

Allergens, IgE, mediators, inflammatory mechanisms

Analysis of rat urine proteins and allergens by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

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Background: In rats, urine has been identified as a major source of the allergens that cause laboratory animal allergy, an important occupational health problem.

Methods and Results: Urinary proteins and allergens of Wistar rats were studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. Proteins excreted by male and female rats during puberty were similar and of low molecular weight. In adulthood, moderate increases in diffuse staining of 26 and 21 kd occurred in female urine. In males the 17 kd protein increased dramatically and the 23 and 21 kd proteins moderately. The urine excretion of high-molecular-weight proteins (75 to 63 kd) increased with age in males (females not studied). Immunoblot studies with six sera showed allergens in urine of male and female rats of all ages, three of which were present in all urine (75, 68, and 21 kd). Three allergens (17, 16, and 15 kd) in female urine may be allergenically similar to the 17 kd allergen in adult male urine. Adult male urine allergens were studied further with sera from 83 rat-hypersensitive subjects. Major allergens were identified at 23, 21, and 17 kd, and all sera had IgE to one or more of these proteins. Twenty-seven percent had IgE to 68 and 63 kd allergens. Minor allergens were identified at 75, 51, and 44 kd.

Conclusions: Rat urine is an important source of the major allergens associated with rat hypersensitivity. Age and sex markedly influence the protein and allergenic constituents of rat urine. (*J ALLERGY CLIN IMMUNOL* 1993;92:298-305.)

Key words: Laboratory animal allergy, rat urine, proteins, allergens, age, sex

Laboratory animal allergy is recognized as an important occupational health problem, affecting approximately 30% of those people working with small animals.¹⁻⁷ Urine has been identified as a major source of the responsible allergens in rat-sensitive subjects.⁸

Previous studies of rat urinary proteins have distinguished a major protein with the electrophoretic mobility of an α_{2u} -globulin and other bands of prealbumin and albumin.⁹⁻¹¹ These pro-

Abbreviations used

a:	Adult rat
CRIE:	Crossed radioimmuno-electrophoresis
F:	Female rat
M:	Male rat
p:	Pubertal rat
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate-buffered saline
SDS:	Sodium dodecyl sulfate

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Received for publication May 28, 1992; revised Jan. 13, 1992; accepted for publication Feb. 5, 1993.

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0091-6749/93 \$1.00 + .10 1/1/46300

teins were further characterized by Longbottom,¹² who found the α_{2u} -globulin had an approximate molecular weight of 17 kd (isoelectric point values of 5.4, 5.6, and 5.8) and the prealbumin an approximate molecular weight of 21 kd (isoelectric point values 4.5 and 4.6).

The allergenicity of rat urine has been demonstrated in numerous epidemiologic studies by skin

prick testing and RAST,³⁻⁶ and the low-molecular-weight constituents (α_{2u} -globulin and prealbumin) identified as the most potent allergens.^{8, 12} Walls and Longbottom¹³ studied the allergenicity of several rat-derived materials by crossed radioimmunoelectrophoresis (CRIE) with the use of 14 rat-sensitive sera. Urinary α_{2u} -globulin (Ag 13) and prealbumin (Ag 4) were found to be "major" allergens and a further 17 minor urinary allergens were described including albumin (Ag S1) and six unidentified serum proteins.¹³ This technique is widely applied for allergen characterization but the results obtained can be influenced by the specificity of the antiserum and the relative concentrations of the antigen and antiserum used.^{14, 15}

We have applied the electrophoretic technique of Laemmli¹⁶ to examine the influence of age and sex on the urinary proteins excreted by rats. The application of Western blot techniques or immunoblotting to investigate the allergenicity of male and female rat urine during and after puberty and a study of male rat urinary allergens with 83 individual rat allergic sera are described for the first time.

METHODS

Patients

Serum was obtained from 146 patients attending occupational allergy clinics and was stored at -20°C . All subjects had allergic symptoms associated with laboratory animal exposure.

Measurement of specific and total IgE

Specific IgE (RAST). Paper discs (Schleicher and Schuell, from Anderman Company Ltd., Kingston-upon-Thames, United Kingdom) were activated with cyanogen bromide (Sigma Chemical Co., Poole, Dorset, United Kingdom). Three milligrams of lyophilized rat urine (collected from 3-month-old males in a metabolic cage) or mouse urine (collected in a similar manner) were coupled per 300 mg of activated discs by the method of Ceska et al.¹⁷ Rabbit epithelia and guinea pig epithelia discs were purchased (Kabi Pharmacia, Uppsala, Sweden).

