

Prostatic kallikrein: A new major dog allergen

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Background: Dog dander is an important cause of respiratory allergy, but the spectrum of known dog allergens appears incomplete. Two lipocalins, Can f 1 and Can f 2, and serum albumin, Can f 3, have been characterized in detail but do not fully account for the IgE antibody-binding activity of dog dander extract. Allergen activity has previously been detected in dog urine but not further characterized.

Objective: We sought to identify, characterize, and assess the importance of allergen components in dog urine.

Methods: Dog urine was fractionated by means of size exclusion chromatography and examined for IgE antibody binding. A protein present in one fraction displaying IgE antibody-binding activity was identified by means of N-terminal sequencing and mass spectrometry. A recombinant form of the protein was produced in *Pichia pastoris*. IgE antibody binding to dog allergen components among sera of 37 subjects with dog allergy was determined by means of ImmunoCAP analysis.

Results: An IgE antibody-binding protein was isolated from dog urine and identified as prostatic kallikrein. A closely related or identical protein was detected in dog dander. The recombinant prostatic kallikrein displayed immunologic and biochemical properties similar to those of the natural protein and bound IgE antibodies from 26 (70%) of 37 sera of subjects with dog allergy, 14 of which reacted to none of Can f 1, Can f 2, or Can f 3. The dog allergen identified here was found to cross-react with human prostate-specific antigen, a key culprit in IgE-mediated vaginal reactions to semen.

Conclusion: Prostatic kallikrein is a new major dog allergen. (J Allergy Clin Immunol 2008;122:1101-1107.)

Key words: Allergen, recombinant allergen, dog dander, allergy, IgE antibody, Can f 1, Can f 2, Can f 3, Can f 5, kallikrein, lipocalin

Dog dander is a common cause of respiratory allergy, with symptoms including rhinitis, conjunctivitis, bronchial inflammation, and asthma. Dog allergens can be detected not only in homes where dogs are kept as pets but also in other places, such as schools and day care centers, where dogs are normally not present.¹

Extracts of dog hair and dander contain a complexity of allergenic and nonallergenic proteins.^{2,3} Three dog allergens have thus far been identified and studied in detail: Can f 1, Can f 2, and Can f 3. The former 2 are both members of the lipocalin

Abbreviations used

DPK:	Dog prostatic kallikrein
MALDI-TOF:	Matrix-assisted laser desorption/ionization time-of-flight
MBP:	Maltose-binding protein
MS:	Mass spectrometry
MW:	Molecular weight
PMF:	Peptide mass fingerprint
PSA:	Prostate-specific antigen
RPC:	Reversed-phase chromatography
SEC:	Size exclusion chromatography

protein family and have been purified and expressed as recombinant proteins.^{4,5} Can f 3, dog serum albumin, is a relatively conserved protein showing extensive cross-reactivity to other mammalian albumins.⁶

Can f 1 is the most important of the known dog allergens, binding IgE antibodies from approximately 50% of subjects with dog allergy. About 20% display IgE antibody binding to Can f 2, most of whom are also sensitized to Can f 1.⁷ Although 30% to 40% of adult subjects with dog allergy show IgE antibody binding to Can f 3,^{3,8} the specific clinical relevance of sensitization to mammalian serum albumins is uncertain. The frequent occurrence of IgE antibody binding to dog dander extract in the absence of detectable IgE reactivity to Can f 1, Can f 2, or Can f 3 implies the existence of dog allergens yet to be identified. The presence in dog dander of a third IgE-binding lipocalin, designated Can f 4, has been reported.⁷ However, as no molecular characterization of this molecule has yet been reported and it is not available in recombinant form, it is presently difficult to assess its importance as an allergen.

It has been known for a long time that major allergens from rodents, including mice and rats, are present in the animals' urine and such allergens have been isolated and extensively characterized.⁹⁻¹³ IgE antibody-binding activity has also been reported to exist in the urine of cats and dogs,¹⁴ but no allergen has thus far been purified from the urine of these animals and characterized at a molecular level.

In this study we set out to study urinary allergens in dogs. We report prostatic kallikrein as a new major dog allergen that is present in both urine and dander. The allergen reported here has received the designation Can f 5 by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee.

METHODS

Protein purification from dog urine

Urine was collected from a 7-year-old male crossbreed between Siberian Husky and Vorsteh, filtered through a 0.45-μm mixed cellulose ester filter

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(Millipore, Billerica, Mass) and applied to a Superdex 75 column (GE Healthcare Life Sciences, Uppsala, Sweden) for size exclusion chromatography (SEC). Fractions from different peaks were analyzed for IgE antibody-binding activity and pooled accordingly. Further protein purification was performed with reversed-phase chromatography (RPC) using a Source 15 RPC column (GE Healthcare Life Sciences) and elution with a 0% to 45% linear gradient of acetonitrile in water containing 0.05% trifluoroacetic acid.

Protein purification from dog dander

Dog dander (Allergon, Valinge, Sweden) was extracted in 20 mmol/L 3-(N-morpholino)-propanesulfonic acid (MOPS) (pH 7.6) and 0.5 mol/L NaCl (MBS buffer), clarified by centrifugation, filtered as described above, and concentrated with a PM-10 Amicon filter (Millipore). The extract was then subjected to SEC on a Superdex 75 column (GE Healthcare Life Sciences) in MBS buffer, and fractions were pooled according to the results of IgE antibody-binding analysis. Further purification was performed by means of RPC, as described above.

