

Cat IgA, representative of new carbohydrate cross-reactive allergens

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Background: Allergens from cat are among the most potent elicitors of allergic disease. Four cat allergens have been identified; however, evidence indicates the existence of additional allergens.

Objective: In this study, we evaluated IgE sensitization to IgA from cat.

Methods: Sera from cat-sensitized patients (n = 81) were analyzed for IgE antibodies to purified cat IgA in the Pharmacia CAP System. Indirect ELISA was performed with cat IgA, cat IgM, and deglycosylated cat IgA. Competitive inhibition ELISA was performed with cat IgA, cat IgM, calf intestine alkaline phosphatase (CIP), and cat serum albumin on solid phase bound cat IgA. IgE reactivity was also evaluated on membrane blotted cat IgA.

Results: Thirty-eight percent (31/81) of the cat-sensitized sera were ImmunoCAP-positive to cat IgA. Indirect ELISA demonstrated a high correlation between IgE reactivity to cat IgA and cat IgM ($r = 0.94$; $P < .001$). Very low responses were observed to deglycosylated IgA. Strong inhibition of cat IgA was observed in all sera after preincubation with cat IgA and cat IgM. Inhibition was also observed in most sera after preincubation with CIP. Immunoblotting demonstrated that the IgE reactivity was mainly directed to the heavy chain of IgA.

Conclusion: This study has revealed a new allergen, cat IgA, containing a novel group of cross-reactive epitopes depending on carbohydrates also present on IgM and partially on CIP.

Clinical implications: This new group of cross-reactive carbohydrate IgE epitopes should be taken into consideration when diagnosing patients with suspected animal allergy.

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Key words: Cat allergy, IgA, IgM, cross-reactive, carbohydrates, cat allergen

Abbreviations used

ALP: Alkaline phosphatase
CIP: Calf intestine alkaline phosphatase
CSA: Cat serum albumin
HRP: Horseradish peroxidase
kU_A/L: Kilounits of allergen-specific IgE per liter
PVDF: Polyvinylidene fluoride

The domestic cat (*Felis domesticus*) is among the most common and potent sources of allergens, causing allergic disease on a worldwide basis.¹ Identification and characterization of single allergenic molecules has greatly facilitated the exploration of associated pathomechanisms as well as diagnosis and treatment of allergy. So far, 4 cat allergens have been described: the major allergens Fel d 1 (secretoglobulin)² and Fel d 4 (lipocalin)³ and the minor allergens Fel d 2 (albumin)⁴ and Fel d 3 (cystatin).⁵ However, analyses of cat dander extract suggest that many more allergens remain to be characterized.⁶ Thirty years ago, Ohman et al⁷ reported evidence for cat γ -globulins as allergens, but the only immunoglobulin of listed allergens is the food-related cow and milk allergen Bos d 7.⁸

Immunoglobulins in cat were first investigated in 1968 by identification of similarities in immunoelectrophoretic patterns in cat, rabbit, and human serum.⁹ The concentrations of IgA and IgM in healthy adult cats have been estimated to 2.6 and 2.0 mg/mL in serum and 0.54 and 0.13 mg/mL in saliva, respectively.¹⁰

In several food and plant allergens, IgE reactivity to the glycan moiety has been demonstrated, causing cross-reactivity between otherwise related and unrelated glycoproteins.¹¹⁻¹³ IgE reactivity has also been demonstrated to the glycan moiety in insect venoms^{11,14,15} and seafood.¹⁶

In this study, we evaluated cat IgA as a new allergen. Our assumption was that IgA in saliva of cats, transferred to the fur by licking and spread as airborne particles, may cause sensitization.¹⁷ We previously made an attempt to investigate the allergenicity of cat IgA and other animal immunoglobulins.¹⁸ The results revealed that there was a complete mutual cross-reactivity between cat IgA and cat IgM. Further investigations demonstrated that the reaction was not mediated by human IgE but by a factor in serum cross-linking animal immunoglobulins and calf intestine alkaline phosphatase (CIP). The factor was

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further studied and shown to be heterophile human IgM antibodies with specificity for the carbohydrate moiety of animal proteins.