Serum (50 μL) was incubated overnight per disc in duplicate. Bound allergen-specific IgE was detected with the use of ^{125}I anti-IgE (Kabi Pharmacia) and results expressed as a percentage of the total iodine 125 anti-IgE added.

Specific IgE to rat urine was measured in all sera ($n = 146$) and to mouse urine, rabbit epithelia, and guinea pig epithelia in eight sera.

Total IgE. The total amount of IgE was determined in eight sera with a paper radioimmunosorbent test kit (Kabi Pharmacia) according to the manufacturer's instructions.

Antigen preparation

Urine was collected from Wistar rats by the use of a metabolic cage (Techniplast, Varese, Italy). The effect of sex and puberty was studied with urine collected from male (M) and female (F) rats that were 6 to 8 weeks old (pubertal, p) and 12 weeks old (adult, a). Appropriately pooled urine was filtered (grade 1 paper, Whatman International Ltd, Maidstone, Kent, United Kingdom), dialyzed against distilled water, and freeze-dried.

The influence of age was examined with urine collected daily from male rats for 9 months beginning at age 3 months. Urine was pooled into 1-month age groups, filtered, and concentrated with an Amicon CH2A hollow-fiber system (Amicon Ltd, Gloucestershire, United Kingdom), and processed as previously stated.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in a Protean II xi vertical slab gel electrophoresis cell (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, United Kingdom). A 15% resolving gel and 3% stacking gel were prepared according to the method of Laemmli¹⁶ with the use of reagents purchased from Bio-Rad and BDH (Merck Ltd, Dagenham, Essex, United Kingdom). Electrophoresis was done at 18 mA per gel for 1 hour and 30 mA per gel for 4 hours.

Samples were dissolved in sample buffer (0.0625 mol/L TRIS HCl, 3% wt/vol SDS, 10 mmol/L iodoacetamide, 0.1 mg/ml bromophenol blue, and 0.1% vol/vol glycerol) for 20 minutes at 60°C (4 mg/ml). Twenty microliters of each rat urine sample was loaded per well and 5 μL of low-molecular-weight marker proteins (Pharmacia Biotechnology) were subjected to electrophoresis in parallel.

The proteins were fixed, stained with Coomassie Brilliant Blue (Merck Ltd., Diagnostics Div., Dorset, United Kingdom) (0.1% wt/vol Coomassie Brilliant Blue, 50% vol/vol methanol, 10% vol/vol acetic acid), and the gel destained. 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.

Immunoblotting

Preparation of solid phase. Gels for immunoblotting were prepared as previously described. The molecular weight markers were excised and stained and the remaining gel blotted overnight onto nitrocellulose (0.45 μm pore size, Bio-Rad) by the method of Towbin et al.¹⁸ and 0.1 mA. The nitrocellulose was blocked with TRIS washing buffer (0.5 mol/L sodium chloride, 0.02 mol/L TRIS, and 0.1% vol/vol Tween 20 [Merck Ltd., Diagnostics Div.]) and dried.

Incubation with serum and anti-IgE label.

(1) *Analysis of allergens in Ma, Mp, Fa, and Fp urine:* Sera (20 ml) was incubated overnight with nitrocellu-

