

Identification of some rabbit allergens as lipocalins

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Summary

Background Rabbits are frequently used as laboratory animals or kept as domestic pets. Rabbit serum albumin and a 17-kDa protein referred to as Ory c 1 have previously been reported as allergens. Several other allergenic proteins have been recognized by crossed immuno-electrophoresis but have not been characterized.

Objective The aim of this study was to characterize the allergenic proteins present in rabbit saliva, urine and fur on the basis of molecular size and, where possible, to determine their amino acid sequences.

Methods Extracts from the male New Zealand white rabbit were used for developing specific direct RAST and RAST inhibition assays. Proteins in the extracts were separated by SDS-PAGE and the individual allergens identified by immunoblotting with serum from rabbit-allergic individuals. The N-termini of four allergens were sequenced.

Results Saliva was the most potent extract. In total, 26 protein bands were recognized as allergens in the three extracts: 12 in saliva, seven in urine and seven in fur. Their molecular weights ranged from an 8-kDa species in saliva to an 80-kDa protein in urine. The N terminal sequences of an 18 kDa and a 21-kDa species in saliva, were identified as lipocalins with sequence similarity to a recently described odourant binding protein.

Conclusions This is the first evidence that allergens from the rabbit are members of the lipocalin superfamily of proteins, suggesting that similar mechanisms may be involved in eliciting the allergic response to rabbits. The 18 kDa allergen from saliva may be the previously named rabbit allergen, Ory c 1.

Keywords: rabbit allergens, RAST, SDS-PAGE, immunoblots, N-terminal sequencing, lipocalins

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Introduction

Type 1 hypersensitivity is commonly experienced by people in regular contact with furry animals in laboratory and domestic settings [1]. Urine, fur, dander and dust from animal housing have been demonstrated to be sources of many of the allergens [2,3]. Cross reactivity between allergens from various animal species may account for allergies in some individuals [4–8].

Rats, mice, rabbits and guinea pigs are the most frequently used laboratory animals. The prevalence of allergy to rats and mice has been widely studied and the major allergens have been identified including structural analyses [9–12]. The rat allergens Rat n 1.01 and Rat n 1.02 and mouse major urinary proteins (mouse MUPs) are members of the lipocalin superfamily of small extracellular proteins in common with the major allergens Can f 1 from dog [13], Bos d 2 from cattle [14] and Equ c 1 from horses [15] and the minor dog allergen Can f 2 [13]. Flower has defined the structurally conserved regions that lie close to each other on the surface of lipocalin molecules forming a

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cell surface receptor binding site. Their amino acid sequence homology is not extensive. Within the core, the lipocalins typically carry small hydrophobic molecules of which some are pheromones [16].

Little is known about the nature of allergens from rabbits and guinea pigs. Crude extracts of rabbit urine or hair/dander are most commonly used for skin prick testing (SPT) [17,18] and urinary or epithelium preparations are used in RAST assays [9,17] to determine allergy to rabbits. Sensitivity to rabbit pelt extracts has been reported by SPT using a protein fraction of 18–38 kDa [19]. More extensive studies by Longbottom and colleagues, using crossed immunoelectrophoresis (CIE), identified several allergens in saliva, fur, urine, dander and dust [20–22]. They termed the major 17 kDa glycoprotein in saliva and urine Ag1. This has been referred to by Schou as Ory c 1 but there are no structural data to characterize this molecule [2]. Serum albumin as well as a second major allergen termed Ag2 and several other bands on CIE were also recorded as allergens.

In this study we have examined the molecular sizes of allergens in rabbit extracts and have determined the N-terminal structure of four of the proteins. As a first step to identify serum samples for use in recognition of rabbit allergens, we established a series of RAST assays to measure specific IgE antibodies to rabbit saliva, urine, fur extracts and to serum albumin. A commercially available kit was used for epithelium measurements. Potency and cross reactivity of the rabbit extracts was studied by RAST inhibition experiments. Following separation of proteins in the rabbit extracts by SDS-PAGE and Western blotting, allergenic proteins were identified using serum samples with high levels of IgE antibodies. After further SDS-PAGE of extracts and blotting onto polyvinylidene difluoride (PVDF) membranes, the N-terminal sequences of four allergens were determined and their amino acid sequence alignments compared with those in the Swiss-Prot database. Two of these proteins contained a sequence motif characteristic of members of the lipocalin superfamily.

Materials and methods

Rabbit extracts

Rabbit urine, fur and saliva were collected from a male New Zealand white rabbit between 3 and 6 months of age. Urine was collected either by cystocentesis directly from the bladder or as a voided sample by palpating the bladder and collecting directly into a storage jar as it was expressed. The urine was stored at -20°C until use. Individual samples collected by each method were pooled and centrifuged (15 min at $2250 \times g$, 4°C). The decanted urine was dialysed extensively in Spectra/Por 3 tubing (cut-off of

3.5 kDa; Spectrum, Gardena, CA, USA) against deionized water for 48 h at 4°C , then freeze-dried and stored at -20°C until use.

