

Original article

Dual sensitization to rat and mouse urinary allergens reflects cross-reactive molecules rather than atopy

Background: Sensitization to rats and mice can develop in laboratory animal workers exposed to only one species. Reasons for this dual sensitization are unclear but may reflect a genetic predisposition to developing allergy (atopy) or alternatively cross-reactivity between rat and mouse urinary allergens. We examined cross-reactivity between rat and mouse urine and the effect atopy has on dual sensitization in laboratory animal workers.

Methods: In a cross-sectional study the frequency of sensitization to rat and/or mouse was analysed in 498 employees exposed to both rat and mouse at work and 220 to rat only. RAST inhibitions, western blots and blot inhibitions were carried out on a subset of five individuals to assess cross-reactivity.

Results: Fourteen per cent of workers were sensitized to rats and 9% to mouse. Over half (62%) of rat sensitized individuals were also mouse sensitized and the majority (91%) of mouse sensitized individuals were also rat sensitized. IgE cross-reactivity was demonstrated between rat and mouse urine using RAST inhibitions. Rates of atopy did not differ between rat only sensitized individuals compared with those sensitized to both species. Sensitization to cats and rabbits was more common amongst those with dual sensitization.

Conclusions: Dual sensitization to rat and mouse reflects IgE cross-reactivity rather than atopy. Individuals with dual sensitization are more likely to be sensitized to other animal allergens. These findings will have implications for individuals working with only one rodent species who develop sensitization and symptoms to be aware of the potential for allergy to other species.

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Individuals working with laboratory animals are at risk of developing occupational allergy and asthma. Rats and mice frequently used in animal research are the most common causes of laboratory animal allergy (LAA). Sensitization to both rat and mouse can develop in employees exposed to only one species, although the reason for this dual sensitization is unclear. It may reflect a predisposition to developing allergy and this is supported by the fact that atopy is a major risk factor for the development of LAA (1–6). Alternatively because rat and mouse allergens are closely related immunological cross-reactivity may exist between allergens from these two species.

The major rat and mouse allergens, respectively, Rat n 1 and Mus m 1, belong to the lipocalin family of proteins and share 66% homology in their amino acid sequence (7). Since Rat n 1 and Mus m 1 share a high degree of sequence homology and an overall 3D structure they may also share cross-reactive IgE binding determinants. However, several very early studies failed to find evidence of cross-reactivity between rat and mouse allergens (8–10)

although one study did observe a partial identity between rat, mouse and guinea pig urine in a single rat allergic patient's serum (11).

For the individual working with either rat or mouse and becoming sensitized to the urinary allergens, it is important to elucidate the degree of cross-reactivity between the allergens from both species, as this may have an important impact on their employment. Moreover identifying cross-reactive structures may also allow for the development of efficient modes of immunotherapy in which specific cross-reactive epitopes can be used to treat multiple allergies.

In a large cross-sectional study of laboratory animal workers, we investigated atopy and the frequency of sensitization to rat and/or mouse allergens in association with specific exposures in order to understand whether sensitization to both species reflected atopy or immunological cross-reactivity. Using RAST, RAST inhibition and western blotting techniques, we investigated the degree of cross-reactivity between rat and mouse urine in a subset of individuals from this cross-sectional study.

Materials and methods

Subjects and exposure assessment

A cross-sectional study of 776 individuals exposed to laboratory rats and mice in the workplace was carried out between September 1999 and May 2001. Blood was taken from 718 individuals, all of whom had current or past exposure to rats at the time of the survey. We identified those who had also been exposed to mice at some stage in their career ($n = 498$; 'rat and mouse exposed') and compared these with the remaining 220 employees ('rat only exposed'). Information on current and previous employment was collected using a detailed questionnaire. Dates of first and most recent handling of rats were recorded so that an estimate of duration of contact with rat proteins could be made.

The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospital.

Skin prick testing

For skin prick tests we used physiological saline, 1% histamine, cat, grass pollen and *D. Pteronyssinus* (Allergopharma, Reinbek, Germany) and in-house extracts of rat and mouse urines as previously described (12). A positive response was regarded as a wheal size of ≥ 3 mm. Atopy was defined as a positive response to tests with one or more common aeroallergens. Individuals were also tested for their sensitivity to rabbit epithelium, guinea pig epithelium and dog dander (Allergopharma).