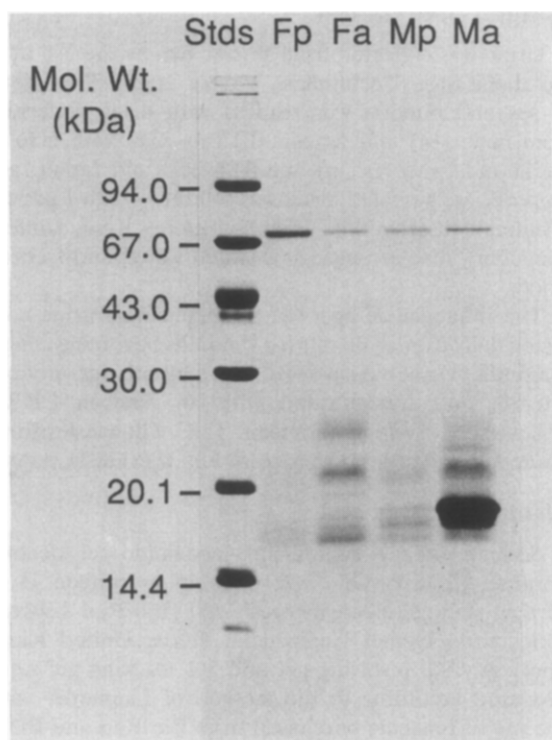


FIG. 1. Analysis of rat urine proteins by SDS-PAGE (15% resolving gel, nonreduced conditions) stained with Coomassie Brilliant Blue. Tracks loaded with 40 μ g of Fp urine, Fa urine, Mp urine, and Ma urine. *Mol. wt.*, molecular weight; *kDa*, kilodaltons; *Stds*, standards.

lose sheets (4 $\times$ 12 cm). The immunoblots were washed with TRIS washing buffer (three times) and distilled water (one time) before incubation overnight with ^{125} I anti-IgE (Pharmacia) diluted to 1 to 1.5 $\times$ 10⁵ cpm/ml (10 ml). After further washing the immunoblots were dried, exposed to x-ray film (Hyperfilm, Amersham, United Kingdom) in an x-ray cassette (Everything X-Ray Ltd, Watford, Herts, United Kingdom) fitted with intensifying screens (Quanta III, Du Pont Co., Wilmington, Del.) at -70° C for 24 hours, and developed.

(2) *Screen of sera sensitive to Ma urine:* Sera with more than 21% binding in the rat urine RAST were diluted (phosphate-buffered saline [PBS], 0.3% wt/vol human serum albumin, 0.1% wt/vol sodium azide) to give an optimum RAST binding of 10%. One nitrocellulose strip (0.5 cm wide) was incubated with serum (2 ml) overnight. All strips were treated as before with 2-ml reagent volumes and exposed for 24, 60, and 140 hours.

The molecular weights of allergens were manually determined and the relative intensity of the radiostaining graded as follows: band visible after 140 hours, 1; band visible after 60 hours, 2; band visible after 24 hours, 3; medium staining after 24 hours, 4; and strong staining after 24 hours, 5.

RESULTS

Variation in rat urine proteins with sex and age

The proteins present in the urine of male and female rats by SDS-PAGE (nonreduced) are shown in Fig. 1. Little protein was found in the urine of Fp rats; one strongly staining band (68 kd) and five faintly staining bands (20 to 15 kd) were visible (Fig. 1, *Fp*). With urine from sexually mature female rats, diffuse staining appeared in the 26 kd and 21 kd regions and minor bands were again visible at 20, 17 ($n = 2$), 16, and 15 kd (Fig. 1, *Fa*). Similarly, less protein was found in Mp than in Ma urine. The bands seen in Mp urine were similar to those in the Fp urine but with an additional faint diffuse staining region at 26 kd and stronger staining of the bands in the 20 to 15 kd region (Fig. 1, *Mp*). Ma urine showed a dramatic increase of the 17 kd band and two minor bands of similar molecular weight. An increase in the staining of the diffuse bands at 23 kd and 21 kd was also seen (Fig. 1, *Ma*).

Fig. 2 shows the protein profiles by nonreduced SDS-PAGE of male rat urine with increasing age. The high-molecular-weight proteins in male rat urine increased with age of the rat in contrast to the low-molecular-weight proteins. Trace amounts of a 68 kd protein were visible at 3 to 4 months of age, but became the most predominantly staining band in the urine sample from 11- to 12-month-old rats (Fig. 2, *track 11*). Other high-molecular-weight constituents (75 kd and 63 kd) behaved similarly. Two additional proteins were visible in the urine sample from the oldest rats at more than 94 and 54 kd.

Variation in rat urine allergens by age and sex

Six sera were selected that had IgE to the 17 kd allergen ($n = 2$, sera A and B), all the low-molecular-weight allergens ($n = 2$, sera C and D), and the low- and high-molecular weight constituents ($n = 2$, sera E and F) of Ma urine for the study of the influence of age and sex of the rat on rat urinary allergens. All sera detected allergens in the urines tested (Fig. 3; three sera shown). Sera A and B had IgE to three components in female rat urine (17, 16, and 15 kd). Sera C and D showed diffuse radiostaining at 21 kd in all urine, which was particularly dense in Ma, Fa, and Mp urine. Sera E and F confirmed that the 75 and 68 kd allergens were present in all the urine samples.