Protein analysis

SDS-PAGE analysis of reduced (4% β -mercaptoethanol) and nonreduced protein samples was performed with a 10% NuPAGE gel (Invitrogen, Carlsbad, Calif) and Mark12 (Invitrogen) as molecular weight (MW) markers. After electrophoretic separation, proteins were visualized by Coomassie Brilliant Blue staining. N-terminal sequence analysis of extracted protein bands was performed with a Hewlett-Packard G1000A instrument (Hewlett-Packard, Palo Alto, Calif). Analytic SEC was performed on a Superdex 75 HR 10/30 column (GE Healthcare Life Sciences) equilibrated with MBS. MW calibration of the column was performed with the low-MW gel filtration calibration kit (GE Healthcare Life Sciences).

For peptide mass fingerprint (PMF) analysis by means of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), sample preparation of RPC-purified urinary protein in solution, including reduction, alkylation, and trypsin digestion, was performed essentially as previously described,¹⁵ using a Bruker Daltonics Autoflex 2 instrument (Bruker Daltonics, Bremen, Germany). Tandem MS (MS/MS) analysis was performed to identify selected peptides. The MSDB database was searched with a Mascot server (Matrixscience, London, United Kingdom) to identify proteins matching the PMF and MS/MS results obtained.

In-gel trypsin digestion of individual protein bands from SDS-PAGE was performed essentially according to the method of Shevchenko et al.¹⁶ Sample preparation and PMF was performed as described above.

Recombinant dog prostatic kallikrein

A synthetic gene encoding dog prostatic kallikrein (DPK) was designed based on the reported amino acid sequence of the protein (accession no. P09582). After oligonucleotide assembly and PCR amplification, the gene was inserted between the *Xho*I and *Sal*I sites of vector pPICZ α A (Invitrogen), adding a C-terminal hexahistidine tag. After verification of the construct by DNA sequencing of both strands, the plasmid DNA construct was linearized by means of *Sac*I digestion and transformed into *Pichia pastoris* strain X-33 for homologous recombination into the chromosomal *AOX1* locus. The strain was grown at 30°C in rich broth medium using a 7-L bioreactor (Belach Biotechnik, Solna, Sweden). Expression was induced and maintained by feeding methanol to the culture to a steady-state concentration of 0.1% (vol/vol). After 70 hours, the culture was harvested, and the cells removed by centrifugation. The supernatant was applied to a Streamline 25 Chelating column (GE Healthcare Life Sciences) charged with NiSO₄. Column washing was performed with 60 mmol/L imidazole in 20 mmol/L Tris-HCl (pH 8.0) and 0.15 mol/L NaCl and the recombinant protein eluted with 500 mmol/L imidazole in the same buffer. Further purification of the recombinant protein was performed by means of cation exchange chromatography in 20 mmol/L 2-(N-morpholino)-ethanesulfonic acid (MES) (pH 6.0) using a SP Sepharose FF column (GE Healthcare Life Sciences). After washing with 0.15 mol/L NaCl, the protein was eluted with 0.3 mol/L NaCl, and fractions were pooled according

to SDS-PAGE results. The protein concentration was determined from absorbance at 280 nm, applying a calculated extinction coefficient of 1.46 per mg/mL.

Subjects with dog allergy and control subjects with pollen allergy

Sera from 37 subjects with dog allergy from Spain (n = 23), Sweden (n = 10), and North America (n = 4) were used in the study (see Table E1 in this article's Online Repository at www.jacionline.org). All patients had a doctor's diagnosis of dog allergy, with symptoms such as asthma, rhinoconjunctivitis, and urticaria; a positive skin prick test response; and a positive specific IgE test result (ImmunoCAP; Phadia, Uppsala, Sweden) to dog dander extract. For control purposes, sera of 44 subjects with pollen allergy without diagnosed or reported symptoms of dog allergy were used. All samples and clinical data were collected under the approval of the local ethics committee at each center.

Specific IgE antibody measurements

IgE antibody-binding activity of purified recombinant and natural allergens was examined by using regular and experimental ImmunoCAP tests (Phadia). Experimental ImmunoCAP tests were prepared as previously described.¹⁷ Purified human prostate-specific antigen (PSA) for analysis of cross-reactivity was purchased from Millipore. IgE inhibition experiments were performed by preincubating serum samples with purified natural or recombinant DPK or, as negative controls, sample diluent (Phadia), the nonallergenic maltose-binding protein (MBP) from *Escherichia coli*, rCan f 1, or rBet v 1 before measurement of IgE antibody binding to the ImmunoCAP solid phase carrying immobilized allergen. All experiments were performed with a final inhibitor concentration of 100 μ g/mL. Results were calculated as mean values of duplicate determinations and expressed as percentages of IgE antibody binding from samples preincubated with serum diluent.

RESULTS

Detection and purification of an IgE antibody-binding protein from dog urine

Separation of dog urine proteins by means of SEC resulted in 3 major peaks (Fig 1, A). Analysis of IgE antibody-binding activity in each peak, using serum samples of 8 subjects with dog allergy, revealed a strong activity in peak 2. The IgE antibody-binding protein preparation resulted in a band of approximately 28 kd in SDS-PAGE under nonreducing conditions. On reduction of the protein sample, the 28-kd band disappeared while 2 bands of approximately 18 and 10 kd appeared, suggesting that the unreduced 28-kd band was made up of the 18- and 10-kd species (Fig 1, B) linked to one another by 1 or more disulfide bridges. Fractions comprising peak 2 were pooled, and further purification by RPC resulted in 1 dominant peak retaining the IgE antibody-binding activity (not shown).

