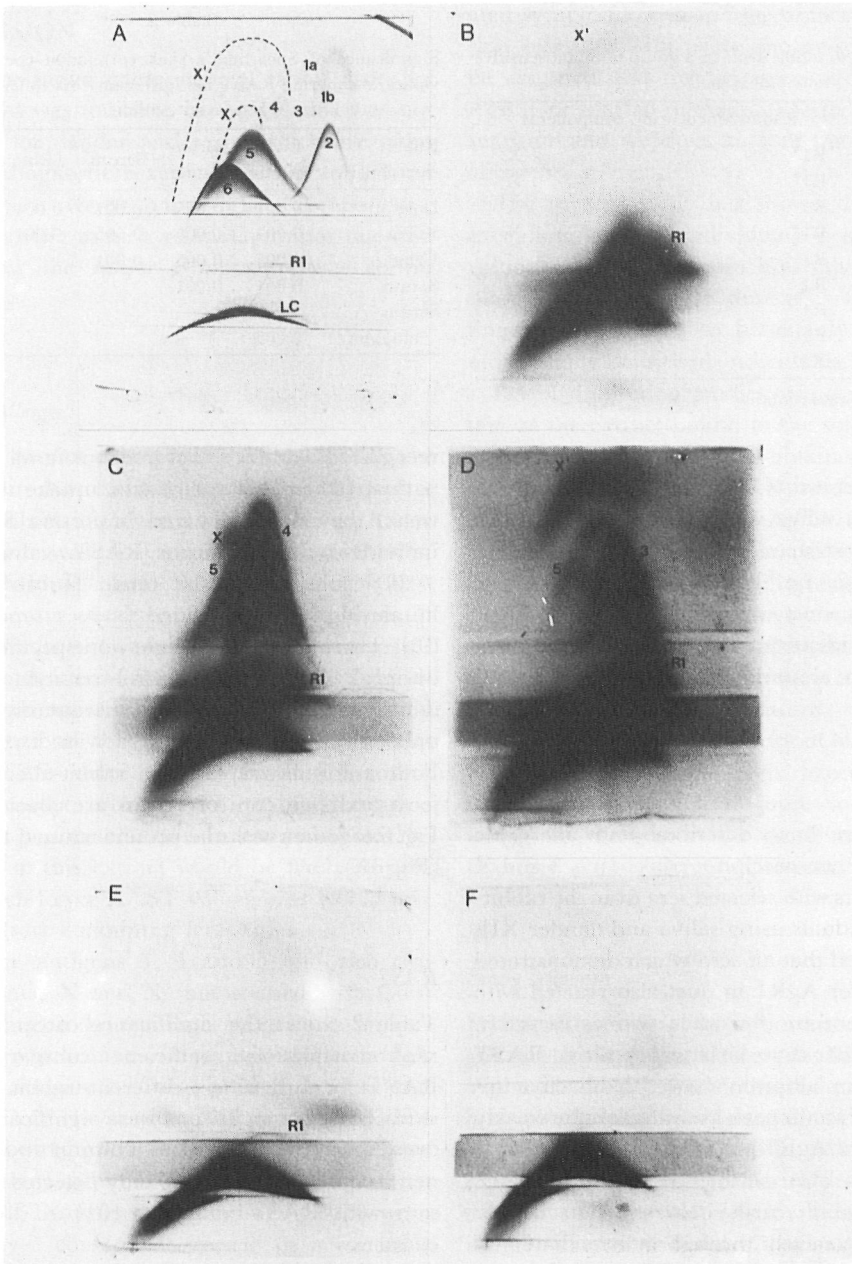


Fig. 6. (A) XIE reference pattern of rabbit urine containing three intermediate gels (anti-rabbit serum; anti-Fab fragment and anti-AgR1 - to clarify the remaining components in the main anti-urine containing window) and XRIE radiographs for four rabbit-sensitive patients (B-E) and one non-atopic subject (F). Similar peaks are numbered.

Table 1.