On the basis of this knowledge, we continued to investigate the biochemistry and IgE binding properties of purified cat IgA using SDS-PAGE, Pharmacia CAP System, immunoblotting, ELISA, and allergen inhibition assays using sera from cat IgE-sensitized patients. The results revealed that cat IgA represents a new carbohydrate cross-reactive allergen that exists in cat dander and serum. Cross-reactivity was shown to glycoproteins, including IgM, and partially to CIP. Our data demonstrate predominant IgE binding to the glycan moiety of the α -chain, the first carbohydrate epitopes described in mammalian allergens.

METHODS

Patient sera

Sera from 81 patients IgE sensitized to cat dander, e1 ImmunoCAP (Phadia AB, Uppsala, Sweden; median, 5.1 kilounits of allergen-specific IgE per liter [kU_A/L]; range, 0.36–52 kU_A/L), were obtained from the Department of Clinical Immunology, Karolinska University Hospital, Stockholm, Sweden.

The study was approved by the ethics committee of the Karolinska Institutet, Stockholm, Sweden.

Purification and analysis of proteins

IgA was purified from cat serum as described.¹⁸ Cat serum albumin (CSA) was purified from cat serum by Blue Sepharose HP and Q Sepharose ion exchange chromatography (GE Healthcare, Uppsala, Sweden) following the manufacturer's instructions and subsequent size exclusion chromatography. Cat IgM and CIP were purchased from Europa Bioproducts (London, United Kingdom) and Sigma (St Louis, Mo), respectively. Deglycosylation of cat IgA was performed by PNGase F (New England Biolabs, Beverly, Mass) following the manufacturer's instructions. The immunoglobulins were analyzed by 15% SDS-PAGE gels using denaturing conditions and Coomassie blue staining.¹⁹ Cat dander extract was produced as described.¹⁸

ImmunoCAP

Cat IgA ImmunoCAP (MIAB, Uppsala, Sweden) was used to determine the concentration of IgE antibodies to cat IgA (ImmunoCAP assay equipment, Phadia AB). An extended measuring range, 0.2 to 100 kU_A/L , with added calibrator 0.2 kU_A/L , was used. The background remained below 0.2 kU_A/L when normal serum spiked with 1000 kU_A/L myeloma IgE was tested. The analyses were, with this exception, performed according to the manufacturer's instructions. The results were expressed in kU_A/L , and a positive test was defined as $\geq 0.2 \text{ kU}_A/\text{L}$.

ELISA

IgE responses to cat IgA, IgM, and deglycosylated IgA were evaluated by indirect ELISA in the 21 sera with $\geq 0.35 \text{ kU}_A/\text{L}$ to cat IgA CAP (range, 0.38–15.6 kU_A/L) essentially as described.²⁰ Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.5 μg protein per well followed by blocking (PBS with 1% BSA). Patient sera diluted 1:10 in dilution buffer (PBS with 0.5% BSA) were added, followed by rabbit antihuman IgE (1:2000; MIAB) and by horseradish peroxidase-conjugated donkey antirabbit immunoglobulins (1:2000; Jackson ImmunoResearch Laboratories,

West Grove, Pa). Finally, substrate was added, and the color reaction was monitored. Values twice the background were considered positive. Median values were used to calculate the difference between IgE reactivity to cat IgA and deglycosylated cat IgA.

Competitive inhibition ELISA was performed essentially as described for the indirect ELISA, but with a previous step of overnight preincubation of sera with proteins. Nine sera, randomly selected among those tested in ELISA (range, 0.38–15.6 kU_A/L to cat IgA), were diluted 1:10 with either dilution buffer or dilution buffer containing a final concentration of 100 $\mu\text{g}/\text{mL}$ cat IgA, IgM, CIP, CSA, or recombinant Fel d 1.²⁰ After preincubation, the samples were added to cat IgA-coated plates.

An in-house ELISA was developed to examine possible CSA contamination in cat IgA and cat IgM. ELISA plates were coated with 0.5 μg anticat albumin (ICL, Newberg, Ore) per well followed by blocking (PBS with 1% BSA). Serial dilutions of CSA, cat IgA, and cat IgM were added to the plate followed by biotinylated (EZ-Link Sulfo-NHS-LC-Biotin; Pierce, Rockford, Ill) anticat albumin (1:500). Thereafter, streptavidin-alkaline phosphatase (ALP) (1:2000) and ALP substrate (Sigma) were added, and reading was performed at 405 nm.

