

Research paper

Interference in immunoassays by human IgM with specificity for the carbohydrate moiety of animal proteins[☆]

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Abstract

In allergen characterization common immunoassays such as ELISA and immunoblotting are often used to evaluate human IgE binding of sera from allergic individuals. In an attempt to find and characterize new cat allergens, heavily stained bands were found at 67 and 90 kDa on blotted cat dander extract. Further characterization confirmed these bands to be cat IgA and IgM heavy chains. Analyses in ELISA of purified cat immunoglobulins linked the human IgE binding to cat IgA and IgM and also revealed complete mutual cross-reactivity between the two isotypes. IgE reactivity was found to be directed to carbohydrates of the immunoglobulins and to IgM from 7 out of 9 animal species, but not to human immunoglobulins. Further investigations revealed that the reaction was not mediated by human IgE but by a factor in the serum cross-linking animal immunoglobulins and alkaline phosphatase. The factor was further studied and shown to be human IgM. This IgM fits into the heterophile antibody classification and is directed against carbohydrates on animal immunoglobulins and on calf intestine alkaline phosphatase.

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1. Introduction

Molecules that induce the production of IgE antibodies and have the property to elicit allergic symptoms in sensitized subjects are defined as allergens. Identification and characterization of single allergenic molecules

has greatly facilitated the exploration of associated pathogenic mechanisms, as well as diagnosis and treatment of allergy. The domestic cat (*Felis domesticus*) is among the most common and potent sources of allergens, causing allergic disease on a worldwide basis (Platts-Mills et al., 1997). Analyses of cat dander extract suggest at least eight IgE binding components (Löwenstein et al., 1985). However, so far only four cat allergens have been described, namely the dominant allergen Fel d 1 (secretoglobulin) (Ohman et al., 1974), Fel d 2 (albumin) (Ohman et al., 1973), Fel d 3 (cystatin) (Ichikawa et al., 2001) and Fel d 4 (lipocalin) (Smith et al., 2004).

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting are common techniques used for allergen characterization and they are also frequently used in other research fields. Both techniques rely on a substrate transfer by enzyme labelled antibodies in the

Abbreviations: ALP, alkaline phosphatase; GαR, goat anti-rabbit; HRP, horseradish peroxidase; OD, optical density; PVDF, polyvinylidene difluoride; RαhIgE, rabbit anti-human IgE; RαhIgM, rabbit anti-human IgM; rFel d 1, recombinant Fel d 1; SEC, size exclusion chromatography.

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last step, which confirms that antibodies have attached to an antigen. ELISA was first described in 1971 (Engvall and Perlman, 1971; van Weemen and Schuurs, 1971). The most commonly used enzymes in ELISA are calf intestine alkaline phosphatase (ALP), horseradish peroxidase (HRP) and, to a lesser extent, β -D-galactosidase (van Weemen, 1985). ALP and HRP linked antibodies have been shown to be equally efficient in ELISA (King and Kochoomian, 1979). For immunoblotting, the method introduced by Towbin (Towbin et al., 1979) is still used today and ALP is reported to work better than HRP (Bestagno et al., 1987).

In this study, we aimed to identify and characterize two novel cat allergens, IgA and IgM, which were detected by immunoblotting and ELISA. However, when appropriate controls were made, it was found that the reactions were not IgE mediated. Interference in the antigen–antibody interaction in the immunoassays was observed and was further investigated.

2. Materials and methods

2.1. Patient sera and non-allergic controls

Serum was obtained from 2 groups of cat IgE sensitized patients ($n=30$, median 2.3 kU_A/l, range 0.35–38 kU_A/l and $n=52$, median 45.5 kU_A/l, range 29–350 kU_A/l respectively), patients sensitized to at least one of either cat, cow or dog ($n=40$, median 1.4 kU_A/l, range 0.35–21.45 kU_A/l), and negative controls ($n=19$, non-cat IgE sensitized). All sera were analysed by the Pharmacia CAP System™ (Pharmacia Diagnostics, Uppsala, Sweden) following the instructions of the manufacturer.