Identification of the IgE antibody-binding protein as prostatic kallikrein

PMF analysis by means of MALDI-TOF MS of in-solution trypsin-digested purified urinary protein resulted in a significant match ($P < .05$) to prostatic kallikrein from dog (accession no. P09582).¹⁸ PMF analysis of the in-gel digested 28-kd protein band, excised from a nonreducing SDS-PAGE gel, yielded a significant match ($P < .05$) to the sequence of the same database entry.

The reduced 18- and 10-kd protein bands, excised and extracted from an SDS-PAGE gel, were identified by means of

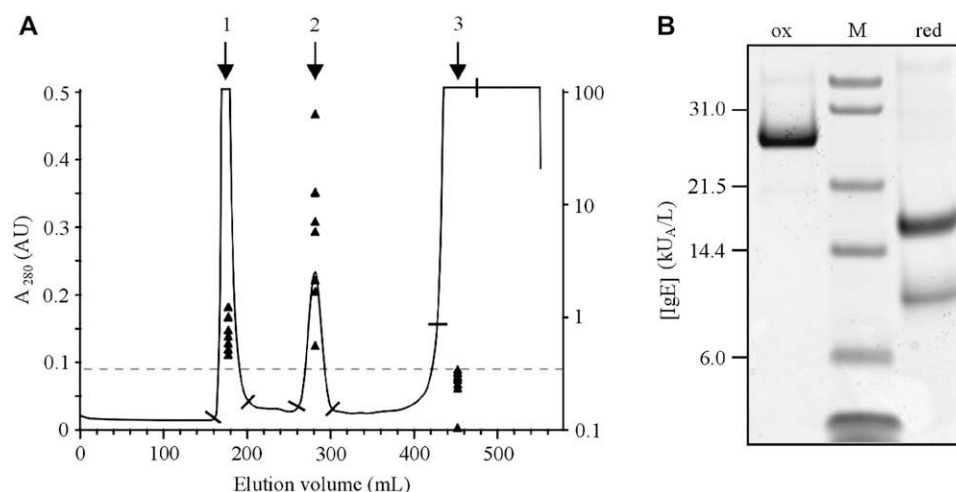


FIG 1. Separation and analysis of dog urinary proteins. **A**, Fractionation by means of SEC. Fractions comprising each of the 3 peaks (arrows, labeled 1-3) were pooled as indicated, and their bindings of IgE antibodies (right axis) from 8 selected sera are indicated as black triangles. **B**, SDS-PAGE of peak 2, displaying the highest IgE antibody-binding activity. Nonreduced (ox) and reduced (red) samples were analyzed. Lane M, MW marker proteins.

N-terminal sequencing. The analysis revealed the amino acid sequences AVIRPGEDRS and IIGGRExLKN (x: unresolved residue), respectively, matching residues 108 to 117 and 25 to 34 in the DPK precursor sequence of accession no. P09582, corroborating the results of the PMF analyses. Taken together, the results identified the major constituent of the IgE antibody-binding protein preparation from dog urine as prostatic kallikrein, also known as urinary kallikrein or arginine esterase.

Assessment of DPK-like IgE antibody-binding activity in dog dander

An IgE inhibition experiment was performed to examine whether dog dander contains determinants recognized by DPK-reactive IgE antibody. Serum samples from 4 dog dander-sensitized subjects (A-D) were each incubated either with DPK purified from dog urine, serum diluent, or MBP. All samples were then analyzed for IgE antibody binding to ImmunoCAP tests carrying immobilized dog dander extract. The results of the inhibition experiment are shown in Fig 2, A. DPK purified from dog urine was found to completely or extensively abolish IgE antibody binding to dog dander extract for sera A and B and partially for serum C. No inhibition by DPK was observed for serum D. Consistent with this result, analysis of direct IgE antibody binding to immobilized allergens in ImmunoCAP revealed predominant reactivity to DPK in sera A and B; reactivity to DPK, rCan f 1, and nCan f 3 in serum C; and reactivity to rCan f 1 and rCan f 2, but not to DPK, in serum D (not shown). Neither serum diluent nor the negative control protein MBP showed an inhibitory effect on IgE antibody binding to dog dander extract. The results demonstrated that epitope structures capable of binding DPK-reactive IgE antibody are present in dog dander and are not confined to urine.

Partial purification and analysis of DPK from dog dander

Having detected the presence of a DPK-like IgE antibody-binding activity in dog dander extract, an attempt was made to

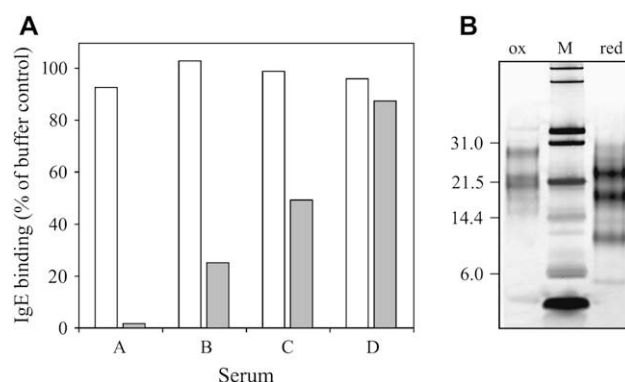


FIG 2. Analysis of DPK in dog dander. **A**, Inhibition of IgE antibody binding to dog dander extract by MBP (negative control; open bars) or natural DPK (gray bars) in 4 dog-reactive sera (A-D). **B**, SDS-PAGE analysis of partially purified DPK from dog dander. Nonreduced (ox) and reduced (red) samples of the preparation were analyzed. Lane M, MW marker proteins.

purify the corresponding protein. By using a 2-step purification procedure, a preparation was obtained that gave rise to one band at 28 kd and a double band at 21 to 22 kd in SDS-PAGE of an unreduced sample (Fig 2, B). On reduction, 3 major bands were seen at 23, 18, and 10 kd (Fig 2, B).