Fur was cut from the outer 1 cm of the rabbit's coat and stored at -20°C until protein extraction [23]. Briefly, the fur was washed in dichloromethane and then methanol before cutting into 1 mm segments and grinding in liquid nitrogen using a mortar and pestle. After air drying, 10 mg portions of fur were extracted overnight at ambient temperature in 1 mL of a solution containing 100 mM DTT, 8 M urea and 50 mM TRIS. Following centrifugation, the supernatant fraction was dialysed, freeze dried and stored at -20°C until use.

Pilocarpine (2 mg/kg) was administered subcutaneously to the rabbit and saliva was collected with cotton bud swabs. Saliva was extracted from the swabs with phosphate buffered saline (PBS) containing 10 mM phosphate, 100 mM diNaEDTA, 150 mM NaCl, 0.1% (w/v) sodium azide at pH 7.6 for 30 min at ambient temperature. After repeating the PBS extraction, the saliva extracts were pooled and filtered through Whatman No.1 filter paper, dialysed, freeze dried and stored at -20°C until use.

Total protein determination

Total protein content of extracts was determined by a modified Lowry assay [24] using BSA (BioRad, Sydney) as the standard.

Serum samples

Blood samples were collected from subjects participating in an epidemiological study of the incidence and prevalence of allergy to laboratory animals and the serum stored at -20°C until use [25]. Skin prick testing was performed by standard techniques with 5% histamine and buffered normal saline as positive and negative controls, respectively. The rabbit urine extract was prepared in-house [25] and the hair extract used was a commercial preparation (Hollister-Stier, Spokane, USA). Wheal diameter was measured after 15 min by clinicians and nurses skilled in the technique. A response was classified as positive when the wheal measured at least 50% of the histamine [26]. The largest wheals observed for hair and urine were 6 mm and 9 mm, respectively.

Serum samples from 24 subjects who were SPT-positive to rabbit urine and/or hair extract and 11 who were SPT positive or showed symptoms on exposure to other furry animals — rats, mice, cats, dogs, guinea pigs or horses — were studied. Serum from 16 subjects who were classified as non-atopic on the basis of lack of reaction to common and animal allergens on skin prick testing, and were symptom-free, formed the negative control group.

Measurement of specific IgE by RAST

RAST assays were developed and optimized in-house for measurement of IgE antibodies to rabbit urine, saliva, fur and serum albumin (RbSA). Briefly, Whatman no. 54 filter paper discs were activated [27] with cyanogen bromide (Sigma Aldrich, Sydney) and stored in air-tight containers at 4 °C. When needed, activated discs were coupled individually in 9 mm id × 75 mm polystyrene tubes with 75 µL urine, saliva or fur extracts. After removal of the extracts, discs were washed three times with 0.1% Tw-TBS buffer (25 mM TRIS AR, 150 mM NaCl AR, 0.1% v/v Tween 20 (BioRad), pH 7.45) and each disc blocked with 1 mL wash buffer for 1 hour. This wash buffer also served as diluent buffer in the assays. After blocking, discs were washed three times and diluted serum (1 : 4; 75 µL) was added to coated discs for 21 h at ambient temperature. After serum aspiration and washing, approximately 20 000 cpm ¹²⁵I-sheep/mouse anti-IgE (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) in 100 µL buffer were added for a further 21 h incubation at ambient temperature. The disc-bound ¹²⁵I-anti-IgE was counted in a Wallac 1260 Multigamma counter II for 5 min. Non-specific binding (NSB), assessed by adding serum to extract-free discs, was subtracted from specific binding tubes. For each serum sample, the counts on allergen coated discs minus NSB were calculated as a percentage of the total added counts (%B/T). All samples were assayed in duplicate.

For albumin RAST assays, after coupling RbSA (Sigma) to the discs, unfilled binding sites were blocked with 5% w/v casein (Sigma). In these and epithelium assays (purchased from Bioclone, Sydney, Australia), 50 µL undiluted serum was incubated with coated discs for 3 h and approximately 100 000 cpm ¹²⁵I-labelled goat anti-IgE (Bioclone) was added as tracer for a further 21 h. The same washing and counting procedures, as well as calculation of results, were used for all RAST assays.

RAST inhibition assays

In each inhibition assay, serum at the same dilution used in each RAST assay was pre-incubated with fur, saliva or urine extracts or RbSA for 3 h at ambient temperature. The quantities of added inhibitors ranged from 10 pg to 100 µg protein of each extract. Serum with added diluent was run in parallel as control for each sample. Seventy-five microlitres of serum-inhibitor incubates were added to duplicate tubes containing the appropriate allergen-coated discs for 3 h (RbSA), or overnight (fur, saliva and urine) at ambient temperature. Discs were washed three times and the assays continued, incubating with 100 µL ¹²⁵I-labelled anti-IgE, washing and counting as for the appropriate RAST assay. The percentage inhibition of binding was calculated using

the following formula:

$$\begin{aligned} &\% \text{ inhibition} \\ &= \frac{\text{cpm in serum (control)} - \text{cpm in serum incubated with inhibitor}}{\text{cpm in serum (control)}} \\ &\times 100 \end{aligned}$$

The quantity of each extract leading to 50% inhibition was regarded as a measure of inhibitor potency.