Results of XRIE to rabbit urine in a group of rabbit sensitive individuals

Patient	Radiostained urine components				
RS	R1*				X ¹
AB	R1*	3*	4	5	X ¹
ZN	R1	3		5	X ¹
LP	R1*		4	5	X ¹
IL	R1				
GS	R1				
RA	R1				
AW	R1	3			
BC	R1				

*Denotes strong radiostaining

ed). The other urine component present in the dust and also saliva was shown to be Ag2.

Fur and dust shared two components, AgR1 and dust Ag2, i.e. both the fur components were found in dust extracts (Fig. 4 A-C). Dander showed identity with three components; AgR1, serum albumin and IgG (Fig. 4 D-F).

XRIE

Previously, we have described four allergenic dust components, precipitin peaks 1, 2, 3 and X (9). XRIE tests with selected sera from the rabbit-allergic individuals using saliva and dander XIE profiles showed that all sera which demonstrated specific IgE for AgR1 in dust also reacted with this component in the other two extracts. In XRIE of dander those sera with positive RAST results to serum albumin showed radioactive uptake on the albumin peak as well as on the weakly protein stained AgR1 peak (Fig. 5).

XRIE was also performed using the XIE profile of rabbit urine as seen in Fig. 6. Removal successively through intermediate gels of anti-serum and anti-light chain component (8) facilitated viewing of radioactive uptake on other precipitin peaks, and inclusion of a further third intermediate gel containing anti-dust to remove AgR1 also clarified the results since uptake on the broad AgR1 peak tended to mask other components. Nine of the original 14 sera were tested using this profile and the results are shown in Table 1. Five urine components were

Table 2.

Significance of Spearman's rank correlation coefficient (*P* values) comparing RAST for IgE against six different rabbit derived extracts

Rabbit extract	Light chains	Serum albumin	Serum	Urine	Saliva
Dust	0.001	0.005	0.001	0.001	0.001
Saliva	0.001	0.005	0.005	0.001	
Urine	0.005	0.005	0.001		
Serum	0.001	0.001			
Serum albumin	0.005				

recognised as minor allergens but in all but one patient (where the radioactive uptake was very weak) they only appeared important for those individuals whose urine RAST values were > 20 % binding of the total ¹²⁵I rabbit anti-human IgE counts added (values reported in (9)). There was a very slight non-specific uptake on peak 6 with the control sera due to the density of the precipitate, consequently similar uptake with the allergic sera was disregarded. Four autoradiographs from rabbit-allergic subjects and one control serum are illustrated in Fig. 6 together with the protein stained template (Fig. 6A).

RAST

Table 2 shows the significance of Spearman's rank correlation coefficient comparing the RAST for IgE in six different rabbit derived extracts. All comparisons were significant, however; specific IgE to serum albumin and components of whole serum was only detected in those sera with RAST values > 10 % to the other extracts.

RAST inhibition

On a dry weight basis inhibition curves of a RAST for IgE against rabbit room dust produced by extracts of fur and saliva more closely paralleled that of the dust self inhibition than did those of urine and albumin (not shown).

DISCUSSION

Whilst the major component of rabbit dust, fur and saliva was found to be AgR1, this was not the case for dander and urine which are often used as diagnostic reagents for this animal. In urine there were many other components, AgR1 proving to be a relatively minor one and in dander the major component was serum albumin.

Urinary allergens

As only two of the urinary components were detected in dust extracts, the finding that urine did not inhibit the dust RAST as well as fur or even saliva was not surprising. Nevertheless, high individual urine RAST values ($> 30\%$ binding) were obtained. Individual analysis of XRIE results showed that rabbit-sensitive individuals with urine RAST values $< 20\%$ binding were demonstrating radioactive uptake only on AgR1, and that unless the RAST results were $> 20\%$ there was no binding to other urinary components. RAST to bladder urine was not performed, but investigation of the response to this extract would be likely to produce much lower RAST values – as would any urine extract containing less AgR1.