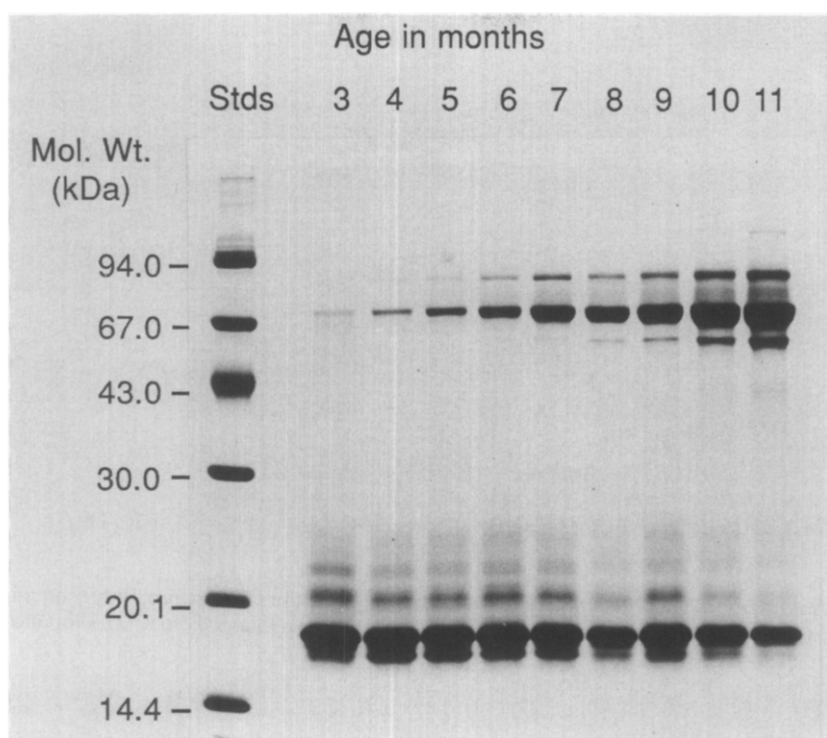


FIG. 2. Analysis of urine proteins from adult male rats of increasing age by SDS-PAGE (15% resolving gel, nonreduced conditions) stained with Coomassie Brilliant Blue. Tracks loaded with 40 μ g urine; *track 3* shows results with urine from rats 3 to 4 months old and remainder of tracks continue in monthly increments to *track 11*, which shows results in urine from rats 11 to 12 months old. *Mol. wt.*, Molecular weight; *kDa*, kilodaltons; *Stds*, standards.

Analysis of Ma urine allergens by RAST and immunoblotting

RAST. Fifty-four sera had rat urine specific IgE of more than 21% and were diluted (range, 1 in 2 to 1 in 80). Fig. 4, *tracks 10, 11*, and 22, show sera with RAST binding of 23.5%, 49.6%, and 29.8%, respectively, and the loss of definition is seen. These were further diluted and retested.

The geometric mean of RAST values for sera with a positive immunoblot to Ma urine was 8.7% ($n = 83$; range, 1.0% to 20.7%) and for the negative immunoblot sera was 0.7% ($n = 63$, range 0.2% to 14.4%). Ten of the sera with a negative immunoblot result had RAST values of 1.5% or higher. Further tests on eight of these sera showed all had raised total IgE levels (greater than 250 IU/ml) and three had raised specific IgE to other animal species (Table I).

Immunoblotting

Sera from 146 subjects were used to examine the allergens in Ma urine by immunoblotting. A positive result was defined as one or more bands

visible after 140 hours of exposure at -70° C. Eighty-three (57%) sera were positive to Ma urine by this criterion. Occasionally, several bands were visible in the 17 kD region (Fig. 4, *tracks 13* and 16), which we did not differentiate between for the purpose of this study. Eight radiostaining bands (allergens) were detected, and the molecular weights and detection frequencies are shown in Table II.