Gel electrophoresis and western blotting

Rabbit urine, fur and saliva extracts and molecular weight markers (BioRad or Pharmacia low molecular weight, 97.4 kDa–14.4 kDa, or pre-stained Novex broad range standards, 250 kDa–4 kDa) were separated by one-dimensional SDS PAGE using 10–20% TRIS/Glycine ready gels (BioRad). Both reduced and non-reduced samples were studied using run conditions according to Laemmli [28]. At the end of the run, bands were fixed and stained with 0.1% w/v Coomassie Blue R-250 for visualization of proteins. A solution of 40% v/v methanol, 10% v/v acetic acid was used as fixative, staining solvent and destain.

For immunoblotting studies, SDS-PAGE-separated proteins were transferred to 0.2 µm nitrocellulose membranes (BioRad) in a BioRad Mini Trans-Blot unit according to Towbin *et al.* [29]. Spare binding sites on the nitrocellulose were blocked with 0.1% Tween-TRIS washing buffer for 2 h, then washed four times with deionized water. Half centimetre-wide strips of separated proteins were incubated with 1 : 2 dilutions of sera from rabbit allergic subjects. After a 16-h incubation with serum, the strips were washed and incubated overnight with ¹²⁵I-anti-IgE (Pharmacia) diluted to 60000 cpm. The strips were again washed and air-dried before exposure to X-ray film (Kodak) in an X-ray cassette with intensifying screen for either 1, 3 or 7 days at – 80 °C before development.

N-terminal sequencing

For amino acid sequencing studies, SDS PAGE-separated proteins were transferred onto 0.2 µm PVDF membrane (BioRad). Before protein transfer, gels were soaked in blotting buffer containing 10 mM CAPS, 0.1 mM thioglycolate at pH 11, with 10% v/v methanol for 10 min. The PVDF membranes were prepared for blotting by soaking sequentially in 100% methanol, deionized water and blotting buffer. Proteins were transferred from the gel to the membrane in the transfer cell using 100 V for 20 min in chilled blotting buffer.

Coomassie blue G-250 stained protein bands were excised from PVDF membranes and subjected to direct

Edman degradation for 10–20 residues using a PE Applied Biosystems Procise 494 sequenator (Foster City, CA, USA). Alternatively, Coomassie blue stained bands were excised from a SDS PAGE gel and incubated overnight in extraction solution (50 mM DTT, 100 mM sodium acetate, 0.1% w/v SDS) at 37 °C. After an ABI ProSorb membrane was moistened with 15 µL of methanol, extracted protein was applied. The membrane was extensively washed before being allowed to dry and used for N-terminal sequencing.

Statistical analysis

Parallelism of responses in RAST inhibition assays was determined by the method of Rodbard *et al.* [30].

Results

The protein content of rabbit extracts were assessed by Lowry assay to be 16% w/w for saliva, 55% for urine and 66% for fur.

Anti-IgE binding in RAST assays was classified as significant when it exceeded three standard deviations above the mean binding of the 16 non-atopic (control) subjects. Grouped RAST data from these and rabbit-sensitive subjects are shown in Table 1(a). Only one of the 11 individuals sensitive to furry animals had a significant RAST measurement, with 4.9% binding to epithelium.

Although a number of the 24 SPT-positive subjects had raised RAST levels towards several allergen extracts, in none were the levels elevated to all of the allergens tested. The data for the five serum samples with the highest levels of antibodies to various extracts are shown in Table 1(b). These five subjects had all been sensitive to both urine and hair extracts from rabbits on SPT. The RAST inhibition studies using serum from subjects 1 (a, b, c) and 2 (d) are presented in Fig. 1. Saliva was highly potent at inhibiting binding of specific IgE antibodies to discs coated with saliva, fur and urine extracts. Urine, fur and albumin were generally more potent at inhibiting binding to discs coated with homologous extracts than to saliva-coated discs. These results suggest that saliva contains more allergenic proteins than either fur or urine. The parallel inhibition observed with two different extracts, as in the case of saliva and urine (Fig. 1, a), urine with saliva and with fur (Fig. 1, b), fur and saliva (Fig. 1, c) and albumin with urine (Fig. 1, d) suggests the presence of common allergenic components [31].

SDS-PAGE analysis under reducing conditions revealed that saliva, urine and fur contain a range of proteins with molecular masses varying from a diffuse band at 120–250 kDa in urine down to 4.4 kDa in fur (Fig. 2). The majority of bands in saliva are smaller than 34 kDa with many appearing as doublets. A number of low molecular weight proteins are also visible in fur. There was no

Table 1a. Specific serum IgE binding (%B/T) to rabbit extracts

Subjects Group size	%B/T	Saliva	Urine	Fur	Albumin	Epithelium
Non-atopics <i>n</i> = 16	Mean + 3SD	0.00 16	0.19 16	0.14 16	0.86 16	2.43 16
Furry animal positive <i>n</i> = 11	Raised level	- 0	- 0	- 0	- 0	4.9 1
Rabbit-positive <i>n</i> = 24	Range of raised levels	0.06–16.7 13	0.21–3.75 7	0.17–1.95 4	0.98–28.1 7	3.01–24.4 7

Table 1b. Specific serum IgE binding (%B/T) to rabbit extracts from five rabbit sensitive subjects. SPT sensitivity* to urine and hair are also shown.