The 3 reduced protein bands were excised from the gel and subjected to in-gel digestion and analyzed with MS. Although the 23-kd band was identified by PMF as Can f 1, peptides $m/z = 1632.98$ and $m/z = 1004.52$ from the 18- and 10-kd bands, respectively, were identified by means of MS/MS as sequences derived from DPK (accession no. P09582).

Furthermore, the 18- and 10-kd bands were extracted from excised gel bands and subjected to N-terminal amino acid sequencing. The resulting sequences, AVxRPGE_xDR_x and xIGGRE_xLKN (x: unresolved residues), matched residues 108 to 116 and 26 to 34 of the DPK precursor sequence (accession no. P09582). The results demonstrate that a protein identical or closely related to DPK is present in dog dander.

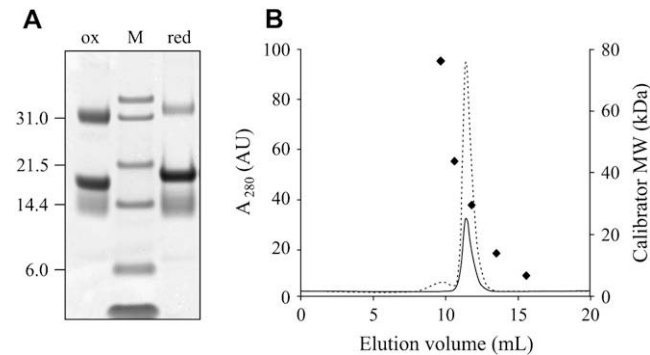


FIG 3. Biochemical analysis of DPK. **A**, SDS-PAGE of nonreduced (*ox*) and reduced (*red*) samples of recombinant DPK. *Lane M*, MW marker proteins. **B**, Analytic gel filtration of purified recombinant (*hatched line*) and natural (*solid line*) DPK on Superdex 75. Elution volumes of 6.5-, 13.7-, 29-, 43-, and 75-kD calibrator proteins are marked by *black diamonds*.

Production and characterization of recombinant DPK

In order to verify the identification of the IgE antibody-binding component of dog urine and investigate its importance as an allergen, DPK was produced as a recombinant protein using *P pastoris* as expression host, purified, and analyzed for IgE antibody-binding activity.

Although the synthetic DPK gene construct was designed to direct the production of a single polypeptide chain, SDS-PAGE of the protein purified from *P pastoris* culture medium indicated a partial cleavage into 2 polypeptide chains (Fig 3, A), resembling the processing of natural DPK.¹⁹ Indeed, N-terminal sequencing revealed that the recombinant protein had been cleaved at the very same position as the natural molecule (data not shown). The recombinant protein showed a clearly higher apparent MW in SDS-PAGE than the natural protein. The difference was particularly evident for the small subunit, which displayed an indistinct band appearance around 14 kD (Fig 3, A).

A sample of the preparation was subjected to analytic SEC in parallel with natural DPK purified from dog urine to assess the aggregation state and integrity of the recombinant protein under physiologic conditions. As shown in Fig 3, B, the chromatogram was dominated by a single symmetric peak in both cases, corresponding to an MW of 33 to 34 kD, as defined by the calibrators used. The analysis demonstrated that the recombinant protein, despite its partial processing, was held together in solution and existed in a homogeneous aggregation state, with an elution volume nearly identical to that of the natural protein.

The immunologic activity of recombinant DPK was assessed in comparison with that of the natural protein purified from dog urine. The 2 proteins were immobilized separately on the ImmunoCAP solid phase, and their IgE antibody binding examined using serum samples from 37 subjects with dog allergy. As can be seen in Fig 4, the 2 datasets showed a very strong correlation ($r = 0.999$), demonstrating that the recombinant allergen closely resembled its natural counterpart with respect to IgE antibody-binding activity. The results also provide proof that the IgE antibody-binding activity observed in dog urine was due to DPK and not a copurifying component. Assay specificity of the experimental recombinant DPK ImmunoCAP test was demonstrated by the negative results obtained on testing a negative control serum spiked with up to 3000 kU/L of myeloma IgE (see Table

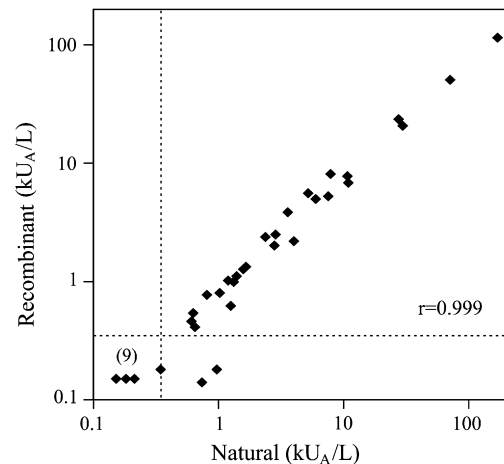


FIG 4. Comparison of IgE antibody-binding activity of natural and recombinant DPK. Sera from 37 subjects with dog allergy were used. *Dotted lines* indicate the 0.35 kUA/L cutoff level. Values of less than 0.15 kUA/L were set to 0.15 kUA/L. Nine samples tested less than 0.35 kUA/L to both allergen preparations.