Measurement of specific IgE

Specific IgE to extracts of rat and mouse urine were measured using the radioallergosorbent test (RAST) as described previously. Results are expressed as the percentage binding of the total ^{125}I anti-IgE added. A percentage binding of $\geq 2\%$ was considered positive (13).

Rat and mouse urine preparation

Urine was collected from male Wistar rats and adult male mice (strain unknown) by the use of a metabolic cage (Techniplast, Varese, Italy). The pooled urine was filtered (grade 1 paper, Whatman International Ltd, Maidstone, Kent, UK) and concentrated with the use of an Amicon CH2A hollow-fiber system and dialysed against distilled water and freeze dried. Rat and mouse urine extracts were stored at -20°C until needed.

RAST inhibitions

Self-inhibitions were carried out by inhibiting the binding of specific IgE to rat urinary protein using rat urinary protein as an inhibitor and likewise for mouse urinary protein. Competitive RAST inhibitions were conducted to determine the inhibition of binding of IgE to rat urinary protein using mouse urinary protein as an inhibitor and also the inhibition of binding of IgE to mouse urinary protein using rat urinary protein as an inhibitor. The inhibitions were carried out using serum from five individuals from the survey population who had specific IgE ($> 10\%$ binding in the RAST) to both rat and mouse urinary protein and whose volume of plasma was sufficient to complete the inhibition experiments. The plasma (50 μl) was incubated overnight at room temperature with 1 mg/ml of the inhibiting allergen. Uninhibited samples were set up in parallel whereby plasma was incubated with PBS alone overnight. Inhibited

and uninhibited samples were then incubated for 16 hours with discs containing rat or mouse allergen. The discs were then washed and processed as above in the RAST assay.

Electrophoresis

Rat and mouse urinary protein were separated by NuPAGE[®] Bis-Tris gel system using a 4–12% acrylamide gel according to manufacturer's instructions (Invitrogen Life Technologies, Paisley, UK). Reduced samples of rat and mouse urinary protein (10, 15, 20, 25 $\mu\text{g}/\text{ml}$) were electrophoresed in parallel with molecular weight markers. Gels were stained with colloidal blue according to the manufacturer's instructions. The relative mobilities of the marker proteins were plotted against the log molecular weight (in kilodaltons), and the molecular weight of the protein bands were interpolated manually.

Western blotting

Rat and mouse urinary proteins (10 $\mu\text{g}/\text{ml}$) were separated by NuPAGE[®] Bis-Tris gel system and then transferred to a nitrocellulose membrane using the NuPAGE western blotting procedure according to manufacturer's instructions (Invitrogen Life Technologies). The membranes were cut into 6 mm wide strips, blocked for nonspecific binding with blocking buffer (PBS/1% bovine serum albumin (BSA)/0.05% tween) for 3 h at room temperature and probed with 3.5 ml aliquots of plasma [diluted 1 : 5 with PBS/0.3% human serum albumin (HSA)] for 16 h at room temperature with shaking. The strips were washed three times with washing buffer (5.8% NaCl/0.5% Tris/0.2% Tween) and then probed with ^{125}I anti-human IgE (Sweden Diagnostics Ltd, Milton Keynes, Little Chalfont, UK) diluted 1 : 10 with PBS/0.3% HSA for 16 h at room temperature with shaking. Following a further three washes they were then dried and exposed to Hyperfilm MP (Amersham Pharmacia Biotech, UK) at -70°C for 10 days.

Inhibition studies were carried out by incubating the inhibitor allergen at a concentration of 1 mg/ml with the plasma in equal volumes for 16 h prior to incubation with the nitrocellulose strip.

Statistical methods

Differences in sex, rates of atopy and prevalence of skin test positive results between exposure and sensitization groups were investigated using chi-squared tests and Fisher's exact tests. Mann–Whitney tests were used for continuous data, such as duration of exposure and level of IgE. All analyses were undertaken using SAS statistical software (SAS, Cary, NC, USA).

Results

Subjects

Laboratory animal employees were categorized according to their exposure and sensitization status (Tables 1 and 2). Those exposed to both rats and mice had a longer median duration of exposure than rat only exposed individuals (10.5 years vs 4.2 years, $P \leq 0.001$), (Table 1).