Serum #	Saliva	Urine	Fur	Albumin	Epithelium	SPT* Urine	SPT* Hair
1	16.7	3.75	1.95	0	0	9	5
2	2.5	2.9	0	28.1	13.7	4	4
3	5.6	1.5	0.2	0	3.8	4	4
4	0.1	0	0	1.4	24.4	4	3
5	0.5	0	0	0.8	16.4	5	6

*Wheal diameter in mm.

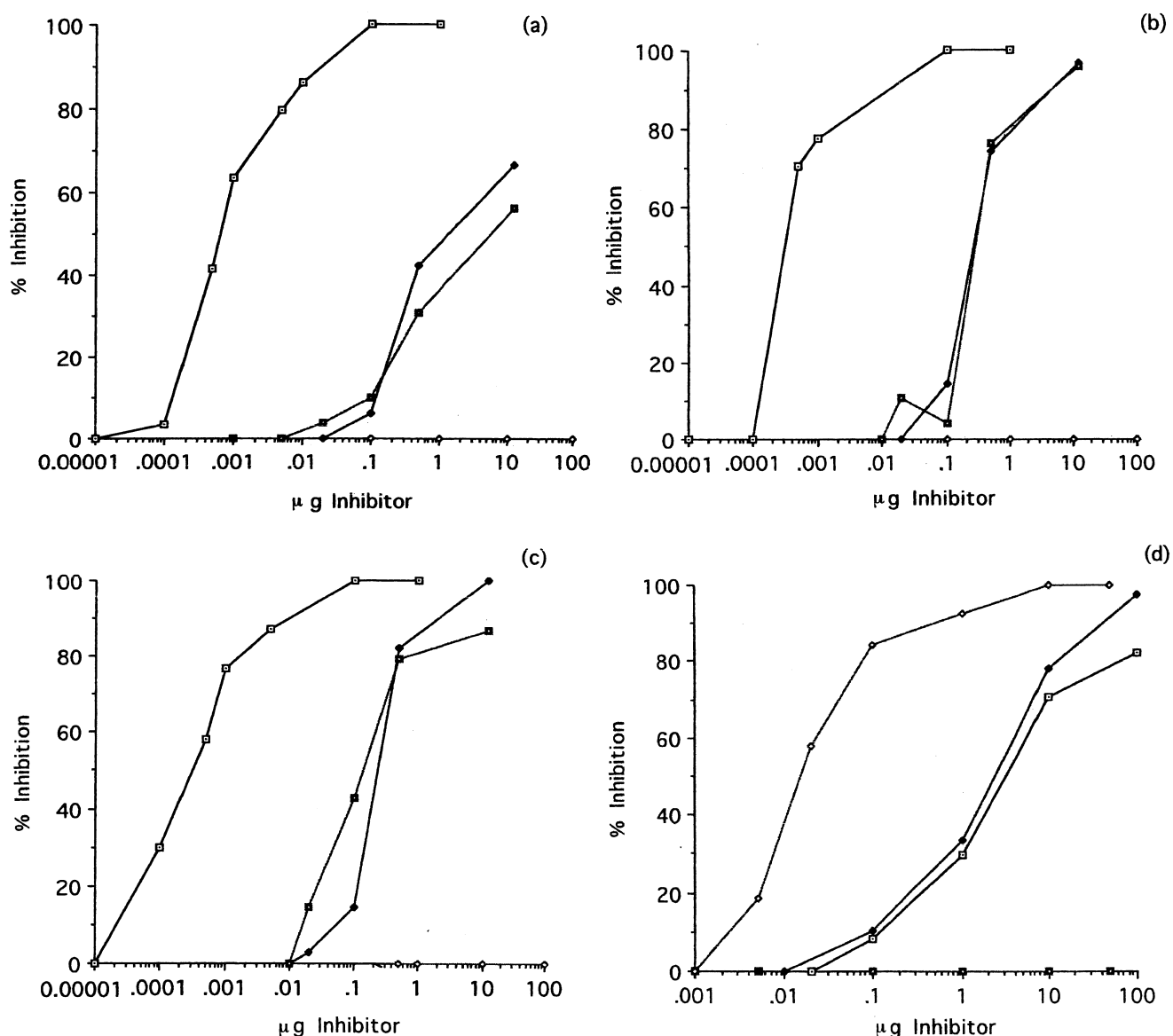


Fig. 1. Dose dependent inhibition of serum IgE binding by rabbit extracts (□—□ saliva, ◆—◆ urine, ■—■ fur, ◇—◇ albumin) to activated discs coated with (a) saliva (b) urine (c) fur and (d) rabbit serum albumin. Serum 1 was used in the studies reported in Figures a–c and serum 2 in Figure d.

difference in the protein array of voided urine and that of urine collected by cystocentesis (data not shown).