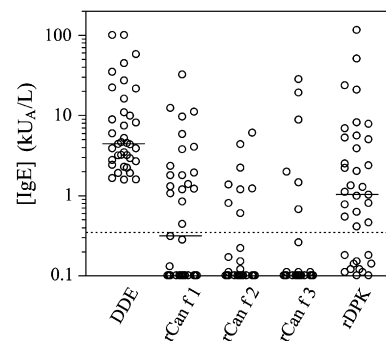


FIG 5. Concentration of specific IgE antibody to dog dander extract (*DDE*), rCan f 1, rCan f 2, nCan f 3, and recombinant DPK among 37 subjects with dog allergy. *Horizontal bars* indicate median values. The *dotted line* indicates the 0.35 kUA/L cutoff level. Values of less than 0.1 kUA/L were set to 0.1 kUA/L.

E2 in this article's Online Repository at www.jacionline.org). Furthermore, among the control subjects with pollen allergy, no serum showed a positive response to recombinant DPK in the absence of a positive response to dog dander (see Table E3 in this article's Online Repository at www.jacionline.org).

Component-resolved analysis of specific IgE antibody responses in subjects with dog allergy

The levels of specific IgE antibody to dog dander extract, rCan f 1, rCan f 2, nCan f 3, and recombinant DPK among 37 subjects with dog allergy were determined by ImmunoCAP measurements (Fig 5 and see Table E1). Of the tested sera, 26 (70%) showed IgE reactivity to recombinant DPK, and 18 (49%) to rCan f 1. Both rCan f 2 and nCan f 3 appeared as minor allergens among the subjects studied, binding IgE antibody from only 8 (22%) and 6 (16%), respectively. Fourteen (38%) of the 37 sera reacted to recombinant DPK alone. On average, among all recombinant DPK-reactive sera, the level of IgE antibody binding to recombinant DPK amounted to 54% of that to dog dander. The corresponding

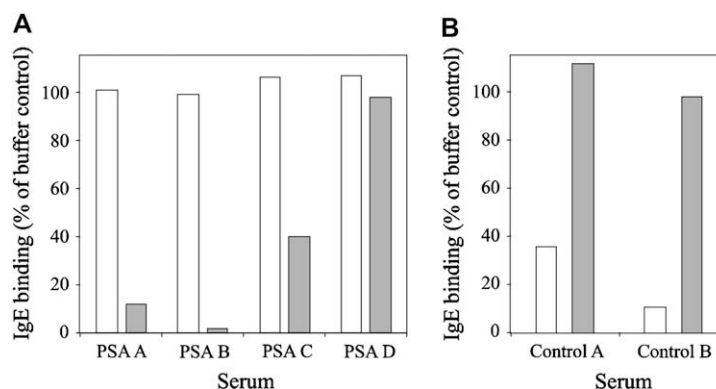


FIG 6. Analysis of cross-reactivity between DPK and human PSA. **A**, IgE antibody binding to immobilized PSA after preincubation of 4 PSA-reactive sera (PSA A-D) with rCan f 1 (negative control, open bars) or recombinant DPK (gray bars). **B**, Control experiment with DPK- and PSA-negative sera reactive to Can f 1 (Control A) or Bet v 1 (Control B). IgE antibody binding to immobilized rCan f 1 or Bet v 1 after serum preincubation with rCan f 1 or rBet v 1 (open bars) or recombinant DPK (gray bars).

relative levels of IgE antibody binding to rCan f 1, rCan f 2, and nCan f 3 were 45%, 25%, and 47%, respectively, among sera specifically reactive to those allergens. Only 2 of the 37 sera tested lacked IgE reactivity to all 4 dog allergens tested. The IgE antibody binding to recombinant DPK showed no significant correlation to any of the other dog allergens, demonstrating that the immune response to DPK is independent from sensitization to Can f 1, Can f 2, or Can f 3.

The occurrence of DPK-specific IgE antibody in atopic individuals not allergic to dog was examined by testing sera of 44 subjects with pollen allergy without diagnosed or reported symptoms of dog allergy, with all dog allergen components available and a range of pollen extracts (see Table E3). Seven (16%) of those sera had a positive response to dog dander extract, with one exception at levels of 2 kUA/L or less, 6 of which also showed IgE antibody binding to recombinant DPK. No serum negative to dog dander showed a positive response to recombinant DPK.

Cross-reactivity between DPK and human PSA

Four PSA-reactive sera were identified and used in an IgE inhibition experiment to examine whether DPK might cross-react with human PSA, with which it shares structural similarity. The results of the experiment are shown in Fig 6. IgE antibody binding to PSA was extensively inhibited by the dog allergen in 2 of the 4 sera (88% and 98%, respectively), whereas partial inhibition (60%) was obtained in 1 serum and no inhibition occurred in 1 serum (Fig 6, A). No inhibition of IgE antibody binding to PSA was caused by rCan f 1 (Fig 6, A), and recombinant DPK caused no inhibition of IgE antibody binding to rCan f 1 or Bet v 1 in sera reactive to those allergens (Fig 6, B). The control experiments demonstrated that the IgE inhibition activity of recombinant DPK was immunologic and specific.