All positive sera bound to one or more of the low-molecular-weight constituents of male rat urine (23, 21, and 17 kD). Forty-two (50.6%) sera had IgE to all low-molecular-weight bands (Fig. 4, *track 5*), 16 (19.3%) sera to the 17 kD and 23 kD bands (Fig. 4, *track 3*), and 15 (18.1%) sera to the 17 kD band alone (Fig. 4, *tracks 9* and 21). IgE binding to the 21 kD or 23 kD bands, or both, in the absence of binding to the 17 kD band was uncommon (10.8%) (Fig. 4, *track 24*). The 17, 21, and 23 kD allergens fulfill the criteria for "major" allergens as described by Lind and Lowenstein.¹⁴

Twenty-six (31.3%) sera had IgE to the high-molecular-weight constituents of Ma urine (75,

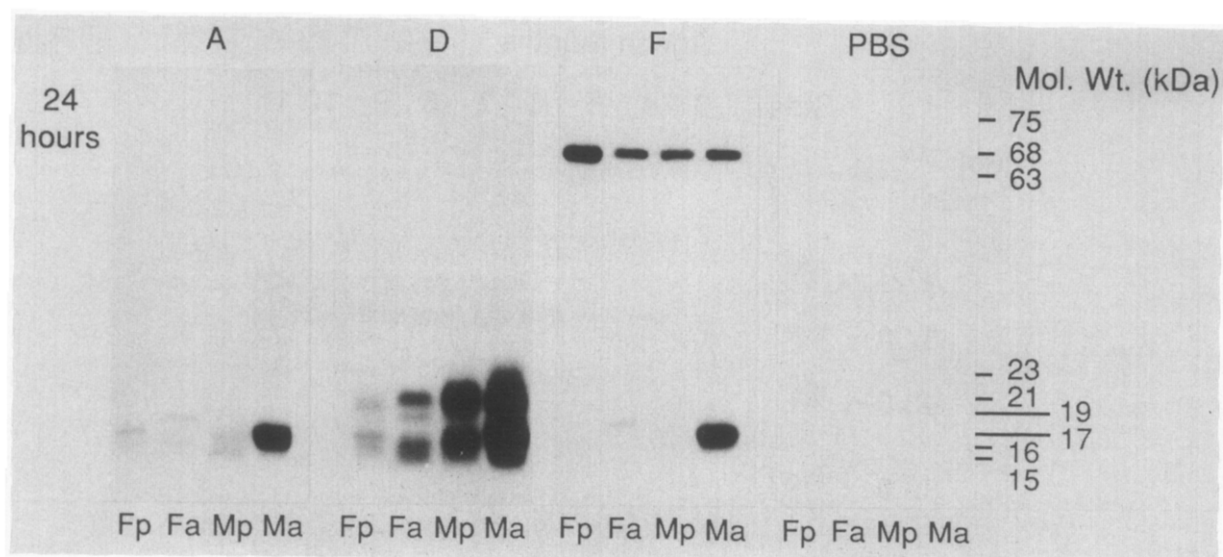


FIG. 3. Autoradiographs of rat urine immunoblots after 24 hours of exposure. Representative sera A, D, and F, and negative control PBS are shown. *Mol. wt.*, Molecular weight; *kDa*, kilodaltons.