Serum samples 1 and 3 were used for immunoblotting studies of protein extracts electrophoresed under both reducing and non-reducing conditions (Fig. 3). At the 1 : 2 dilution used, only serum sample 1 showed detectable binding to proteins in all three extracts under both sets of conditions. Sample 3 bound only to proteins in reduced urine. The relative intensity of radiostaining was graded on the basis of detection interval. Bands observed at 24 h were classified as strong, at 70 h as intermediate and at 7 days as weak (Table 2). Twelve allergens were recognized in

saliva, seven in urine and seven in fur. There were more strongly allergenic bands present in saliva with some of these differing by only 1 kDa from others present in urine and fur. Bands at 13 and 15 kDa were detected as strongly allergenic in both urine and fur. Of the other strong bands observed at 19 and 23 kDa in fur, there was a weaker 23 kDa counterpart present in saliva.

Three discrete allergen bands from saliva and one from urine, were excised from PVDF blots for N-terminal sequencing. Their sequences and homology to other proteins was determined by reference to the Swiss Prot data base (Table 3). The strongly allergenic 18 and 21 kDa

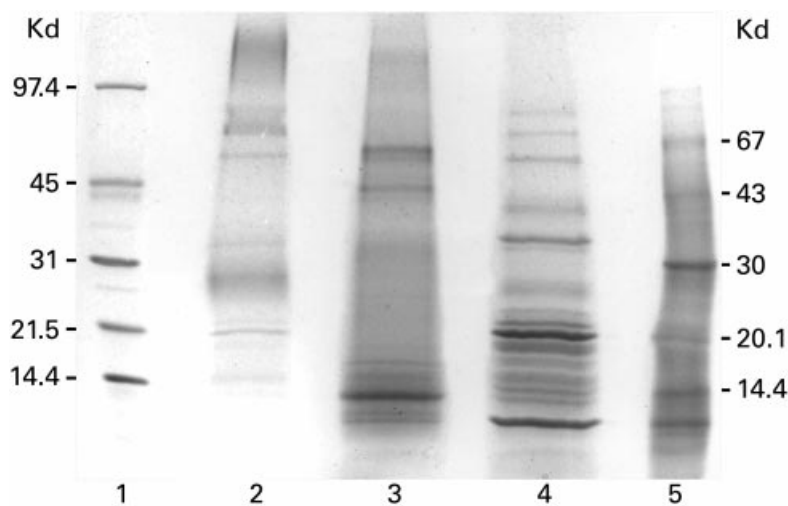


Fig. 2. Rabbit extracts separated by SDS-PAGE under reducing conditions and stained with Coomassie Blue R-250. Lane 2 = urine; lane 3 = fur; lane 4 = saliva; lanes 1 and 5 contain Pharmacia and BioRad molecular weight markers, respectively.

proteins from saliva and the homologous proteins listed are all members of the lipocalin superfamily.

Sequencing of the 13 and 15 kDa proteins of fur was also attempted. These proteins had been difficult to transfer to PVDF and appeared to be blocked at the N-terminus. By an alternative approach [32] their amino acid contents were obtained and the AACompliment 2 database (<http://expasy.proteome.org.au/tools/aacompl>) was searched to identify proteins of similar amino acid content. The results of this search contained many different proteins, which did not allow any conclusive identification of these allergens (data not shown).

Discussion

This study has revealed for the first time that some rabbit allergens are members of the lipocalin superfamily. Of the two lipocalins identified in rabbit saliva, the 18 kDa molecule is most likely Ag1 (Ory c 1) previously detected by Price and Longbottom using CRIE and SDS-PAGE as a major component of saliva, fur, dander and dust [21]. Its sequence shows significant homology with odourant binding proteins from several species. Although it is tempting to suggest that the strong allergen we have detected at 19 kDa in fur could be the same protein,