2.2. Reference sera and serum pools

For the purpose of inter-assay comparisons in ELISA, negative and positive reference pools were produced and included in each run. A positive reference was prepared from 10 sera selected from the 52 IgE sensitized subjects positive to cat IgA (pool I). These sera were also used in immunoblotting. A second serum pool (pool II) was prepared from the 49 of the 52 patients that were positive in cat IgA ELISA. This pool was used in inhibition experiments and for size exclusion chromatography (SEC). For preparative serum SEC a HiLoad™ 16/60 Superdex™ 200 pg FPLC column, equilibrated in PBS was used at a flow rate of 1 ml/min and 2 ml fractions were collected. A third serum pool (pool III) was prepared from the 18 of the 30 IgE sensitized patients that were positive in cat IgA ELISA. All serum pools were also used for analyses of cat IgM responses. The ne-

gative reference was prepared from 19 non-cat IgE sensitized individuals by extensive adsorption over a mixture of cat immunoglobulins bound to a 1 ml *N*-hydroxysuccinimide-activated HiTrap™ column (Amersham Biosciences, Uppsala, Sweden).

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

2.3. Purification of immunoglobulins

A pool of sera collected from 5 cats was kindly donated by the National Veterinary Institute (SVA, Uppsala, Sweden). Affinity purification of cat IgA from serum was performed as described (Rönnmark et al., 2002) using (Z_{IgA1})₂-His₆ affibody (kindly provided by Prof. Per-Åke Nygren, Royal Institute of Technology, Stockholm, Sweden). IgG was purified from cat serum using HiTrap Protein A as described by the manufacturer (Amersham Biosciences). The cat IgG and IgA were further purified by two consecutive runs of preparative SEC. The fractions containing the desired immunoglobulins were pooled and concentrated using an Amicon cell (Amicon, Bedford, MA, USA) with an YM-3 filter (Millipore, Bedford, MA, USA). IgM antibodies from all species were purchased from Europa Bioproducts (Cambridge, UK). The protein concentration was measured by BCA™ Protein Assay (Pierce, Rockford, IL, USA) using Bovine Plasma Gamma Globulin (Bio-Rad, Hercules, CA, USA) as standard.

2.4. Extraction of cat dander allergens

A total of 1.2 g cat dander (Allergon, Ängelholm, Sweden) was mixed with 300 ml 16 mM KH₂PO₄ buffer (pH 7.4) with 25 mM NaCl and extracted for 1 h, end over end, at 4 °C. After centrifugation at 7000 $\times g$ for 1 h at 4 °C in a J2-21 centrifuge (Beckman Coulter, Fullerton, CA, USA), the soluble proteins were concentrated in a 200 ml Amicon cell with a YM-3 filter (Millipore). Finally, the buffer was changed to 10 mM NaCl using NAP 10 column (Amersham Biosciences). The extract was stored at –20 °C until use.

2.5. Indirect ELISA

IgE responses to animal immunoglobulins and for control purpose to human myeloma IgG1, IgA and IgM (kindly provided from Pharmacia Diagnostics AB, Uppsala, Sweden) were analysed using serum from IgE sensitized patients and non-sensitized controls by ELISA as described (Grönlund et al., 2003). Briefly, 96-well micro-titre plates (Nunc, Roskilde, Denmark) were

coated with 0.5 µg of purified immunoglobulin per well followed by blocking with bovine serum albumin. Patient sera diluted 1/2 in PBS were incubated in duplicate, followed by rabbit anti-human IgE (RøhlIgE) (MIAB, Uppsala, Sweden) and ALP conjugated goat anti-rabbit immunoglobulins (GαR-ALP) (Dako, Glostrup, Denmark). For control purpose, streptavidin-ALP (Amersham Biosciences) or GαR-ALP was added after the serum incubation, and the RøhlIgE was omitted. Finally, ALP substrate (Sigma) was added and the colour reaction monitored at 405 nm. Values were considered positive when the IgE optical density (OD) responses exceeded 3 times the OD of the negative reference. Inter-assay variation was not allowed to exceed 10% between runs.

For HRP ELISA, essentially the same protocol was used but all NaN₃ was removed from the buffers. Donkey anti-rabbit HRP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, dilution 1/2000) was used as secondary antibody. O-phenylenediamine tablets (Sigma, 10 mg) dissolved in 5 ml 0.05 M phosphate/citrate buffer and 10 µl hydrogen peroxide (Sigma, 30 % wt.) was used as substrate. The colour reaction was measured at 450 nm. Serum from a patient with IgE myeloma diluted to an IgE concentration of 25 000 kU/l was coated with 0.25 µg/ml of IgE per well and used in the ELISA to compare ALP and HRP signals.