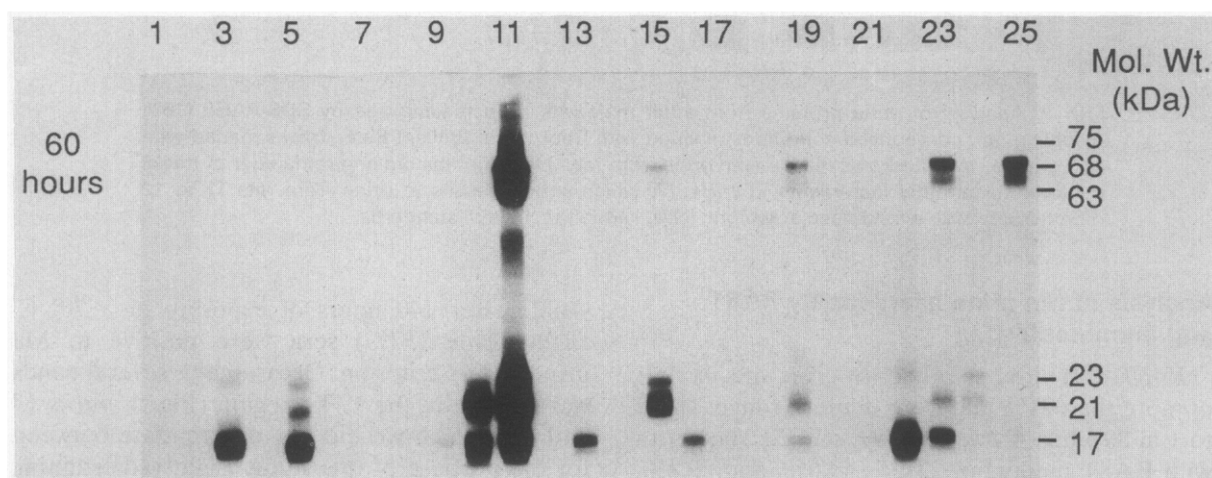


FIG. 4. Autoradiographs of Ma urine immunoblots after 60 hours of exposure. *Track 1*, negative control PBS; *track 2*, negative control, nonallergic serum; and *tracks 3 through 25*, individual sera. *Mol. wt.*, Molecular weight; *kDa*, kilodaltons.

68, and 63 kd), often with radiostaining visible after 24 hours (Table II). Eleven (42.3%) sera had IgE to both the 68 kd and 63 kd allergens (Fig. 4, *tracks 15 and 23*), and 11 sera had specific IgE to all three high-molecular-weight constituents (Fig. 4, *track 19*). All sera that reacted to the high-molecular-weight constituents also bound to low-molecular-weight allergens (Fig. 4, *track 25*).

DISCUSSION

Rat urine has previously been shown to be a potent source of allergens among those sensitive to rats.⁸ We have used modern electrophoretic

techniques to study the influence of age and sex of the rats on the proteins and allergens present in the urine of Wistar rats.

SDS-PAGE analysis under nonreducing conditions showed that the urinary proteins of male and female pubertal rats were similar and that in adulthood both had increased staining of the 21 kd protein. A dramatic increase in the 17 kd protein or proteins and a moderate increase in the 23 kd band was found in Ma urine, which was not seen in Fa urine. The 17 kd protein or proteins excreted in Ma urine existed as one major and two minor bands that were poorly

TABLE I. Total and specific IgE measurements of eight sera that were rat urine RAST positive but negative by immunoblot

Serum	Total IgE (IU/ml)	RAST percent binding			
		Rat urine	Mouse urine	Guinea pig epithelia	Rabbit epithelia
1	15,800	3.4	31.4	10.2	1.8
2	8,360	2.7	0.7	9.5	49.5
3	10,230	1.5	1.7	1.2	18.8
4	2,350	2.5	0.6	2.6	0.5
5	860	9.5	1.7	0.5	0.4
6	430	6.1	0.6	0.6	0.4
7	5,800	14.4	3.8	1.1	0.8
8	280	1.7	1.0	0.4	0.4

TABLE II. Distribution of the male rat urine allergens recognized by 83 positive sera after 140 hours (grade 1 or higher) and 24 hours (grade 3 or higher) of exposure

Molecular weight (kd)	Positive (grade ≥ 1)		Strong staining (grade ≥ 3)	
	No.	%*	No.	%†
75	13	15.7	2	15.4
68	24	28.9	16	66.7
63	22	26.5	12	54.5
51	25	30.1	3	12.0
44	12	14.5	0	
23	65	78.3	21	32.3
21	50	60.2	28	56.0
17	74	89.2	51	68.9

*Percentage of total number positive to rat urine.

†Percentage of number positive to that molecular weight allergen.

separated under the conditions studied. The identical molecular weight and the influence of age and sex suggest that the 17 kd and 21 kd components may be α_{2u} -globulin and prealbumin as described by Roy and Neuhaus,¹¹ Longbottom,¹² and others.^{9, 10}

In this study the end of puberty was taken as the onset of sexual maturity when the male rats could sire and the female rats bear a litter. Roy and Neuhaus¹⁹ have shown that α_{2u} -globulin could be detected in trace amounts in the urine and liver of 40-day-old male rats, which coincided with the onset of puberty and circulating testosterone levels. We have shown that the major change in male rat urine composition occurs with the establishment of full sexual maturity at 8 to 12 weeks of age.