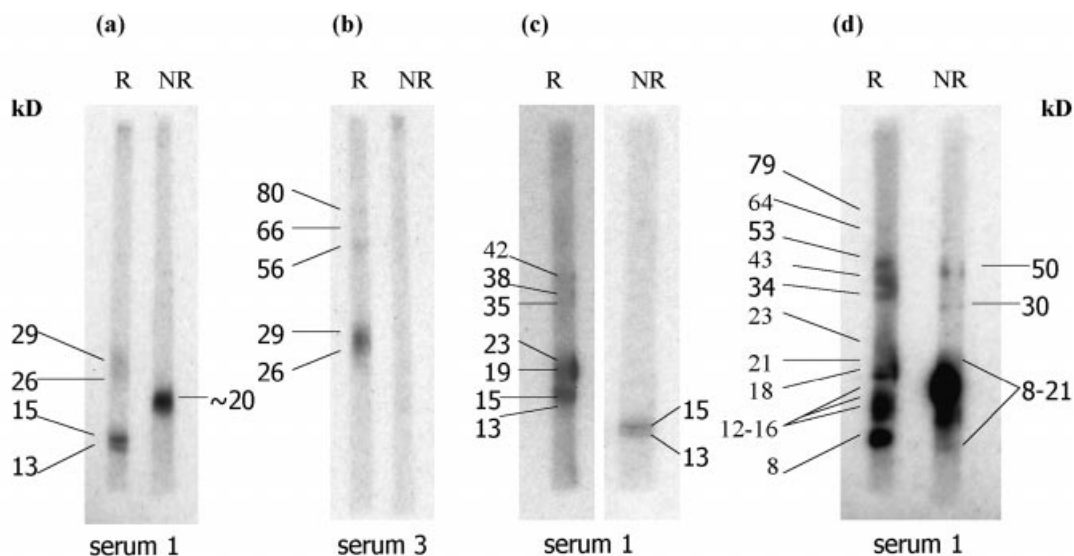


Fig. 3. Autoradiography of immunoblots of urine (a) and (b), fur (c) and saliva (d) rabbit extracts separated by SDS-PAGE under reducing (R) or non-reducing (NR) conditions and probed with either human serum #1 or #3. The molecular weights of proteins identified as allergens are indicated.

Table 2. Allergens identified by autoradiography of immunoblots following incubation with serum IgE antibodies. The strength of binding is classified as strong, intermediate or weak on the basis of time taken for development of signal on the autoradiograph [9]

Allergen classification	Rabbit extract	Serum source	Molecular weight of allergens (kDa)
Strong (24 h)	Saliva	Subject #1	12,14,16,18,21,34,43
	Urine	" 1	13,15
	Fur	" 1	13,15,19,23
Intermediate (70 h)	Urine	Subject #3	26–29
Weak (7 days)	Saliva	Subject #1	8,23,53,64,79
	Urine	" 1	26, 29
	Fur	" 1	35,38,42
	Urine	" 3	56,66,80

confirmation of this is dependent on amino acid analysis. In addition to homology with the listed lipocalins and strong homology with the 18 kDa protein, the N-terminal sequence of the 21 kDa lipocalin allergen is identical to the recently described rabbit odourant binding protein II purified from rabbit nasal mucosa [33]. This may be the protein Ag2 detected in saliva, dust, urine and fur by Warner and Longbottom and recently referred to as Ory c 2 [22,34]. In earlier studies, Ohman *et al.* [19] demonstrated allergenic activity in gel filtered fractions of rabbit pelt in the region of 18–38 kDa. On the basis of molecular size one or both of the sequenced lipocalins may have eluted in this fraction.

When the three serum samples with the highest binding to saliva, urine, fur and albumin were studied in RAST inhibition assays, pre-incubation of serum with as little as 300 pg of saliva resulted in 50% inhibition of binding to discs coated with saliva, urine and fur. These results suggested that all three extracts may contain the major

rabbit allergen(s) although in different proportions. Great care was taken with saliva and urine collections to ensure against contamination by contact with fur. Other investigators [20–22] used metabolic cages but this technique has the potential to lead to contact with fur. In this study urine collected by cystocentesis and by direct collection of voided urine showed identical protein profiles. To ensure that saliva and urine which could adhere to fur during normal grooming or from housing surfaces were absent from fur extracts, the fur was washed with dichloromethane and methanol prior to extraction to remove any adherent hydrophilic and hydrophobic molecules. Having taken these precautions we believe that these studies reflect the true protein constituents of the individual starting materials.

Even when urine and fur were used in inhibition studies with their homologous extracts coating the discs, they were hundreds to thousands of times less potent than saliva in inhibiting IgE antibody binding. The exception was albumin which was highly potent at inhibiting binding of

Table 3. N-terminal amino acid sequences of four rabbit allergens

Rabbit extract	Allergen size (kDa)	N-terminal sequence*	Homologous proteins
Saliva	8	AACPAFVLDSVGFLFDPK 1 10 18	Cat allergen - Fel d 1 Rabbit uteroglobulin
Saliva	18	DVDPAQLSGPWRTSAMAMDN 1 10 20	Rabbit OBP-II Rabbit Sal 21 kDa
Saliva	21	VDPAQVSGSWRTAAIASDDP 1 10 20	Rabbit OBP-II Rb Sal 18 kDa
Urine	13	S DGPAGDRGPP 1 10	Collagen α 2(1) chain (bovine) Collagen α 1(1) chain (rat) Procollagen α 1(1) chain (mouse)

* Underlined amino acids show homology between 18 and 21 kDa proteins.

serum sample #2 to albumin coated discs and over 100 times more potent than saliva. It was, however, ineffective as an inhibitor in the other systems even at concentrations as high as 100 µg. These results suggest that the albumin content of saliva and urine is very low and it may even be totally absent from fur.

Epithelial extract was not available for inhibition studies; however, the serum RAST results suggest that epithelium has a different profile of allergens to those in saliva, urine and fur. Subject #1 with the highest level of IgE antibodies to saliva, fur and urine did not react with epithelium. The IgE antibodies in serum from subjects 4 and 5 were highly reactive to epithelium with only low binding to saliva and albumin. Subject 2 who had the highest binding to albumin also had highly significant binding to epithelium. Further studies are required to elucidate the molecular species in epithelium recognized by rabbit allergic individuals.