DISCUSSION

In this study we have isolated a previously unknown allergen from dog urine and identified it as prostatic kallikrein. Our results further demonstrated that this allergen, or a molecule with very similar structural and immunologic properties, is present also in extracts of dog hair and dander. Finally, we assessed the importance of DPK as an allergen in a population of subjects with

dog allergy. DPK has been named Can f 5 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

After the identification of the main protein component in a fraction of dog urine displaying strong IgE antibody-binding activity, it was of interest to develop a recombinant form of the protein, both to verify the identification and to ensure unlimited supply of the allergen. Efforts first made to express the protein in *E coli* (results not shown) resulted in an insoluble protein that could not be refolded to an active state and that did not undergo autocatalytic cleavage, as did the natural allergen and the recombinant protein subsequently expressed in *P pastoris*. Further, on solubilization and immobilization to the ImmunoCAP solid phase, the *E coli*-expressed recombinant DPK displayed much lower IgE antibody-binding activity than the natural protein preparation from urine and the recombinant DPK produced in *P pastoris*. Similar experiences regarding failure and success in the expression of human kallikreins in *E coli* and yeast have previously been reported.^{20,21}

Recombinant DPK produced in *P pastoris* showed a higher apparent MW in SDS-PAGE than the natural protein purified from urine. Although the intact protein and the larger fragment produced on reduction were expected to gain marginally in size because of the C-terminal hexahistidine tag, the most prominent difference to the natural protein, approximately 4 kd, was displayed by the small fragment. Drawing from its electrophoretic heterogeneity and the fact that it contains a sequence motif for N-linked glycosylation (asparagine residue 55 of the mature polypeptide chain), it appears likely that the size increment observed was due to glycosylation. Interestingly, the natural dog protein has been reported to carry a glycan moiety attached to its small subunit, in this case consisting of no more than 4 to 5 glucosamine residues.^{19,22,23} As recombinant DPK produced in *P pastoris* was found to be fully immunoreactive and displayed features of correct folding and complete solubility, this protein was chosen for further work, regardless of a likely difference in glycosylation as compared with the natural protein.

Recombinant DPK and DPK purified from dog urine displayed comparable IgE antibody-binding properties as solid-phase reagents in the ImmunoCAP assay. Only 2 of 37 sera gave rise to clearly deviating results with the 2 allergen preparations, testing negative with the recombinant protein while showing low-level binding to the purified natural protein, corresponding to an IgE

antibody concentration of 0.7 and 1.0 kUA/L. However, the data available do not allow us to conclude whether this binding represented a true DPK epitope or binding activity of a copurifying component. An important conclusion from the results with recombinant DPK is that they serve to remove all doubt as to the identification of the IgE antibody-binding component detected in dog urine. Additionally, the successful production of recombinant DPK provides for supply of the allergen without a need for collection of dog urine.

The IgE results from the study group of subjects with dog allergy demonstrate that DPK is a major dog allergen, both in terms of frequency and magnitude of IgE antibody binding. Although Can f 1, the most important among previously known dog allergens, barely qualifies as a major allergen in this study, as well as in other reports,⁷ recombinant DPK bound IgE antibody from 70% of the subjects studied here. The fact that almost one third of the patients in this population were sensitized only to DPK further establishes its importance as a major dog allergen. Thus, DPK represents an important addition to the panel of known dog allergens. The positive test results to recombinant DPK in 6 of 44 control subjects with pollen allergy might be due to any of several factors, including subclinical sensitization to dog, serologic cross-reaction unrelated to dog, and underreporting of dog allergy among these subjects, with the latter possibly as a result of no or limited contact with dogs.

Kallikrein has not previously been described as an animal allergen, and its amino acid sequence shows no significant similarity to any known animal dander or urinary allergen. However, DPK and other members of the same protein family from different species (cow, horse, rat, rabbit, mouse, and guinea pig) show an intermediate level of sequence conservation (53% to 59% sequence identity). A certain extent of mammalian pan-allergen characteristics is therefore possible, although experimental evidence of cross-reactivity and relevant protein localization will be required before any conclusion to this effect can be drawn. As expected, no correlation between IgE binding to DPK and Can f 1, Can f 2, or Can f 3 could be observed in our study.

The presence of allergens in urine of mammals has been reported in previous studies.^{9,14} In rats, it has been shown that the highest content of some urinary allergens is present in adult male animals.²⁴ For the purpose of this study, urine was collected from an adult, intact male dog. Even though the protein identified here has no sequence similarity to the major allergen in rat urine, it has been described as an androgen-regulated protein expressed in the prostate,²⁵ suggesting an occurrence restricted to male animals. Indeed, castration of male dogs has been found to drastically reduce the production of DPK.²⁵ Thus, an interesting possibility is that subjects with dog allergy, depending on their specific sensitization profile, might be differently sensitive to male and female dogs. This would be expected, particularly in patients sensitized exclusively to DPK, as were one third of the subjects studied here. Furthermore, as the practice of male dog castration might vary between countries and regions, it is possible that the importance of DPK in dog allergy will be found to differ accordingly. However, we are not aware of any studies systematically examining these aspects of dog allergy and not of any indicative anecdotal evidence.

Because the commercially available dog dander used in this study comprised a pool of material from male and female dogs of different breeds, a preliminary experiment was performed in

which proteins were extracted from the hair of a female dog. Although we could readily detect Can f 1 in this extract, we were unable to detect DPK (not shown). Considering that it is presently unclear how DPK becomes deposited on dog hair or dander, an expanded study will be required to establish whether DPK is consistently absent from the hair and dander of female dogs.