Specific IgE to rat and/or mouse

Specific IgE measurements were carried out to rat and mouse urine for all individuals. 14% ($n = 100$) of the

Table 1. Characteristics and prevalence of sensitization to rat and/or mouse of employees who have been exposed to rat only ($n = 220$) or both rat and mouse ($n = 498$)

	Rat only exposed employees ($n = 220$)	Rat and mouse exposed employees ($n = 498$)	<i>P</i>
Male, n (%)	118 (54)	261 (52)	0.76
Age, median (range) (in years)	33 (18–65)	33 (17–59)	0.90
Duration of exposure to rats, median (range) (in years)	4.2 (0.1–37.1)	10.5 (0.1–41.6)	<0.001
Atopic, n (%)	107 (49)	207 (42)	0.08
IgE level to rat urine % binding (range)	0.6 (0.2–75.0)	0.7 (0.2–67.7)	0.38
Rat IgE negative	192 (87)	419 (84)	0.69
Mouse IgE negative $n = 611$, n (%)			
Rat IgE positive	11 (5)	27 (5)	
Mouse IgE negative $n = 38$, n (%)			
Rat IgE negative	1 (0.5)	5 (1)	
Mouse IgE positive $n = 6$, n (%)			
Rat IgE positive	16 (7)	46 (9)	
Mouse IgE positive $n = 62$, n (%)			

Table 2. Characteristics and prevalence of skin prick test positivity to various allergens in rat only or both rat and mouse sensitized individuals

	Rat only sensitized employees ($n = 38$)	Rat and mouse sensitized employees ($n = 62$)	<i>P</i>
Male, n (%)	24 (63)	33 (53)	0.33
Age, median (range)	31 (18–61)	33 (20–58)	0.28
Duration of exposure to rats, median years (range)	8.9 (0.6–23.0)	9.7 (0.2–30.3)	0.41
Atopic, n (%)	27 (71)	45 (74)	0.77
IgE level to rat urine, % binding (range)	3.9 (2.1–25.9)	10.5 (2.21–75.0)	<0.001
Cat SPT positive, n (%)	7 (18)	27 (44)	0.01
Rabbit SPT positive, n (%)	1 (3)	12 (23)	0.02
Guinea pig SPT positive, n (%)	3 (8)	11 (18)	0.16
Dog SPT positive, n (%)	2 (7)	9 (17)	0.17
Grass pollen SPT positive, n (%)	18 (47)	35 (57)	0.33
House dust mite SPT positive, n (%)	20 (53)	33 (54)	0.89

SPT, skin prick test.

total population were sensitized to rat and 9% ($n = 68$) to mouse. Over half (62%) of those with rat specific sensitization were also sensitized to mouse, whereas for those sensitized to mouse, the percentage of individuals with rat sensitization was much higher (91%). Interestingly of those only exposed to rats, 8% (17/220) were shown to have mouse specific sensitization, this proportion was similar for those exposed to both rat and mouse (10%, 51/498) (Table 1). Individuals who were sensitized

to both rodent species had a higher specific IgE binding to rat urine than individuals who were only monosensitized to rats (10.5 vs 3.9; $P < 0.001$; Table 2). Whether an individual was sensitized to rats only or rats and mice was not dependent on the species of animal with which they were first exposed. Of the 38 rat only sensitized, first exposure information was available for 35 individuals. Twenty (57.1%) were first exposed to rats and 15 (42.9%) were first exposed to mice. Similar proportions were found for rat and mouse sensitized individuals. Of the 58 on which first exposure information was available 34 (58.6%) were first exposed to rats and 24 (41.4%) were first exposed to mice. These proportions were not significantly different ($P = 0.89$). 36.1% (13/36) of those only sensitized to rats and 50.8% (31/62) of those sensitized to both species reported work related chest symptoms. Prevalence of work related eye, nose or chest symptoms were not statistically different between the two groups (data not shown).

Skin prick tests

We found no significant differences amongst the 38 subjects sensitized to rats only and the 62 sensitized to both rat and mouse in terms of prevalence of atopy, gender, age, or duration of exposure (Table 2). However, compared with rat only sensitized individuals, a greater proportion of those with sensitization to both rodent species had positive skin prick tests to cats (44% vs 18%; $P = 0.01$), rabbit (23% vs 3%; $P = 0.02$), guinea pig (18% vs 8%; $P = 0.16$) and dog (17% vs 7%; $P = 0.17$) (Table 2). The percentage of positive skin prick tests to grass pollen or house dust mite did not differ significantly for those individuals sensitized to rats only or both species (Table 2).