Serial urine collections from male rats between 3 and 12 months old showed increased excretion of high-molecular-weight proteins with increasing age. Rats are known to be susceptible to age-

dependent renal changes,²⁰ and serum proteins in rat urine have been identified by both gel filtration¹² and electrophoretic studies.^{10, 11, 21} We have identified three proteins with molecular weights of 75, 68, and 63 kd that are detectable in urine from male rats 3 to 7 months of age.

Like CRIE, immunoblotting is a qualitative method of allergen characterization, and in both techniques the intensity of radiostaining of allergens depends on the concentration of IgE in the serum, the strength of the anti-IgE label used, and the length of autoradiography. An important difference between the two methods is that CRIE uses an antiserum to precipitate the proteins or carbohydrates and hence allergens may only be identified if they are antigenic for the species in which the antisera is raised. Variation in antigen precipitation can occur as a result of coprecipitation¹⁴ or variation in reagent concentration.¹⁵ Immunoblotting is not subject to these variables, but allergens may be vulnerable to the denaturing and

reducing conditions used for SDS-PAGE.²² We have used nonreducing conditions to allow the greatest chance for proteins to assume their native conformations after blotting.²³

In this study, immunoblotting was as sensitive for detecting IgE as RAST, inasmuch as radiostaining was apparent in most sera with RAST values of greater than 1.0%. It has been suggested that false positives may be observed when Tween 20 is used as a blocking agent alone,²⁴ but no false-positive reactions were observed in this study. Ten (7.0%) sera, however, had rat urine RAST values of greater than 1.5% but were repeatedly immunoblot negative. Edwards et al.²⁵ have demonstrated that a total IgE level of greater than 615 IU/ml is sufficient to cause interference and give a false-positive RAST result in a rat urine system. Eight of the 10 sera in our study were studied further and all were found to have raised total IgE levels (greater than 250 IU/ml). Three sera had elevated specific IgE to other animal species.

Immunoblotting studies that used six selected sera demonstrated the presence of allergens in the urine of male and female rats of all ages. The molecular weights of the allergens detected were very similar to those of the proteins analyzed by SDS-PAGE and showed the same age and sex distribution as those proteins. In general the high-molecular-weight allergens (75 and 68 kd) and the 21 kd allergen did not show sex differences and were present in animals of all ages. Sera with strong IgE binding to the 17 kd allergen in male rat urine had IgE binding to 17, 16, and 15 kd components in female rat urine, and further investigation is required to determine if this is true cross-reactivity. Vandoren et al.²⁶ have recently described a heterogenous protein in female rat urine with similar immunologic but different physicochemical properties to male α_{2u} -globulin. Our study would support that female rats are an additional source of allergenic urinary proteins. Sexual maturity did not affect the allergenicity of female rat urine as judged by the number or density of radiostaining bands, but male rat urine showed a great increase in the 17 kd allergen after puberty.

Immunoblotting with sera from 83 rat-sensitive subjects identified eight allergens in Ma urine with molecular weights of 75, 68, 63, 51, 44, 23, 21, and 17 kd. Distinct patterns of IgE reactivity were apparent. All of the sera reacted to one or more of the low-molecular-weight constituents (23, 21, and 71 kd) that were "major" allergens. This is

further evidence that the 21 kd and 17 kd allergens are the allergens prealbumin (Ag 4) and α_{2u} -globulin (Ag 13) described by Walls and Longbottom.¹³

High-molecular-weight glycosylated forms of α_{2u} -globulin have been reported with molecular weights of 75, 50, 45, and 24 kd,²⁷⁻³⁰ the smallest of which has been detected in the urine. Seventy percent of the serum tested had IgE to both the 17 kd and 23 kd allergens but only one serum had IgE to the 23 kd allergen alone. The 23 kd allergen might therefore represent a glycosylated form of α_{2u} -globulin.

Twenty-six sera had IgE that bound to the high-molecular-weight constituents of male rat urine (75, 68, and 63 kd). Although these are not classified as major allergens, most subjects had a positive response after 24 hours. All subjects with IgE to these allergens also had some response to the low-molecular-weight allergens.

Male and female rats of all ages are sources of allergenic urine proteins. Adult male rats are the predominant source of three major allergens with molecular weights of 17 kd (α_{2u} -globulin), 21 kd (prealbumin), and 23 kd, the latter of which is described as an allergen for the first time. Male rats excrete significant amounts of high-molecular-weight proteins in their urine as they age that are highly allergenic for a subset of rat-allergic subjects.

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