In a recently published case report, it was demonstrated that vaginal hypersensitivity to semen was associated with IgE sensitization to human PSA, which is present in seminal plasma.²⁶ As DPK and human PSA share substantial structural similarity (55% to 60% sequence identity), it is possible that sensitization to DPK confers an increased risk of development of such allergic reactions to human seminal fluid. Indeed, in a recent article by Basagaña et al,²⁷ it was demonstrated that the binding of PSA to IgE antibody from a patient with allergic reactions to human semen. In further support of such a linkage, we were able to demonstrate extensive cross-reactivity between DPK and PSA in 3 of 4 PSA-reactive sera identified. Even though IgE antibody recognition of PSA among individuals sensitized to DPK appears to be less frequent than one would expect from the level of sequence similarity between the 2 proteins, it is possible that IgE-mediated reactions to this dog allergen play a role in certain cases of infertility in human subjects.

In conclusion, this study reports the identification of a new major dog allergen, prostatic kallikrein, which forms significant contribution to the panel of previously known dog allergens. DPK, now designated Can f 5, will be important in component-resolved diagnosis of dog allergy and might carry particular significance in relation to allergic vaginal reactions to human semen and complications caused by that condition.

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Clinical implications: Subjects with dog allergy sensitized to prostatic kallikrein might react more severely to male than female dogs. By way of cross-reactivity, IgE-mediated vaginal reactions to human semen might be associated with dog sensitization.

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TABLE E1. Subjects with dog allergy: Demographics, allergy symptoms, and dog allergen sensitization

Subject no.	Age (y)	Country	Symptoms	e5*	rCan f 1*	rCan f 2*	nCan f 3*	rDPK*
1	35	ES	A, RC	>100	0.09	0.12	0.08	>100
2	43	ES	A, RC	5.92	0.28	0.06	0.05	5.26
3	43	ES	A, RC	16.00	0.05	0.07	0.05	23.56
4	37	ES	A, RC	5.20	0.13	0.05	0.06	0.11
5	25	ES	A, RC	3.14	1.92	0.17	0.07	0.41
6	41	ES	A, RC	4.31	0.00	0.01	0.03	2.49
7	39	ES	RC	2.75	0.00	0.01	0.02	2.01
8	42	ES	RC	1.64	0.00	0.02	0.04	0.62
9	19	ES	RC	>100	11.04	2.21	28.20	0.18
10	19	ES	RC	2.20	0.08	0.11	0.11	1.02
11	34	ES	RC	9.86	0.01	0.02	0.02	7.77
12	42	ES	RC	3.43	1.19	0.02	0.01	0.14
13	51	ES	A, RC	1.58	3.75	0.00	0.01	0.15
14	26	ES	RC	2.27	0.04	0.03	0.02	0.09
15	44	ES	RC	10.80	0.03	0.02	19.09	8.12
16	21	ES	RC	4.60	0.02	0.01	0.04	2.19
17	39	ES	A, RC	34.80	0.02	0.02	1.45	20.75
18	32	ES	RC	2.39	1.06	0.02	0.01	1.27
19	23	ES	RC	8.85	0.00	0.01	0.01	6.85
20	23	ES	A, RC	21.50	5.83	0.07	8.76	0.99
21	37	ES	A, RC	3.17	0.04	0.01	0.03	2.37
22	22	ES	A, RC	57.50	12.25	0.22	0.26	50.67
23	23	ES	RC, U	8.07	2.32	0.05	0.11	0.10
24	50	SE	RC	44.50	32.14	0.15	0.08	0.14
25	44	SE	A, RC	27.10	9.60	4.36	0.11	4.99
26	25	SE	RC	2.66	1.21	0.60	0.67	0.11
27	29	SE	A, RC	1.89	0.31	0.07	0.06	0.54
28	27	SE	RC	3.86	1.38	0.09	0.08	0.77
29	32	SE	U	4.38	1.78	1.19	0.08	0.18
30	25	SE	RC	3.19	0.01	0.02	0.08	1.33
31	52	SE	A	3.85	0.44	1.22	0.05	0.46
32	20	SE	A, RC	7.41	1.78	1.36	0.04	3.85
33	29	SE	A, RC	22.10	4.04	6.06	1.97	5.56
34	32	NA	RC	2.90	0.84	0.06	0.08	0.12
35	30	NA	RC	4.50	1.30	0.05	0.05	0.80
36	48	NA	A, RC	1.90	0.00	0.01	0.01	1.11
37	27	NA	RC	1.57	0.09	0.80	0.06	0.12

e5, Dog dander extract; rDPK, recombinant DPK; ES, Spain; A, asthma; RC, rhinoconjunctivitis; SE, Sweden; U, urticaria; NA, North America.

*Allergen-specific IgE antibody in international kilounits per liter; negative = less than 0.35 kUA/L.

TABLE E2. Assay specificity of experimental recombinant DPK
ImmunoCAP test

Test sample	Test result*
Negative serum	0.004
Negative serum + 1000 kU/L myeloma IgE	0.035
Negative serum + 3000 kU/L myeloma IgE	0.064

*Allergen-specific IgE antibody in international kilounits per liter; negative = less than 0.35 kUA/L.