Urinary antigens 3, 4 and 5 and also two components, X and X¹ not stained with Coomassie blue on the template, were those components for which some sera contained specific IgE (urinary antigen 3 is also present in dust and saliva as Ag2 [by tandem XIE not shown]) but all may be regarded as minor allergens. Since bladder urine did not contain AgR1 the positive urine RAST results with binding $< 20\%$ were caused by a component present as a contaminant due to the mode of collection. Therefore, studies which have employed rabbit urine in their assays and diagnostic tests, (Cockroft et al. (1) used rabbit urine as a skin test solution and Edwards et al. (2) measured airborne urinary protein allergens by RAST inhibition with eluted air samples), were possibly largely dependent upon the AgR1 contamination of their urinary extract. As all individuals who associ-

ated symptoms with rabbit contact had positive skin reactions with urine and or serum it must be assumed that the urine extract did contain AgR1, or that all individuals tested were highly sensitive and were reacting to the other minor allergens.

The present study has shown that (with the exception of one individual) positive RAST values to serum albumin are only obtained in those whose RAST to dust is $> 10\%$, indicating these reactions to be mainly confined to more highly sensitised individuals.

RAST inhibition studies using urinary protein as the extract bound to the solid phase will again influence the results obtained in proportion to the amount of AgR1 present. If there is only a small quantity of AgR1 in the urine extract then an air sample (likely to contain high levels of AgR1 (9)) will greatly inhibit the RAST (as XRIE studies have shown a low IgE response to other urinary allergens) and consequently suggest inflated urinary allergen levels from the sampled area. Edwards et al. (2) did suggest that the relative purity of allergen might be a cause of the high levels detected in rabbit rooms but attributed it more to the overall weight of allergen in the crude extract rather than the actual allergens present.

Serum albumin

Serum albumin and its importance as an allergen has been disputed. In rat-allergic patients Wahn et al. (10) found that rat serum caused histamine release from basophils of only eight of 33 rat-allergic patients, and Longbottom (4) has reported that the albumin-rich fraction of rat urine was of only relatively low skin test reactivity. On the other hand, Ohman et al. (7) reported that the Sephadex G-100 fraction of rabbit pelt extract, which had the electrophoretic mobility of serum albumin, showed good skin test reactivity in rabbit-sensitive patients.

In the present study individuals with positive RAST results to serum albumin and whole serum showed radioactive uptake on the albumin peak as well as on the AgR1 in rabbit dander XRIE. The subjects also all had high

RAST values to the other rabbit extracts. Therefore, these results again suggest that serum albumin is an important allergen only for highly sensitised individuals. The serum and serum albumin RAST results showed good overall correlation with the dust RAST values although the patient serum with the highest IgE levels to dust, saliva and urine was not in fact the highest for albumin. This would indicate that there may still be some selective recognition of allergens even though, in general, serum albumin appears to become more important as an allergen the more sensitive the individual.

Antigens 2 and 3

Antigens 2 and 3 were both found to be minor allergens in dust extracts. All but one serum from an individual allergic to rabbits demonstrated specific IgE for Ag3, but since most of the radio-staining was weak it must be regarded as a minor allergen. Ag2 also appeared to be allergenic for several rabbit-sensitive individuals. Both antigens were present in saliva extracts and Ag2 was also in fur and urine extracts. Their presence in these extracts must therefore play some part in the dust RAST inhibition studies, particularly the inhibition by urine which had only low levels of AgR1.

As might be expected from the results of the dust autoradiographs, in the urine XRIE urinary Ag3 (Ag2) was not stained on all the autoradiographs. For this there are only two possible explanations: 1) XRIE should be considered only a semi-qualitative technique (11) and therefore discrepancies in total numbers of patients reacting with a similar antigen in each XRIE series may be expected and/or, 2) the position of Ag2 in the dust profile (Fig. 1A) rendered it particularly susceptible to the process of co-precipitation, particularly with AgR1 and therefore some of the radio-staining seen on the peak may have been due to the trapping of other allergenic molecules. Nevertheless, Ag2 should be considered when assessing sensitivity to rabbits. Ag3 appears to be a salivary-derived allergen since it was not detected in fur, urine or dander and, as stated previously, is only of minor importance.