The larger number and strength of the allergens in saliva identified by autoradiography of immunoblots parallels the high potency of saliva as an inhibitor of IgE binding in RASTs. Of the 12 allergens detected in saliva, three allergens that were clearly separated by Coomassie blue staining were chosen for sequencing. The 20 amino acids at the N-terminal of both the 18 and 21 kDa proteins included the glycine-X-tryptophan motif which is typically located in the N-terminal region of proteins of the lipocalin superfamily [16]. These two proteins had a high homology of 68% to each other as well as 72 and 94% homology, respectively, with the recently described, 21 kDa odourant binding protein-II isolated from rabbit nasal mucosa (rb OBP-II) [33]. Rabbit OBP-II and two other rabbit OBP, termed I and III, bind the odourant 2-isobutyl-3-methoxy-pyrazine in micromolar quantities, a property common to other vertebrate OBPs [35]. This molecule, a low threshold odourant in man, has been used in competition studies with pheromones from male mice to examine their binding to the urinary lipocalins, the MUPs [36] and to rat α2u-globulins [37]. These latter two families of proteins are the major allergens of mice and rats in man just as Bos d 2 and Equ c1 which were shown to have homology to the rabbit salivary proteins are the major allergens from cattle [14] and horses [15].

Homology existed between the 8 kDa saliva allergen and chain 1 of the major cat allergen Fel d 1 after its leader sequence had been cleaved. Fel d1 has a similar molecular weight to the salivary allergen and also has homology to rabbit uteroglobulin a protein involved in progesterone transport [38].

The N terminal amino acid sequence of the 12 kDa urinary protein was rich in proline and had structural homology with a variety of collagen molecules. This could represent a fragment of a collagen molecule

excreted by rabbits. The significance of this finding is unknown.

Recently a 15-kDa allergen separated from a mixed rabbit fur and dander extract was shown to cross-react with IgE antibodies in deer-allergic individuals [8]. It is of note that an allergen of similar size was identified in this study.

It is well recognized that albumins from different species may cause cross-reactions in allergen studies [6,7]. Serum albumin was detected by RAST in a number of serum samples. In albumin RAST assays samples 1 and 3 did not bind and nor was there significant inhibition by albumin when added to saliva, fur and urine RASTs for these subjects. However, sample #2 showed high binding in albumin assays and was studied with SDS-PAGE gel-separated samples of the three extracts at a serum dilution of 1/20. No bands were detected by autoradiography. This unexpected result may be due to the use of too high a dilution of serum but recent studies suggest that treatment of albumin with 2-mercaptoethanol, used for reducing proteins before SDS-PAGE in our study, could have led to a reduction in epitopes available for recognition of IgE antibodies [39]. Future studies will need to take this potential problem into account.

This study is the first to provide molecular sizes of the large range of allergens extracted from rabbit saliva, fur and urine and to determine structures of N terminal sequences by Edman degradation. The finding that two of these allergens are lipocalins is in agreement with the type of allergens frequently described for other animals. Determination of the importance of the 18 and 21 kDa allergens in a larger population of subjects is needed to determine which of the strong bands can be categorized as major allergens. RAST inhibition assays indicate that at least in subject number 1 in this study salivary allergens are far more potent than those from fur and urine. Further studies are also needed to determine the structures of other allergens in all three extracts and which proteins are common to all.

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References

- 1 Gordon S. Allergy to furred animals. Editorial. *Clin Exp Allergy* 1997; 27:479–81.
- 2 Schou C. Defining allergens of mammalian origin. *Clin Exp Allergy* 1993; 23:7–14.