2.6. Competitive inhibition ELISA

Competitive inhibition ELISA was performed essentially as described for the indirect ELISA, but with a prior step of pre-incubation of serum pool II with serial dilutions of an allergen. The serum was diluted 1/2 with either PBS or PBS containing a final concentration of 250, 50, 5, 0.5, 5×10^{-2} , 5×10^{-3} and 5×10^{-4} µg/ml of cat IgA and IgM, respectively, and incubated over night at +4 °C. Recombinant Fel d 1 (rFel d 1) (Grönlund et al., 2003), used as negative control, and cat IgG were incubated with the serum in final concentrations of 250, 50, 5, 5×10^{-2} and 5×10^{-4} µg/ml. Following pre-incubation, the samples were added to cat IgA or IgM-coated plates. The ability of increasing amounts of myeloma IgE (ND) to compete with allergen bound IgE for binding to RøhlIgE, was investigated. The OD-value for serum pool II without inhibitor protein was regarded as no inhibition.

2.7. Immunoblotting

Samples were separated using 15% polyacrylamide gels as described below and transferred to polyvinylidene

difluoride (PVDF) membranes (Immobilon™-P transfer membranes, Millipore) following the manufacturer's instructions. The transfer was performed in a Hoefer® TE 22 Mighty Small™ Transphor tank unit (Amersham Biosciences) followed by blocking and visualization of allergens as described (Johansson et al., 1994). For competitive inhibition immunoblotting experiments, proteins tested for competition with IgE binding were added to serum to a final concentration of 50 µg protein/ml.

To locate cat immunoglobulin heavy chains and albumin, cat dander extract blotted strips were exposed to either affinity purified goat anti-cat IgA α-chain, goat anti-cat IgM µ-chain or goat anti-cat albumin (dilution 1/10 000, Immunology Consultants Laboratory, Newberg, OR, USA), followed by ALP-conjugated anti-goat IgG antibodies (dilution 1/20 000, Sigma) or ALP-conjugated goat anti-cat IgG Fc (dilution 1/2000, Bethyl, Montgomery, TX, USA).

2.8. Pepsin digestion

The fragmentation of cat IgA to F(ab')₂ fragments was performed with pepsin (Sigma) according to the manufacturer's instructions and followed by SEC to obtain the desired 120 kDa fragments.

2.9. Deglycosylation

Cat IgA was deglycosylated by PNGase F (New England Biolabs, Beverly, MA, USA) following the manufacturer's instructions. For control purpose, IgA was treated identically but without the addition of the active amidase, referred to as "sham treated IgA".

2.10. Analysis of immunoglobulins

Purity of animal immunoglobulins was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 5% and 15% homogeneous gels (Gallagher, 1999a,b), with low molecular weight markers (Amersham Biosciences). Samples were denatured at 98 °C for 5 min in SDS sample buffer with or without dithiothreitol (Amersham Biosciences). Purity was also analysed by SEC using a Superose 6™ HR 10/30 column on an FPLC™ system (Amersham Biosciences), equilibrated in PBS, at a flow rate of 0.3 ml/min. For the cat immunoglobulins, competitive inhibition ELISA with affinity purified goat anti-cat IgA α-chain, goat anti-cat IgM µ-chain (dilution 1/20 000), followed by ALP-conjugated anti-goat IgG antibodies (dilution 1/20 000) or ALP-conjugated goat anti-cat IgG Fc (dilution 1/500, Bethyl), was performed to quantitatively