All other allergens found were restricted to a single source material and recognised by only a few sera.

In conclusion from these and previous findings it is apparent that AgR1 is the major rabbit allergen and its presence in any diagnostic material is essential for efficient testing. For the rabbit diagnostic testing, materials derived from urinary and dander sources should not be recommended, as the major allergen is not uniformly present.

Other minor allergens were recognised by highly rabbit-sensitive individuals; however, Ag2 was the only additional dust allergen to be present in any great extent in other extracts and therefore might perhaps be considered when performing quantitative studies of rabbit inhalant allergens. Serum albumin did not appear to be a major rabbit allergen and its presence in low amounts in most extracts was unlikely to be of any great importance. Therefore extracts of rabbit fur or dust are those most recommended for use in investigations of individuals with sensitivity to rabbits.

ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Dr. C. Corker in the initial immunisation of guinea pigs, and of Mr. T. Betts and Mrs. G. Musselwhite in obtaining rabbit source materials. Thanks are also due to Mrs. H. Potucek and Mr. P. Hyett of the Royal Marsden Photographic Department. We thank Ms. Linda Romain for typing this manuscript. This work was supported by a grant from the Wellcome Trust.

REFERENCES

1. Cockcroft A, Edwards J, McCarthy P, Andersson A. Allergy in laboratory animal workers. *Lancet* 827-830, 1981.
2. Edwards RG, Beeson MF, Dewdney JM. Laboratory animal allergy: the measurement of airborne urinary allergens and the effects of different environmental conditions. *Lab Anim* 17, 235-239, 1983.
3. Kroll J. Tandem crossed immunoelectrophoresis. In Axelsen NH, Kroll J, Weeke B, (eds.): *A manual of quantitative immunoelectrophoresis methods and applications*. *Scand J Immunol* 2, Suppl. 1, 57-59, 1973.
4. Longbottom JL. Purification and characterisation of allergens from the urines of mice and rats. In Oehling A, et al. (eds): *Advances in allergology and immunology*, pp. 483-490. Pergamon Press, Oxford, 1980.

5. Lowenstein H. Crossed radioimmunoelectrophoresis In Oehling A. et al. (ed.): *Advances in allergology and clinical immunology*, pp. 473-477. Pergamon Press, Oxford, 1980.
6. Neuman I, Lutsky I. Laboratory animal dander allergy; II. Clinical studies and the potential protective effect of disodium chromoglycate. *Ann Allergy* 36, 23-29, 1976.
7. Ohman Jr JL, Lowell FC, Bloch KJ. Allergens of mammalian origin II. Characterisation of allergens extracted from rat, mouse, guinea pig and rabbit pelts. *J Allergy Clin Immunol* 55, 16-24, 1975.
8. Price JA, Longbottom JL. Allergy to rabbits. I. Specificity and non-specificity of RAST and crossed-radioimmunoelectrophoresis due to the presence of light chains in rabbit allergenic extracts. *Allergy* 41, 603-612, 1986.
9. Price JA, Longbottom JL. Allergy to rabbits II. Identification and characterisation of a major rabbit allergen. *Allergy* 43, 39-48, 1988.
10. Wahn U, Peters T, Siraganian RP. Studies on the allergenic significance and structure of rat serum albumin. *J Immunol* 125, 2544-2549, 1980.
11. Walls AF, Newman Taylor AJ, Longbottom JL. Allergy to guinea pigs: I. Allergenic activities of extracts derived from the pelt, saliva, urine and other sources. *Clin Allergy* 15, 241-251, 1985.
12. Walls AF, Newman Taylor AJ, Longbottom JL. Allergy to guinea pigs: II. Identification of specific allergens in guinea pig dust by crossed-radioimmunoelectrophoresis and investigation of the possible origin. *Clin Allergy* 15, 535-554, 1985.

Address:

Dr. Jill A. Warner
 Child Health
 Level G, Centre Block
 Southampton General Hospital
 Tremona Road
 Southampton S09 4XY
 UK

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.