TABLE E3. Subjects with pollen allergy without diagnosed or reported symptoms of dog allergy: Demographics, and allergen sensitization

Subject no.	Country	e5*	rCan f 1*	rCan f 2*	nCan f 3*	rDPK*	t3*	t9*	g6*	w21*	w6*
1	DE	0.20	0.01	0.01	0.01	0.02	3.71	1.31	81.80	0.12	0.39
2	DE	0.03	0.00	0.01	0.00	0.01	0.01	0.17	14.03	0.03	0.11
3	DE	0.03	0.00	0.01	0.01	0.01	0.16	0.26	0.39	0.25	0.20
4	DE	0.04	0.00	0.01	0.00	0.02	0.01	0.20	12.47	0.05	0.05
5	DE	0.01	0.00	0.00	0.01	0.00	1.32	1.66	2.21	0.03	0.66
6	DE	0.22	0.00	0.00	0.00	0.15	0.02	0.37	15.10	0.05	0.12
7	ES	0.02	0.01	0.01	0.01	0.02	0.00	0.00	0.00	12.59	0.00
8	ES	42.27	1.16	0.07	8.37	113.5	0.05	3.99	9.48	0.29	0.17
9	ES	0.14	0.03	0.02	0.05	0.02	2.68	6.46	25.22	10.04	2.99
10	ES	0.01	0.02	0.00	0.00	0.01	0.00	3.72	0.00	0.01	0.00
11	ES	0.08	0.01	0.02	0.05	0.02	0.01	1.26	39.32	2.14	0.14
12	ES	0.05	0.01	0.01	0.01	0.01	0.18	0.16	0.60	7.94	0.11
13	ES	0.06	0.01	0.01	0.01	0.02	4.93	8.04	31.65	1.74	2.99
14	ES	0.11	0.00	0.01	0.01	0.08	0.09	8.58	16.57	0.91	0.93
15	ES	0.30	0.00	0.01	0.01	0.02	0.01	0.04	0.01	30.86	0.01
16	ES	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.88	0.00
17	ES	0.07	0.00	0.00	0.01	0.01	0.00	0.00	0.00	15.66	0.00
18	ES	0.09	0.00	0.00	0.00	0.01	0.01	11.76	0.06	0.05	0.00
19	ES	0.05	0.00	0.01	0.01	0.01	0.24	0.82	25.13	0.37	0.31
20	ES	0.01	0.00	0.00	0.00	0.01	0.00	7.24	5.21	0.02	0.00
21	ES	0.00	0.00	0.00	0.00	0.00	0.07	0.15	0.00	6.60	0.00
22	ES	0.01	0.00	0.00	0.00	0.00	0.04	0.13	0.38	20.37	0.38
23	ES	0.03	0.00	0.00	0.00	0.01	0.07	3.11	0.04	0.13	0.00
24	ES	0.05	0.00	0.00	0.00	0.01	0.00	0.98	41.13	0.03	0.11
25	ES	0.01	0.00	0.00	0.00	0.00	0.37	11.23	0.02	11.03	0.00
26	ES	1.12	0.00	0.00	0.00	1.33	2.20	18.87	0.01	51.88	0.03
27	ES	0.24	0.03	0.04	0.03	0.04	0.05	5.04	2.99	0.10	0.04
28	ES	0.01	0.00	0.00	0.00	0.01	0.01	0.02	0.01	5.05	0.05
29	ES	0.10	0.01	0.02	0.02	0.01	0.26	4.71	80.33	0.44	3.96
30	ES	0.01	0.00	0.00	0.00	0.00	0.01	7.10	4.43	0.45	0.01
31	ES	0.02	0.00	0.01	0.00	0.01	0.05	51.19	0.07	0.07	0.17
32	ES	1.05	0.00	0.00	0.00	1.13	0.00	1.72	0.02	0.05	0.00
33	ES	0.06	0.02	0.01	0.01	0.02	0.12	37.40	1.87	0.26	0.16
34	ES	0.01	0.00	0.00	0.00	0.01	0.02	0.06	0.51	1.59	0.00
35	ES	0.01	0.02	0.01	0.00	0.01	0.02	0.86	6.35	3.05	0.03
36	ES	2.02	0.00	0.00	0.00	1.99	0.01	0.36	6.58	0.03	0.02
37	ES	1.94	1.53	1.19	1.97	0.58	51.24	129.4	166.3	131.6	74.70
38	ES	0.01	0.00	0.00	0.00	0.00	0.02	0.12	0.21	12.31	0.00
39	ES	0.64	0.02	0.03	0.03	0.61	1.62	3.78	1.81	77.78	0.88
40	ES	0.00	0.00	0.00	0.00	0.00	0.01	0.30	0.00	0.02	0.00
41	ES	0.09	0.00	0.00	0.00	0.07	0.01	5.01	0.00	0.02	0.00
42	ES	0.00	0.00	0.00	0.00	0.00	0.01	5.55	0.00	0.03	0.00
43	ES	1.21	0.04	0.07	0.06	0.06	0.14	5.99	9.14	14.77	0.12
44	ES	0.06	0.04	0.01	0.01	0.02	0.03	0.46	14.00	0.18	0.38

e5, Dog dander extract; rDPK, recombinant DPK; t3, birch pollen extract; t9, olive pollen extract; g6, timothy grass pollen extract; w21, *Parietaria judaica* pollen extract; w6, mugwort pollen extract; DE, Germany; ES, Spain.

*Allergen-specific IgE antibody in international kilounits per liter; negative = less than 0.35 kUA/L.