RAST inhibitions using rat and mouse urinary proteins

Self-inhibition with either rat or mouse urinary protein resulted in over 90% inhibition in all but one subject at the concentration of 1 mg/ml. In general, rat urine was able to inhibit the binding of specific IgE binding to mouse urinary protein (median 68% inhibition, 53.2–89.6) to a greater extent than mouse urine was able to inhibit the binding of specific IgE to rat urinary protein (median 50% inhibition, 35.4–79.1). Inhibition profiles were unique for each individual. An example of a RAST inhibition profile is shown in Fig. 1.

Electrophoresis of rat and mouse urinary protein

Electrophoresis of rat and mouse urinary protein revealed a number of proteins (Fig. 2A,B). Within the rat urine, three strong bands at 9, 17 and 75 kDa and three weaker bands at 21, 23, and 58 kDa were evident. The protein identified in the 17 kDa region existed as one major and two minor bands as previously published (14). One major

and one minor band were identified in the 19 kDa region of mouse urine. A further three weaker bands were revealed with molecular weights of 22, 40 and 75 kDa (15–17).

Western blotting

All individuals bound the major allergens in rat and mouse urinary protein: Rat n 1 (17 kDa) and Mus m 1

(19 kDa) respectively except for one subject whose immunoblot was too weak to detect any binding to mouse allergens (data not shown).

In addition plasma from subject 1 bound additional allergens (Fig. 3A) demonstrating specific IgE binding with the 58 kDa protein in rat urine and the 40 kDa protein in mouse urine.

Inhibition immunoblot

A competitive inhibition immunoblot was carried out on the plasma sample from subject 1, (Fig. 3B,C). Both rat and mouse urine was able to inhibit the binding of specific IgE with rat urine at 17 and 58 kDa. Similarly rat and mouse urinary proteins were able to inhibit specific IgE binding with the range of proteins in mouse urine, although rat urine did not completely inhibit specific IgE binding to the 19 kDa mouse urinary protein.

Sequence alignment of Rat n 1 with Mus m 1 and 3D modelling

The alignment of the major rat and mouse allergens, Rat n 1 and Mus m1 respectively, indicates that the overall sequence homology between the two proteins is 66%. Figure 4 shows that there are a number of short stretches of amino acids along the two molecules in which sequence homology can be observed. Those over five amino acids

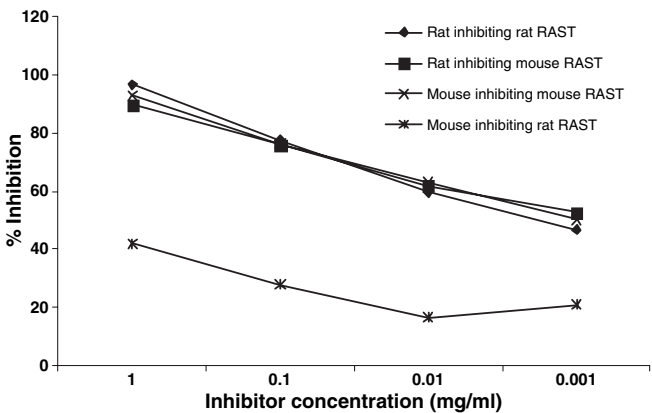


Figure 1. Example of a RAST inhibition experiment from subject 1.