Immunoblotting

Immunoblotting was performed under reducing conditions with cat dander extract, purified cat IgA, or deglycosylated cat IgA as described¹⁸ by using 15% polyacrylamide gels and polyvinylidene fluoride (PVDF) membranes (Immobilon-P transfer membranes; Millipore) following the manufacturer's instructions. One hundred microliters serum from 5 patients with high IgE reactivity to cat IgA, each diluted in 500 μL dilution buffer, was added to IgA blotted PVDF strips. One hundred microliters of a pool consisting of sera from the 21 patients with $\geq 0.35 \text{ kU}_A/\text{L}$ to cat IgA CAP, diluted in 500 μL dilution buffer, was added to cat IgA and deglycosylated cat IgA blotted on PVDF strips. Thereafter, rabbit antihuman IgE (1:2000; MIAB) and subsequently HRP-conjugated donkey antirabbit antibodies (1:2000; Jackson ImmunoResearch Laboratories) were added, followed by substrate (tetramethylbenzidine; Sigma). ALP-conjugated goat anticat light chains (1:2000; Bethyl, Montgomery, Tex) and anticat IgA α -chain (1:20,000; ICL) followed by ALP-conjugated antigoat IgG antibodies (1:40,000; Sigma) were also analyzed on cat IgA blotted strips, followed by substrate (AP Conjugate Substrate Kit; Bio-Rad, Hercules, Calif). Three different batches of cat dander (Allergon, Ängelholm, Sweden) were extracted and blotted on PVDF membranes. To each extract, anticat IgA α -chain (1:5000) with and without overnight preincubation with 50 $\mu\text{g}/\text{mL}$ of purified cat serum IgA was added, followed by ALP-conjugated antigoat IgG antibodies (1:10,000) and substrate as described.

Statistical analysis

Statistical analysis was performed by using the software Statistica version 5.5 by StatSoft (Tulsa, Okla). Spearman rank was used for correlation analysis. A P value $< .05$ was considered significant.

RESULTS

Purification and analysis of proteins

Purified cat IgA and IgM all displayed a protein corresponding to immunoglobulin light chains at 28 kd in SDS-PAGE (Fig 1). An additional weak band was present at 24 kd in the IgA preparation, corresponding to the J-chain. Proteins at 64 and approximately 94 kd appeared at the molecular sizes of the heavy chains of IgA (α -chain) and IgM (μ -chain), respectively (Fig 1). CIP demonstrated

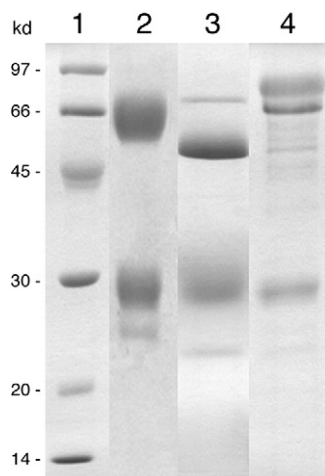


FIG 1. Analyses of size and purity of purified cat immunoglobulins by Coomassie blue stained 15% SDS-PAGE under reducing conditions. *Lane 1*, low molecular weights markers; *lane 2*, IgA; *lane 3*, PNGase F treated IgA; *lane 4*, IgM.

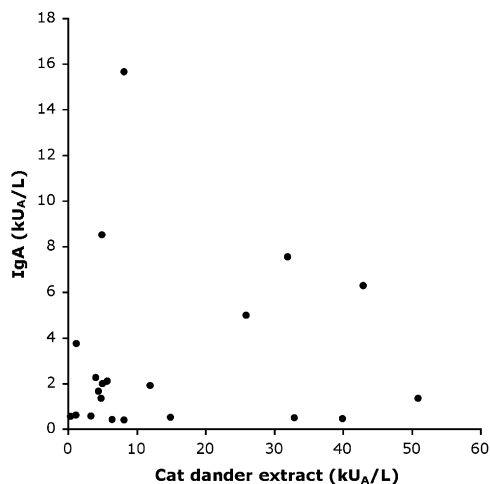


FIG 2. Correlation between IgE reactivity to cat IgA (*y*-axis) and cat dander extract (*x*-axis) among 21 cat-sensitized patients using the Pharmacia CAP System ($r = 0.02$; $P > .05$).

a stained band at the molecular weight of 64 kD, whereas CSA demonstrated a band at 66 kD (data not shown).