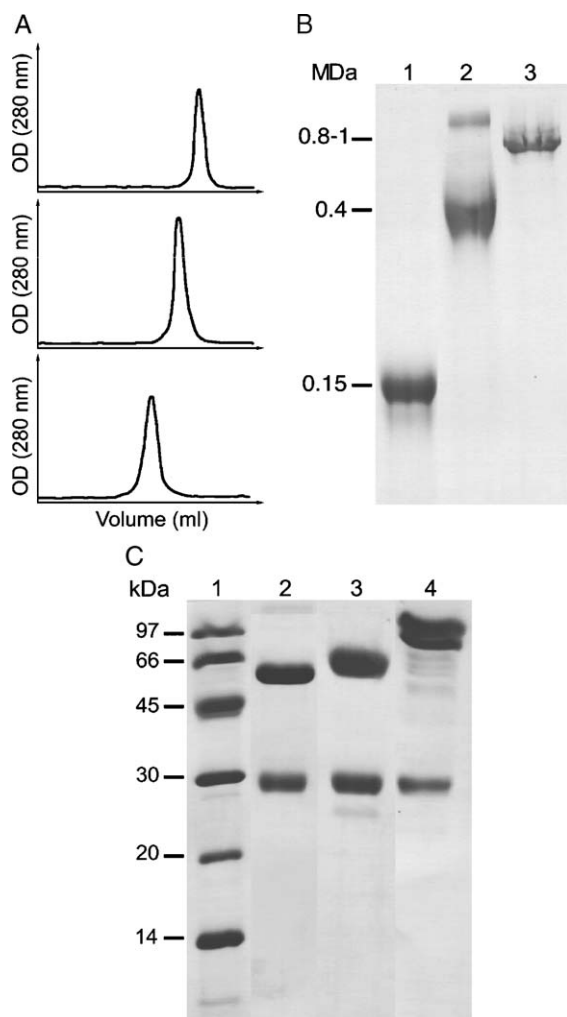


Fig. 1. Analyses of size and purity of purified cat immunoglobulins. Superose 6™ SEC of purified IgG, IgA and IgM (A). 5% SDS-PAGE of cat immunoglobulins under non-reduced conditions (B): lane 1, IgG; lane 2, IgA; lane 3, IgM and 15% SDS-PAGE under reducing conditions (C): lane 1, IgG; lane 2, IgA; lane 3, IgM; lane 4, low molecular weight markers.

reveal the amount of cross-contamination. The respective antibodies were pre-incubated with 50, 5, 0.5 and 0.05 $\mu\text{g/ml}$ of cat IgG, IgA and IgM or with dilution buffer only. Thereafter the mixtures were added to cat IgA and IgM coated plates and developed as described. The different inhibition curves were evaluated to find the concentration required for 50% heterologous inhibition, which was compared to 50% homologous inhibition.

2.11. Affinity chromatography

Rabbit anti-human IgM antibodies (R α hIgM) (Amersham Biosciences) were immobilized on 1 ml

N-hydroxysuccinimide-activated HiTrap columns according to the manufacturer's instruction. For control purpose, the same was made with normal rabbit γ -globulin. Serum pool III was passed through the columns and the flow through was collected. The columns were washed with 10 column volumes of PBS, eluted in 0.5 ml fractions with 0.1 M citrate buffer pH 3.0, the eluted fractions were neutralised with 1 M Tris-HCl, pH 9.0. Pool III, given an arbitrary value of 100 absorbance units (AU) was serially diluted and used as a reference allowing calculations of absolute reactivity to cat IgA.

2.12. Statistical analysis

Statistical analysis was performed using the software Statistica version 5.5 by StatSoft (Tulsa, OK, USA). Spearman Rank was used for correlation analysis.

3. Results

3.1. Purity of cat immunoglobulins

Analytical SEC documented the cat IgG, IgA and IgM preparations to contain very little contamination and only single peaks were found (Fig. 1A). No contamination could be detected by SDS-PAGE following analysis of either reduced or non-reduced samples (Fig. 1B and C).

Competitive inhibition ELISA with affinity purified anti-cat immunoglobulin antibodies revealed about 1% IgA and 1% IgG in the IgM preparation, compared to less than 1% IgG and less than 10% IgM in the IgA preparation.

3.2. Immunoblotting

The human IgE reactivity to cat dander extract, blotted to PVDF strips, was analysed in serum from each of 10 cat IgE sensitized patients, individually (Fig. 2A) or pooled (Fig. 2B). All but one patient (Fig. 2A, lane 7) reacted to an 8 kDa protein, presumably Fel d 1. Furthermore, reactivity was noted to bands around 14–20, 30, 45, 64 and 90 kDa and above. Most notable were the very strong and frequently occurring 64 and 90 kDa bands.