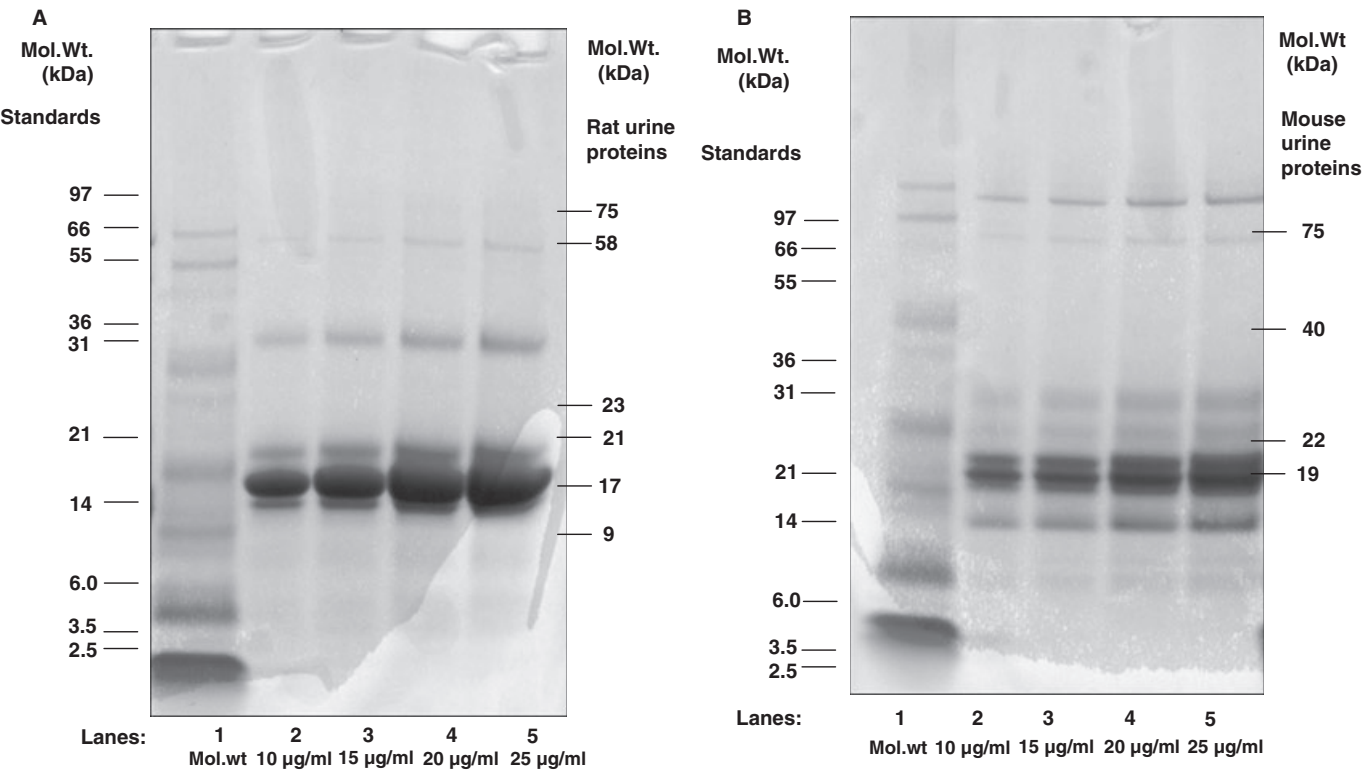


Figure 2. Gel electrophoresis of rat urine (A) and of mouse urine (B), each at four different concentrations.

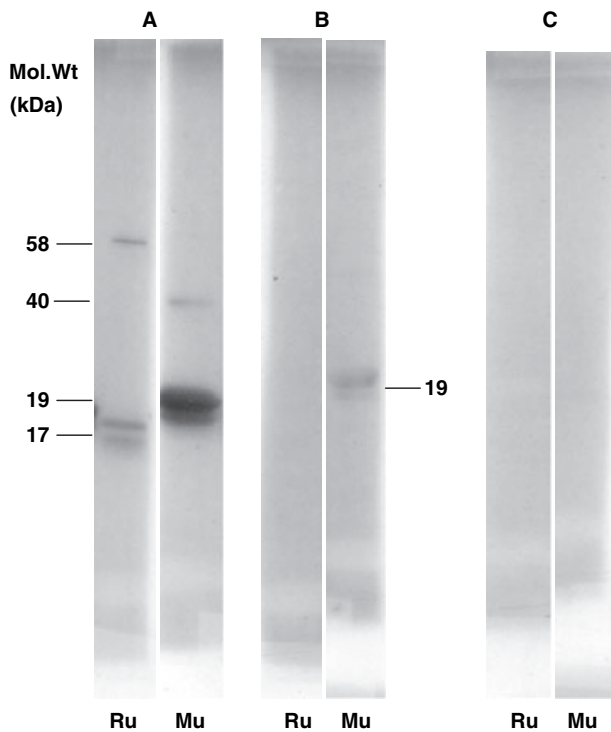


Figure 3. Immunoblot of serum from subject 1 with rat and mouse urine (A), blot inhibition with rat urine (B) and mouse urine as inhibitor (C).