Purified cat IgA contained $< 0.8\%$ CSA and cat IgM $< 0.4\%$.

Analysis of IgE antibodies

Among the 81 cat-sensitized patients, 31 (38%) had ≥ 0.20 kUA/L IgE antibodies to cat IgA (range, 0.21–15.6 kUA/L) analyzed by the Pharmacia CAP System. There was no correlation between the IgE levels to cat IgA and cat dander extract (Fig 2; $n = 21$; $r = 0.02$; $P > .05$).

IgE reactivity to cat IgA, cat IgM, and deglycosylated cat IgA was examined by indirect ELISA in 21 cat IgA CAP-positive sera (range, 0.38–15.6 kUA/L). Seventeen of the 21 sera had no IgE reactivity to deglycosylated cat IgA (median reduction, 90%; Fig 3). Of the 4 sera

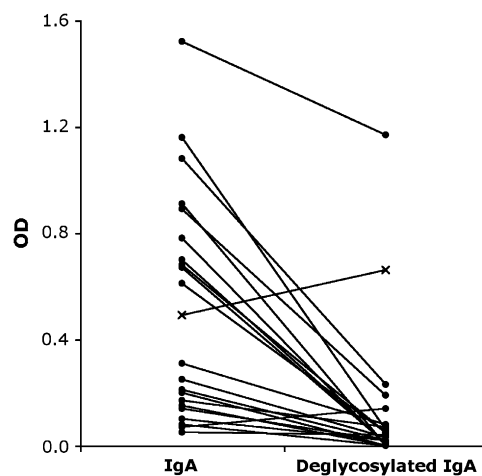


FIG 3. IgE reactivity to natural cat IgA and deglycosylated cat IgA shown as OD values in 21 cat IgA CAP-positive sera (solid circles) and OD values for goat anticat IgA (positive control, crosses).

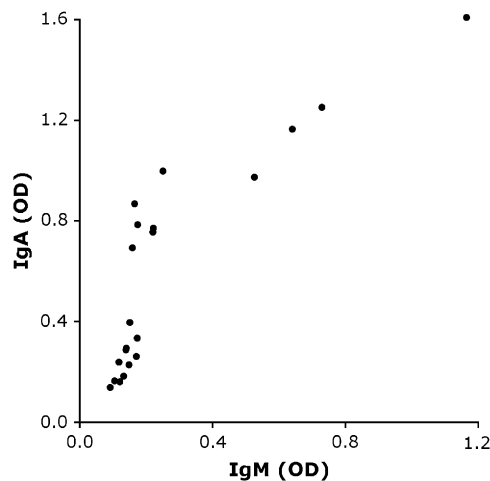


FIG 4. Correlation between IgE reactivity to cat IgA (*y*-axis) and cat IgM (*x*-axis) among 21 cat IgA-sensitized patients using ELISA ($r = 0.93$; $P < .001$).

reacting to deglycosylated IgA, 1 serum preserved the majority (77%), 2 lost nearly all (79%), and the fourth serum had slightly higher reactivity to IgA after deglycosylation. Specific goat anticat IgA α -chain antibodies demonstrated a 35% increased reactivity to cat IgA after deglycosylation (Fig 3).

When comparing the IgE responses in ELISA between cat IgA and cat IgM among the 21 cat IgA CAP-positive sera, a high correlation was observed (Fig 4; $r = 0.93$; $P < .01$). In contrast, there was no or low correlation between either cat IgA or cat IgM and deglycosylated cat IgA ($r = 0.30$; $P > .05$; and $r = 0.46$; $P = 0.04$, respectively).