3.3. Cross-reactivity studies using competitive inhibition immunoblotting

Serum pool I was pre-incubated with cat IgG, IgA, IgM and rFel d 1 (Fig. 2B, lanes 2–5) and the reactivity was compared to no inhibition (Fig. 2B, lane 1). Addition of cat IgG did not have any visible effect on the

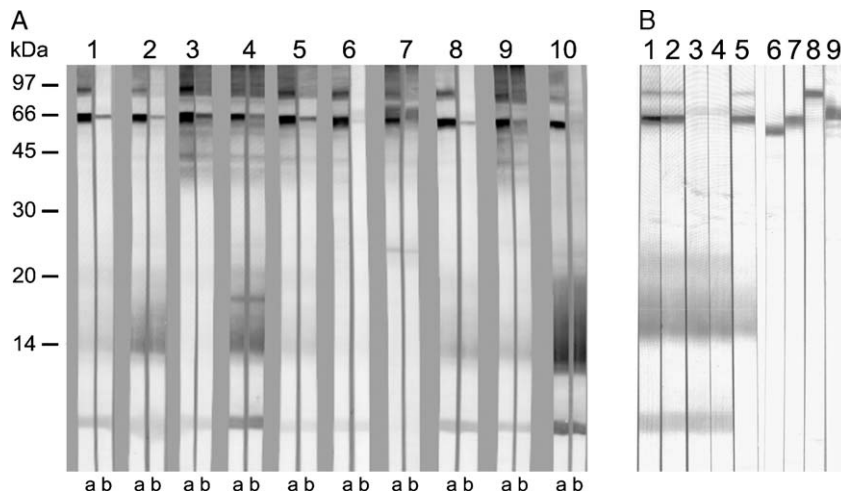


Fig. 2. IgE reactivity to PVDF blotted cat dander extract proteins in serum from 10 individual cat IgE sensitized patients (A), after pre-incubation with dilution buffer (a) and after competition of IgE reactivity by addition of purified cat IgA (b). IgE reactivity to PVDF blotted cat dander extract proteins (B) using the sera as a pool ($n=10$) after pre-incubation with dilution buffer (lane 1) and after pre-incubation with cat IgG (lane 2), IgA (lane 3), IgM (lane 4) and rFel d 1 (lane 5). For the purpose of comparison, specific goat antibodies to cat IgG (lane 6), IgA (lane 7), IgM (lane 8) and albumin (lane 9) were used on the blotted strips.

staining pattern (Fig. 2B, lane 2) while cat IgA eliminated both the 64 and the 90 kDa bands (Fig. 2B, lane 3). Similarly, pre-incubation with cat IgM completely abolished both bands (Fig. 2B, lane 4). Addition of highly purified rFel d 1 abolished bands at 8 kDa and reduced the staining of the broad band between 14 and 20 kDa (Fig. 2B, lane 5). Strips incubated with goat antibodies to cat γ -, α -, μ -chains and albumin, specifically stained bands at 55, 64, 90 and 66 kDa respectively, indicating the 64 kDa band to be α -chain and the 90 kDa μ -chain (Fig. 2B, lanes 6–9). In addition, by adding purified cat IgA to the individual patients' sera, both the bands at 64 and 90 kDa were substantially reduced (Fig. 2A, lane 1b–10b).

3.4. Reactivity to various immunoglobulins

Indirect ELISA revealed human IgE reactivity to cat IgA among 18 of 30 cat IgE sensitized patients, 49 of 52 highly sensitized patients and 9 of 19 non-sensitized controls. Binding of human IgE to cat IgM was found in 20 sera from the 40 patients with IgE sensitization to animal dander. The 20 positive sera were further analysed for reactivity to IgM from different animal species and compared to cat IgM (Table 1). The reactivity to rabbit and mouse IgM was about 50% higher than to cat IgM whereas reactivity to dog and pig IgM was similar to cat IgM. Reactivity to horse and cow IgM was about 30% lower than to cat while no reactivity was detected to hamster or guinea pig IgM. When compared to reactivity to cat IgM, no correlation was

found to guinea pig IgM. Intermediate correlation was found to mouse and hamster IgM, while a strong correlation was noted to IgM from the other species.