in length are labeled as regions 1–9. Part of region 4 and region 5 are located in the second conserved region of the molecules while region 7 is located in the third conserved region. The mapping of these identical residues on the surfaces of Rat n 1 and Mus m 1 (Fig. 5) using the Swissprot PDB viewer (<http://www.expasy.org/spdbv/> accession number P02761 (rat), P02762 (mouse)) demonstrates that the homologous regions 7, 8, 2 and 6 in the amino acid sequence lie next to one another, on both molecules, in their three-dimensional form. This results in a large accessible area which is identical between the Rat n 1 and Mus m 1 molecules. The residues in region 6 are

located on beta strand H of the molecules and regions 7 and 8 are located at the C terminus on the coiled alpha helix and strand I respectively while region 2 lies in loop one at the N-terminus of the molecule.

Discussion

Allergy to both rat and mouse is common amongst individuals working with laboratory animals. We have demonstrated within a workforce of laboratory animal workers that more than half (63%) of rat sensitized individuals also have evidence of specific IgE to mouse urine and the majority of mouse sensitized individuals are also sensitized to rats (90%). We hypothesized that this dual sensitivity could be explained by either a predisposition to allergy, for example, atopy or cross-reactivity between the allergens from these two species.

Rates of atopy did not differ significantly between individuals sensitized to rats only compared with those sensitized to both species, indicating that a predisposition to allergy was not an important factor in whether an individual was likely to have dual sensitization. Furthermore, we observed similar rates of sensitization to mouse in both rat only exposed workers compared with those exposed to both species suggesting cross-reactivity as the most likely explanation for these findings.

We investigated cross-reactivity between rat and mouse urinary proteins using detailed inhibition experiments and observed that rat urine inhibited the binding of specific IgE to mouse urine (53–90%) more strongly than mouse urine was capable of inhibiting specific IgE binding to rat urine (35–79% inhibition). Self-inhibition with the homologous antigen was high. Similarly, immunoblotting results also demonstrated cross-reactivity between the separated proteins of rat and mouse urine to which the individual had specific IgE. Thus, we have demonstrated cross-reactivity between rat and mouse urine using both RAST inhibition and blot inhibition techniques. Furthermore, these results suggest that there are common epitopes within the urinary allergens of rat

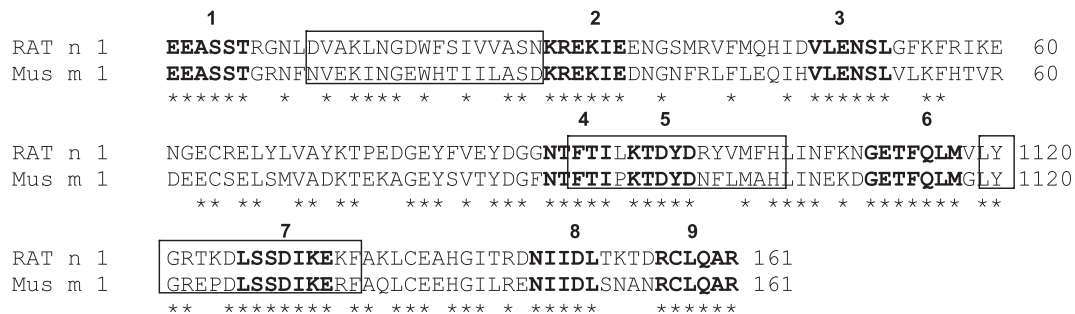


Figure 4. Sequence alignment of Rat n 1 and Mus m 1. Asterisks denote identical residues between the molecules. Letters highlighted in bold and numbered 1–9 indicate identical sequences of more than five amino acids in length. Boxed regions denote the structurally conserved domains of the lipocalins.

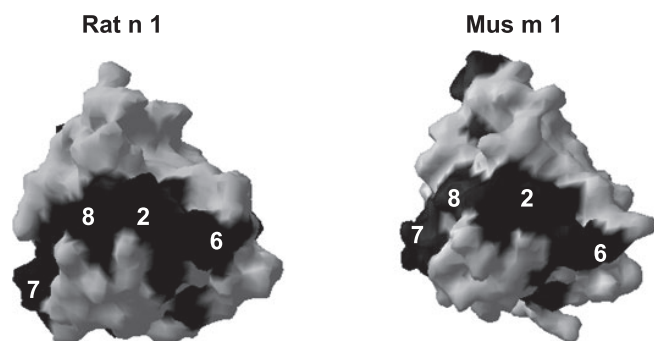


Figure 5. Three dimensional structure of Rat n 1 and Mus m 1 highlighting where the identical linear sequences between the two molecules lie in the conformational form.

and mouse, but proteins from rat urine in particular contain a greater number of unique epitopes.