Competitive inhibition ELISA

Preincubation of 9 cat IgA-positive sera (range, 0.38–15.6 kUA/L) with cat IgA resulted in nearly complete

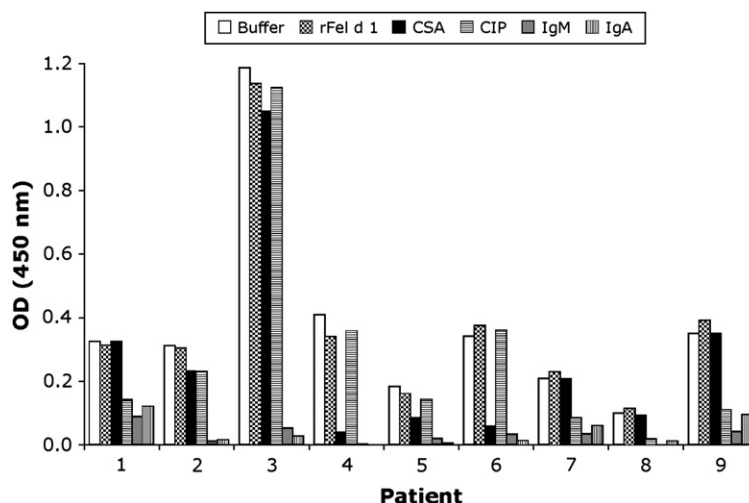


FIG 5. Competition of IgE responses to cat IgA in 9 cat-sensitized patients using ELISA. Y-axis, OD after preincubation with buffer, recombinant Fel d 1, CSA, CIP, IgM, and IgA.

inhibition (Fig 5). Cat IgM was able to inhibit almost all IgE binding to cat IgA, and the degree of inhibition was comparable to the inhibition by cat IgA in all sera. CIP inhibited the IgE reactivity to cat IgA similar to the homologous inhibition in sera 1, 7, 8, and 9. Twenty-six percent inhibition could be observed after preincubation with CIP in serum 2, whereas no effect was observed in the remaining 4 sera. No inhibition was observed after preincubation with CSA in 5 sera, whereas 25% to 85% inhibition was noted in the remaining 4 sera. The control protein Fel d 1 had no inhibitory effect.

Immunoblotting

Polyvinylidene fluoride blotted cat IgA, separated on a 15% SDS-PAGE, demonstrated IgE binding to the α -chain of cat IgA in 5 patients (Fig 6, A, lanes 3-7). One patient also demonstrated IgE reactivity to the light chain of cat IgA (Fig 6, A, lane 5). Goat antibodies directed to cat immunoglobulin light chains and cat IgA α -chains confirmed the sizes of IgA heavy and light chain (Fig 6, A, lanes 1 and 2).

To confirm the presence of cat IgA in cat dander extracts, 3 different batches were examined. Presence of IgA α -chain was verified by staining of a 64-kD band in all 3 batches by antibodies (Fig 6, B, lanes 1, 3, 5). Preincubation with purified cat serum IgA almost completely abolished the goat anticat α -chain bands (Fig 6, B, lanes 2, 4, 6). Using the pool of 21 cat IgA-positive sera demonstrated IgE binding to natural cat IgA but close to no binding to deglycosylated cat IgA (Fig 6, C, lanes 1 and 2). However, polyclonal goat anticat IgA demonstrated similar reactivity to cat IgA before and after deglycosylation (Fig 6, C, lanes 3 and 4).

DISCUSSION

Cat immunoglobulins have been discussed as allergen candidates for decades.⁷ In this study, we have identified

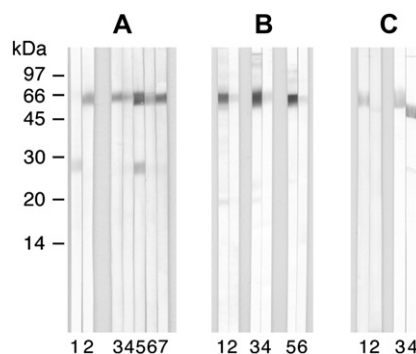


FIG 6. Goat anticat immunoglobulin light chains (lane 1) and goat anticat IgA α -chains (lane 2), IgE reactivity of patients' sera (lane 3-7) to PVDF blotted cat IgA (A). Cat IgA α -chain recognition in 3 different batches of cat dander extract without (lanes 1, 3, and 5) and with (lanes 2, 4, and 6) preincubation with cat IgA (B). IgE reactivity to cat IgA (lane 1) and deglycosylated IgA (lane 2) in a pool of 21 cat IgA-sensitized patients, for control purpose, goat antibodies to cat IgA α -chains on cat IgA (lane 3), and deglycosylated cat IgA (lane 4) (C).