3.5. Cross-reactivity studies using competitive inhibition ELISA

The intra-species cross-reactivity patterns of cat IgG, IgA and IgM were also evaluated by competitive inhibition ELISA using serum pool II. The ability of cat IgA or IgM to compete with human IgE for solid phase bound cat IgA (Fig. 3A) and IgM (Fig. 3B) was dose dependent and reached almost complete inhibition at 250 μ g/ml of added competitor. On the other hand, the capacity of cat IgG to compete was 50 to 100 fold lower (Fig. 3A and B). For control purposes, the major allergen from cat, rFel d 1, was analysed and displayed

Table 1
IgE reactivity to IgM from 8 different species expressed as mean OD for 20 individuals and correlated to reactivity to cat IgM

	OD-mean	Spearman <i>R</i>	<i>p</i> -level
Cat	0.72	–	–
Dog	0.75	0.98	0.00
Pig	0.76	0.97	0.00
Rabbit	1.10	0.93	0.00
Horse	0.55	0.91	0.00
Cow	0.50	0.87	0.00
Mouse	1.15	0.68	0.00
Hamster	0.07	0.60	0.01
Guinea Pig	0.09	0.10	0.67

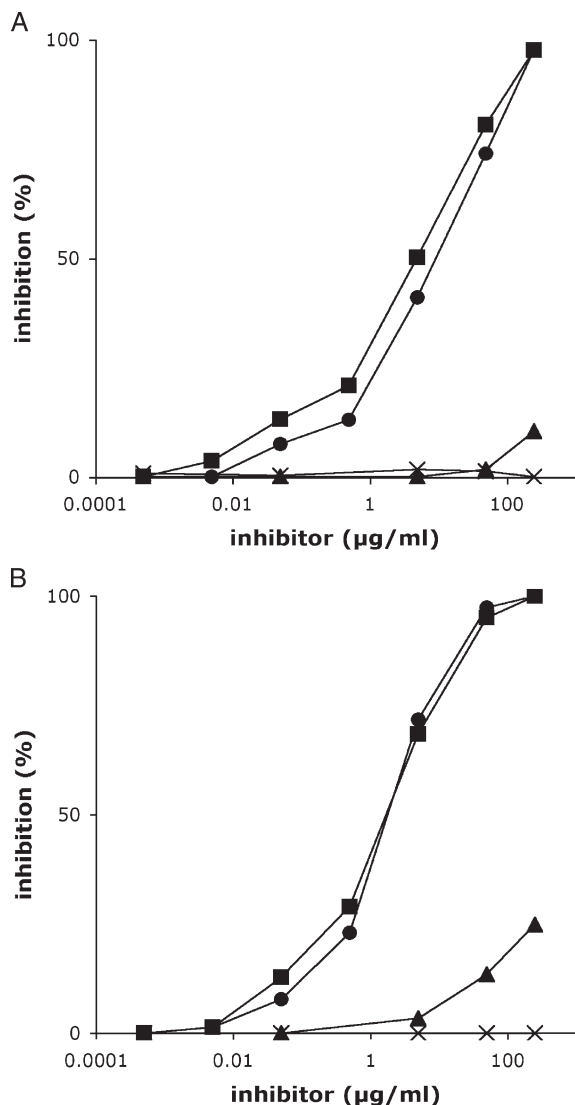


Fig. 3. Competition of IgE responses to cat IgA (A) and cat IgM (B) coated to micro-titre plates in a pool of 49 cat IgE sensitized patients. Displayed on the x-axis is concentration of inhibitor, IgA (●), IgM (■), IgG (▲) and rFel d 1 (×) and on the y-axis percent inhibition.

no competing capacity at any of the concentrations tested.

3.6. Investigation of the influence of carbohydrate structures on cat IgA

The reactivity to cat IgA F(ab')₂ fragments obtained by pepsin digestion was examined in patients with IgE reactivity to cat IgA but no activity was found (data not shown).

Three individual patient sera and pool III were tested in ELISA to deglycosylated cat IgA, “sham treated IgA” and natural IgA. None of the sera reacted with

deglycosylated IgA while they reacted to natural IgA and to “sham treated IgA” (data not shown). Goat anti-cat IgA reacted positively to all three forms of IgA, reactivity to deglycosylated IgA and “sham treated IgA” was similar, although reactivity was stronger to natural IgA.