The B cell epitopes of Rat n 1 have not as yet been determined. Bayard et al. attempted to map the IgE binding regions of Rat n 1 using overlapping peptides but found great individual variation between sera from rat allergic individuals in their binding to these peptides (18) although some were recognized more frequently than others. Of particular interest in our study was the fact that all sensitized individuals exhibited unique inhibition profiles. This may have been the result of differing patterns of IgE binding to the various allergens within the rat and mouse urine by each individual. Taken together, the findings of Bayard et al. and results from our study demonstrate that Rat n 1 and Mus m 1 are likely to contain multiple B cell epitopes some of which are unique and others which are shared between the two allergens.

Mapping of the identical residues on the surfaces of the 3D structure of Rat n 1 and Mus m 1 revealed a substantial area in which several of the identical amino acids in the primary sequence (regions 2, 6, 7 and 8) join together to form a large accessible site for potential IgE binding. The majority of these residues are located at the C terminus of the molecule, with the exception of region 2 which is located at the N-terminus. This is consistent with reports of B cell epitopes being more likely to be located in flexible and protruding regions such as the terminal parts of the protein (19). Interestingly, regions 2 and 7 (Fig. 4) correlated with the peptides that were recognized by IgE antibodies in sera from rat allergic subjects in the study by Bayard et al. (18) and region 7 is located in the conserved region of the molecules, indicating that this area may be a common cross-reactive epitope amongst all lipocalin allergens. Furthermore, the regions within the cow lipocalin allergens, Bos d 2, and Bos d 5 of most importance for IgE binding were located in the carboxy terminal portion of the molecules (20–22). Thus it is likely that both Rat n 1 and Mus m 1 contain a conformational epitope made up of identical linear sequences from the terminal ends of their primary structure to which IgE antibodies are directed, resulting in cross-reactivity between the two proteins.

A recent study demonstrated IgE cross-reactivity between Mus m 1 from mouse and Equ c 1 from horse (23). Mus m 1 and Equ c 1 have only a 43% sequence homology; however they contain an area of similar amino acid sequence which is likely to be the cross-reactive epitope between both molecules. Rat allergen was not included in this study but it is possible given the cross-reactivity between rat and mouse urinary allergens that we have observed that Rat n 1 contains an epitope that is also cross reactive with Equ c 1.

We were interested to find in our study that individuals who were sensitised to both rat and mouse had a significantly higher IgE titre to rat urine compared with those only sensitized to rats. Given that we have demonstrated cross-reactivity between rat and mouse urinary allergens, it is likely that IgE directed to epitopes on mouse allergens that cross react with epitopes on rat allergens are responsible for the IgE titre to rat urine being raised in those with dual sensitization. Furthermore, positive skin prick tests to other animals such as cat and rabbit were more commonly seen amongst those sensitized to both rodent species rather than rats only. These findings indicate that dual sensitization to rat and mouse is associated with immune responses to a broader spectrum of animal allergens.

This study was carried out on a large, carefully defined cross-sectional population and is the first to date to carry out detailed immunological techniques to assess cross-reactivity between rat and mouse urinary allergens. One limitation was that we were unable to determine whether rat only exposed individuals, despite never handling mice in the past or at the time of the study, were also being exposed to mouse allergen within the animal houses where they worked with rats. Nevertheless our study has confirmed that cross-reactivity does indeed exist between rat and mouse urinary allergens and the degree of cross-reactivity is likely to be dependent on the various epitopes being recognized on the major allergens from these two species. Furthermore, we have shown that allergy to mouse in the absence of allergy to rats is uncommon in individuals working with laboratory animals and allergy to other animal species is less common amongst rat monosensitized individuals compared with those sensitized to both rat and mouse. These findings have important implications for those working with laboratory animals. In particular it is necessary for individuals working with only one rodent species who develop sensitization and symptoms to be aware of the potential for allergy to other species. Strategies developed for immunotherapy would need to be aware of the cross-reactive epitopes between Rat n 1 and Mus m 1.

Acknowledgments

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