IgE binding to cat IgA by several immunoassay approaches and demonstrated that the binding is mediated mainly by carbohydrate moieties, notably on the heavy chain.

The identity of cat IgA is supported by the finding of expected bands in SDS-PAGE under reducing conditions, corresponding to molecular sizes of the heavy and light chains of cat IgA.^{21,22} Furthermore, the immunoglobulin identity was also demonstrated by using antibodies specific to cat α -chains in immunoblotting. IgE responses to cat IgA were evaluated in 81 cat dander-sensitized patients by the ImmunoCAP System, and more than $\frac{1}{3}$ were positive. Cat IgA has been designated *Fel d 5* (*F domesticus* allergen 5) by the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee.

We recently demonstrated heterophile IgM antibodies directed to carbohydrates on a wide variety of animal immunoglobulins and also on CIP.¹⁸ Therefore, we wanted to examine whether IgE reactivity to cat IgA could be directed to carbohydrates. We compared the IgE reactivity to cat IgA, cat IgM, and deglycosylated cat IgA in indirect ELISA assays. Deglycosylation of glycoallergens with enzymes such as PNGase F has previously resulted in reduced IgE binding to glycoproteins with epitopes in the glycan moiety.²³⁻²⁶ No IgE reactivity to deglycosylated cat IgA was observed in the vast majority of patients, whereas the control antibody indicated that deglycosylation did not disrupt the protein structure. The results indicate that the major part of the IgE binding epitopes are located in the carbohydrate structures of cat IgA. However, the few cases of IgE reactivity to deglycosylated IgA suggest additional IgE binding epitopes present on the protein backbone structure.

IgE in patient serum samples detected cat IgM as well as cat IgA, and a high correlation between IgE antibodies to cat IgA and IgM was observed. To examine cross-reactivity between cat IgA and IgM, we performed competitive inhibition assays. Preincubation separately with cat IgA or IgM completely or almost completely inhibited IgE binding to cat IgA in all patients tested, indicating that cat IgA and cat IgM share similar or identical carbohydrate epitopes. CIP, which has previously been demonstrated to share human IgM carbohydrate epitopes with cat IgA and cat IgM,¹⁸ was also tested for inhibitory properties. Interestingly, substantial inhibition was noted in 5 of 9 patients, indicating that cat IgA and CIP share IgE epitopes depending on carbohydrate moieties. Strong inhibition with CSA to solid phase bound cat IgA was observed in 2 patients. The reason for this is unclear but could be posttranslational modifications other than glycosylation or the minute CSA contamination of cat IgA.

We also evaluated antibody binding to cat IgA and cat dander extract in immunoblotting experiments. The results indicate that the majority of cat IgA-sensitized patients react to the α -chain and only a few to the light chains. Bands of the α -chains are partially overlapping with albumin¹⁸; it is therefore possible that reactivity to bands in this area occasionally has been misinterpreted as albumin reactivity.²⁷

Presence of IgA in cat dander would suggest a possible route of sensitization. Therefore, we investigated the existence of cat IgA in 3 different batches of cat dander by using specific anticat IgA α -chain antibodies. The cat IgA content in cat dander extract was alike in all batches and could be inhibited with our purified serum IgA.

The similar IgE responses between cat IgA and CIP are an example of cross-reactivity between unrelated glycoproteins, something that has also been observed among the cross-reactive carbohydrate determinants.¹¹⁻¹³ The carbohydrate structure of CIP has been solved by mass spectrometry²⁸ but has so far not been solved specifically for cat IgA or cat IgM.

In conclusion, we have described a new allergen, cat IgA, which was detected in nearly 40% of cat-sensitized

individuals. The main IgE reactivity was found to be directed against an epitope involving a carbohydrate moiety on the heavy chain. The carbohydrate epitopes were shared by cat IgA and IgM and partially also by CIP. This is the first report on cross-reactive IgE binding to an epitope involving and depending on carbohydrates in mammalian allergens.

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