3.7. Examination of IgE involvement

In order to examine the involvement of RαhIgE, irrespective of the presence of human IgE anti-cat IgA, increasing amounts of purified myeloma IgE was added to sera from pool III at the RαhIgE step in an indirect cat IgA ELISA. The reactivity decreased less than 10% and the decrease was not dose dependent (data not shown). Also, omitting RαhIgE decreased the reactivity of pool III less than 3.4% in the indirect ELISA (data not shown).

3.8. Cross-binding of cat IgA, animal IgM and alkaline phosphatase

Addition of SEC fractions of serum pool II to a cat IgA coated ELISA plate, followed by adding either streptavidin-ALP or GαR-ALP and thereafter development, omitting the RαhIgE, revealed that the response to cat IgA existed in the fractions corresponding to the void volume (data not shown). Due to the high molecular weight of the cat IgA binding factor, a hypothesis that human IgM was cross-binding the carbohydrates on cat IgA, GαR-ALP or streptavidin-ALP, was put forward. To investigate the hypothesis, pool III was passed through a rabbit RαhIgM coupled column. It was shown that the activity decreased from 52 to 24 AU in the flow through of the anti-human IgM and that 18 AU of activity was found in the first two eluate fractions. However, when pool III was passed through a column with rabbit-γ of irrelevant specificity, no decrease in activity was shown in the flow through and no activity was found in the eluate.

3.9. Comparison between ALP and HRP

Pool III displayed clearly positive signals in both the HRP and ALP systems, although 60% stronger reactivity was observed in the ALP ELISA. The removal of RαH IgE antibodies totally blanked the HRP system, whereas the ALP system still displayed clearly positive signals (data not shown).

4. Discussion

IgE sensitization to an allergen is the key factor for the development of allergy. Although allergy to cat is

common, so far only two major and two minor allergens have been characterized in cat dander extract, even though at least eight IgE binding components have been suggested by immunoblotting and other techniques. We aimed to identify and characterize new cat allergens by using common techniques for allergen characterization, i.e. ELISA and immunoblotting. Immunoblotting was performed on PVDF blotted cat dander extract in an effort to evaluate IgE binding proteins. In addition to known allergens, in our study heavily stained bands were found at 90 and 64 kDa. Albumin as the binder was ruled out since the 64 kDa band was slightly smaller than albumin, sized 66–68 kDa. However, immunoglobulin heavy chains of IgA (α -chains) did correspond to the size of the 64 kDa band and IgM (μ -chains) corresponded to the 90 kDa band, and were therefore assumed as possibly representing hitherto unidentified cat serum allergens. Immunoglobulins are present in high concentrations in cat saliva and serum (Harley et al., 1998), which would make them potential allergens. Accordingly, cat IgG, IgA and IgM were isolated. Data from SEC and non-reducing SDS-PAGE support previous reports of cat IgG as a 150 kDa monomer, IgA as a 350 kDa dimer and IgM as a 0.8–1 MDa multimer (Vaerman, 1970; Vaerman, 1973). Each preparation displayed two bands in SDS-PAGE under reducing condition, corresponding to the well-known molecular sizes of the heavy and light immunoglobulin chains. In addition, identification of the immunoglobulins was confirmed using antibodies specific to cat γ -, α - and μ -chains in immunoblotting.

Further characterization of the IgE binding revealed, to our surprise, complete mutual cross-reactivity between cat IgA and IgM. In addition, a broad reactivity to IgM from different animals was observed, high reactivity was seen to 6 of 8 species tested. However, no reactivity was noted to human IgM or any other human immunoglobulin. These results suggest the presence of surface-exposed IgE binding structures on the Fc part, or in the hinge region, of animal immunoglobulins. This finding was disturbing, since the heavy chains of different immunoglobulin classes do not have the same amino acid composition and do not share major antigenic epitopes. However, the broad activity to IgM from different animal species, the complete cross-reactivity between cat IgA and cat IgM and the lack of binding to F(ab')₂ fragments lead us to investigate whether the IgE reactivity might be directed against common immunoglobulin structures such as carbohydrate side chains. When the lack of activity to deglycosylated cat IgA indicated that the observed IgE binding was directed to carbohydrates, the broad reactivity was no longer surprising. However, IgE reactivity has, to the best of

our knowledge not previously been reported to animal carbohydrates, only to plant carbohydrates (Batanero et al., 1996). These findings lead to a thorough investigation of the involvement of IgE. The addition of purified myeloma IgE to R α hIgE did not block the reactivity, and removal of R α hIgE did not decrease the reactivity in the indirect ELISA more than marginally. These control experiments show that most of the reactivity was independent of IgE.