- 3 Gordon S, Tee RD, Newman Taylor AJ. Analysis of the allergenic composition of rat dust. *Clin Exp Allergy* 1996; 26:533–41.
- 4 Boutin Y, Herbert H, Vrancken ER et al. Allergenicity and cross-reactivity of cat and dog allergenic extracts. *Clin Allergy* 1988; 18:287–93.
- 5 Huwiler B, Wuthrich B. A case of fallow deer allergy. Cross-reactivity between fallow deer and horse allergy. *Allergy* 1992; 47:574–5.
- 6 Spitzauer S, Pandjaitan B, Soregi G et al. IgE cross-reactivities against albumins in patients allergic to animals. *J Allergy Clin Immunol* 1995; 96:951–9.
- 7 Hilger C, Kohnen M, Grigioni F et al. Allergic cross-reactions between cat and pig serum albumin. Study at the protein and DNA levels. *Allergy* 1997; 52:179–87.
- 8 Spitzauer S, Valenta R, Muhl S et al. Characterization of allergens from deer: cross-reactivity with allergens from cow dander. *Clin Exp Allergy* 1997; 27:196–200.
- 9 Gordon S, Tee RD, Newman Taylor AJ. Analysis of rat urine proteins and allergens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. *J Allergy Clin Immunol* 1993; 92:298–305.
- 10 Bayard C, Holmquist L, Vesterberg O. Purification and identification of allergenic α_2 -globulin species of rat urine. *Biochim Biophys Acta* 1996; 1290:129–34.
- 11 Robertson DHL, Cox KA, Gaskell SJ et al. Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. *Biochem J* 1996; 316:265–72.
- 12 Bocskei Zs, Groom CR, Flower DR, Wright CE et al. Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. *Nature* 1992; 360:186–9.
- 13 Konieczny A, Morgenstern JP, Bizinkauskas CB et al. The major dog allergens, Can f 1 and Can f 2, are salivary lipocalin proteins: cloning and immunological characterization of the recombinant forms. *Immunology* 1997; 92:577–86.
- 14 Mantylarvi R, Parkkinen S, Rytönen M et al. Complementary DNA cloning of the predominant allergen of bovine dander: a new member of the lipocalin family. *J Allergy Clin Immunol* 1996; 97:1297–303.
- 15 Gregoire C, Rosinski-Chupin I, Rabillon J et al. cDNA cloning and sequencing reveal the major horse allergen Equ c 1 to be a glycoprotein member of the lipocalin superfamily. *J Biol Chem* 1996; 271:32951–9.
- 16 Flower DR. Multiple molecular recognition properties of the lipocalin protein family. *J Mol Recognition* 1995; 8:185–95.
- 17 Venables KM, Tee RD, Hawkins ER et al. Laboratory animal allergy in a pharmaceutical company. *Br J Ind Med* 1988; 45:660–6.
- 18 Renstrom A, Malmberg P, Larsson K et al. Prospective study of laboratory-animal allergy: factors predisposing to sensitization and development of allergic symptoms. *Allergy* 1994; 49:548–52.
- 19 Ohman JL, Lowell FC, Bloch KJ. Allergens of mammalian origin. II Characterization of allergens extracted from rat, mouse, guinea pig and rabbit pelts. *J Allergy Clin Immunol* 1975; 55:16–24.
- 20 Price JA, Longbottom JL. Allergy to rabbits I. Specificity and non-specificity of RAST and crossed-radioimmuno-electrophoresis due to the presence of light chains in rabbit allergenic extracts. *Allergy* 1986; 41:603–12.
- 21 Price JA, Longbottom JL. Allergy to rabbits II. Identification and characterization of a major rabbit allergen. *Allergy* 1988; 43:39–48.
- 22 Warner JA, Longbottom JL. Allergy to rabbits. III Further identification and characterisation of rabbit allergens. *Allergy* 1991; 46:481–91.
- 23 Herbert BR, Chapman ALP, Rankin DA. Investigation of wool protein heterogeneity using two-dimensional electrophoresis with immobilised pH gradients. *Electrophoresis* 1996; 17:239–43.
- 24 Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193:265–75.
- 25 Bryant DH, Boscatto LM, Mbolo PN et al. Allergy to laboratory animals among animal handlers. *Med J Aust* 1995; 163:415–8.
- 26 Bernstein IL. Proceedings of the task force on guidelines for standardizing old and new technologies used for the diagnosis and treatment of allergic diseases. *J Allergy Clin Immunol* 1988; 82:487–526.
- 27 Ceska M, Eriksson R, Varga JM. Radioimmunosorbent assay of allergens. *J Allergy Clin Immunol* 1972; 49:1–9.
- 28 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- 29 Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1974; 76:4350–4.
- 30 Rodbard D, De Munson PJ, Lean A. Improved curve-fitting, parallelism testing, characterization of sensitivity and specificity, validation, and optimization for radioligand assays. In: *Radioimmunoassay and Related Procedures in Medicine*, 1. IAEA-SM-220. Vienna, International Atomic Energy Agency, 1978:460–505.
- 31 Chard T. An Introduction to Radioimmunoassay, Related Techniques, 4th edn. Amsterdam: Elsevier, 1990.
- 32 Wilkins MR, Pasquali C, Appel RD et al. From proteins to proteomes: large scale identification by two-dimensional electrophoresis and amino acid analysis. *Bio/Tech* 1996; 14:61–5.
- 33 Garibotti M, Navarrini Pisanelli AM, Pelosi P. Three odorant-binding proteins from rabbit nasal mucosa. *Chem Senses* 1997; 22:383–90.
- 34 Bush RK, Wood RA, Eggleston PA. Laboratory animal allergy. *J Allergy Clin Immunol* 1998; 102:99–112.
- 35 Pelosi P. Odorant-Binding Proteins: Structural Aspects. *NY Acad Sci*, 1998; 855:281–93.
- 36 Bacchini A, Gaetani E, Cavaggioni Q. Pheromone binding proteins of the mouse, *Mus musculus*. *Experientia* 1992; 48:419–21.
- 37 Cavaggioni A, Findlay JBC, Tirindelli R. Ligand binding

- characteristics of homologous rat and mouse urinary proteins and pyrazine-binding protein of calf. *Comp Biochem Physiol* 1990; 96B:513–20.
- 38 Morgenstern JP, Griffith IJ, Brauer AW et al. Amino acid sequence of Fel d I, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci USA* 1991; 88:9690–4.
- 39 Restani P, Fiocchi A, Beretta B et al. Effects of structure modifications on IgE binding properties of serum albumins. *Int Arch Allergy Immunol* 1998; 117:113–9.