To reveal what factor in human serum was responsible for the cross-reactivity, we first investigated its molecular size. SEC revealed the factor directly linking cat IgA and G α R-ALP to be a very high molecular weight component, eluting in the void in SEC. The use of streptavidin-ALP instead of G α R-ALP in the assays yielded the same results, which supported the notion that the factor can directly cross-bind cat IgA and ALP. Further investigations revealed the factor in human serum to be IgM. Considering that the cross-linking IgM factor has a high prevalence regardless of serum origin, and that the immunoassay technique used here has been extensively used, it is surprising that this phenomenon has, to the best of our knowledge, not been reported earlier.

Pre-adsorption of patient sera using a matrix consisting of carbohydrates could have removed the IgM causing the problem. However, such pre-adsorption would also have removed potentially interesting IgE since both IgE and IgM could be directed to the same molecules and even to the same allergenic epitopes on these molecules. Therefore pre-adsorption of the sera was not considered an option in this study. Furthermore, dilution of the patient sera was not considered, because allergen-specific IgE is present in only minute quantities. Deglycosylation of the antigen is a third option, but also has drawbacks, the main concern being that it would remove or destroy possible carbohydrate-related allergenic epitopes. We proposed a simpler solution, namely to change the enzyme from ALP to, for example, HRP. Removal of the R α hIgE yielded background signals for the HRP ELISA, but not for the ALP ELISA, which indicates that the HRP system is working as expected, while the ALP ELISA is not. All other controls performed as assumed in the HRP system. HRP, however, is known to contain epitopes cross-reacting with allergen structures from plants, called cross-reactive carbohydrate determinants (CCDs) (Batanero et al., 1996). IgE, and most likely antibodies of other isotypes, can be directed to the carbohydrate structure of these allergens. This could cause similar cross-reactivity as demonstrated in this study; antibodies could cross-link solid phase bound CCDs and HRP directly.

Therefore, HRP as well as ALP should be analysed for possible cross-reactivity when used in immunoassays. An alternative to glycosylated enzymes is to use the *E. coli* produced non-glycosylated enzyme β -D-galactosidase, which would eliminate any concern of carbohydrate cross-linking.

The positive signals, using a pool of serum in the HRP ELISA, which could be inhibited by removal of anti-IgE, indicates that at least some individual sera have IgE antibodies directed to cat IgA, and this will be further investigated.

We noticed a small cross-reactivity between cat IgG on the one hand, and cat IgA and cat IgM on the other with inhibitions 50–100 fold lower than that of IgA and IgM. This suggests that cat IgG and presumably IgG from other species can be causing the same undesirable binding as IgM from various mammals and cat IgA, but to a lower extent. However, in a very sensitive assay, this could affect the result. To avoid any unsolicited binding, the use of unglycosylated antibodies, as for example Affibody® molecules, instead of IgG antibodies may be considered.

The IgM antibodies we have described here fit into a group of human antibodies called heterophile antibodies. These antibodies are almost always of IgM class and react with antigenic epitopes present in two or more species and they seem to have no well-defined immunogen (Kaplan and Levinson, 1999). Heterophile antibodies have been known for a long time to interfere in immunoassays and give rise to false positive results (Foucard et al., 1975; Hunter and Budd, 1980). They have, to the best of our knowledge, not previously been demonstrated to be specific for carbohydrates on animal proteins. However, carbohydrate specific IgG antibodies have actually been reported to interfere in immunoassays (Hamilton and Adkinson, 1985). Interference by antibodies in immunoassays is a well known phenomenon which can have significant consequences (Kricka, 2000; Ismail et al., 2002) and it is therefore important that appropriate controls are included in the assay and that high quality antibodies are used (Shields and Turner, 1986).

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