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Fine specificity of B-cell epitopes on *Felis domesticus* allergen I (*Fel d* I): Effect of reduction and alkylation or deglycosylation on *Fel d* I structure and antibody binding

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The repertoire of B-cell epitopes on the major cat allergen, Fel d *I, was analyzed with monoclonal antibodies (MoAbs) in topographic mapping studies and in immunoassays with antigen derived from other cat* (Felidae) *species. Four essentially nonoverlapping epitopes on* Fel d *I, designated Fd1A to D, were defined by use of 15 anti* Fel d *I MoAbs in cross-inhibition radioimmunoassay. Only MoAbs directed against epitope Fd1B bound to putative* Fel d *I homologues in hair and dander extracts from seven other feline species (*Panthera *species, [*n = *5],* Leptailurus serval, *and* Leopardus pardalus*). Quantitative monosaccharide analysis showed that* Fel d *I was a glycoprotein, containing high levels of fucose, as well as glucosamine, galactose, and mannose. Binding of MoAbs and human IgG or IgE antibody to native, reduced and alkylated or deglycosylated* Fel d *I was compared by means of immunoprecipitation and immunoassay, and the effects of these treatments on the structure of* Fel d *I were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis. On reduction and alkylation,* Fel d *I dissociated into 14 kd and 3.2 kd peptides, and deglycosylation with trifluoromethane sulfonic acid produced a 12 to 14 kd peptide. These procedures resulted in a 100- to 1000-fold loss in murine or human antibody binding activity and caused significant loss of secondary structure, as judged by circular dichroism spectroscopy. Treatment with potassium hydroxide also caused a marked loss in antigenic reactivity. In contrast, enzymatic deglycosylation generated a 9 kd peptide, which showed strong reactivity with murine and human antibodies, comparable to native* Fel d *I. The results show that MoAbs define a broad repertoire of B-cell epitopes on* Fel d *I, one of which is expressed by other cat species. These epitopes are conformational and do not appear to involve oligosaccharide residues. (J ALLERGY CLIN IMMUNOL 1994;93:22-33.)*

Key words: *Cat allergen, epitopes, monoclonal antibodies, glycoproteins, asthma*

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Abbreviations used

CD:	Circular dichroism
Fel d I:	*Felis domesticus* allergen I
MoAb:	Monoclonal antibody
MW:	Molecular weight
PBS-T:	Phosphate-buffered saline containing 0.05% Tween-20
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis
TFMS:	Trifluoromethanesulfonic acid

Immediate hypersensitivity to the domestic cat *(Felis domesticus)* has been extensively studied with the use of a major protein allergen produced in cat salivary and sebaceous glands, *Felis domesticus* allergen I (*Fel d* I).[1-7] Most individuals with cat allergy (>90%) produce IgE (and IgG) antibodies to *Fel d* I, and immunoabsorption studies show that *Fel d* I accounts for 60% to 95% of the IgE binding activity of cat dander extracts.[8-10] IgE antibodies to other proteins in cat dander extracts have been identified with serologic techniques.[3, 5, 9, 11] However, *Fel d* I is the most important cause of sensitization and has proved to be a consistent marker for immunologic and clinical studies of cat allergy. These studies include comparisons of IgG and IgE antibody responses during cat immunotherapy[8, 12, 13]; analysis of the particle size and concentration of cat allergen in the air[14-18]; epidemiologic studies on cat allergens as risk factors for acute asthma[19-21]; and assessment of environmental control procedures to reduce allergen exposure.[22-24]

Fel d I was originally purified from cat pelt extracts and shown to be a ~36 kd dimer composed of two 17 kd subunits.[1, 2] The development of affinity purification techniques and monoclonal antibody (MoAb)–based immunoassays for *Fel d* I made it possible to purify the allergen directly from house dust extract and to determine its partial amino acid sequence.[10, 25-28] Recently, the primary structure of *Fel d* I has been established by protein sequencing and complementary DNA cloning. Each 17 kd subunit comprises two polypeptide chains of 70 and 92 amino acid residues (chains 1 and 2, respectively), one of which (chain 1) shows sequence homology to rabbit uteroglobulin.[29] This structural information is essential for defining both B-cell and T-cell epitopes on *Fel d* I and for studies on antigen recognition and processing.

Previous studies identified two B-cell sites on *Fel d* I with the use of MoAbs and demonstrated antigenic cross-reactivity between *Fel d* I molecules from different members of the cat family *(Felidae)* with human antibodies.[10, 25, 26, 30] Proliferative T-cell responses to *Fel d* I have also been reported.[31, 32] In the present study we analyzed the epitope specificity of a large panel of murine IgG anti-*Fel d* I MoAbs and also compared the effects of structural modification of *Fel d* I (reduction and alkylation and various deglycosylation procedures) on the binding of MoAbs and human IgG and IgE antibodies. The MoAbs define four nonoverlapping epitopes on *Fel d* I, only one of which appears to be conserved among other cat species. The results also show that *Fel d* I is a fucose-rich glycoprotein and that removal of carbohydrate side chains generates polypeptides with different antibody binding activities.

METHODS

Cat allergens

Fel d I was purified from house dust extract by affinity chromatography over MoAb Fd1A immunosorbent and by size exclusion high-performance liquid chromatography, as described previously.[10, 25] Hair and dander samples from seven other cat species (*Panthera onca, P. tigris longipilis, P. leo, P. pardus kotiya, P. uncia, Leptailurus serval,* and *Leopardus pardalus*) were collected from animals kept at the Natura Artis Magistra Zoo, Amsterdam, The Netherlands, and extracted at 5% wt/vol in 37 mmol/L phosphate buffer, pH 6.8. The extracts were coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden), as described previously.

MoAbs

Two of the anti-*Fel d* I MoAbs, Fd1A and Fd1B, were produced as part of previous studies.[10, 25] A second panel of anti-*Fel d* I MoAbs was produced from a BALB/c mouse, which had been immunized intraperitoneally three times with 50 μg *Fel d* I in Complete Freund's Adjuvant, at 10- to 14-day intervals and had been given an intrasplenic boost with 20 μg *Fel d* I four days before fusion. Immune spleen cells were fused with SP2O myeloma cells as described by Chapman et al.[33] Hybrids were initially screened for production of IgG antibodies to *Fel d* I by ELISA, with microtiter plates coated with *Fel d* I–rich house dust extract. They were subsequently selected for cloning on the basis of antigen binding radioimmunoassay (RIA) with iodine 125–labeled *Fel d* I and on solid-phase inhibition radioimmunoassay to identify antibody specificities that were either similar to or different from MoAbs Fd1A and Fd1B. The inhibition radioimmunoassays were modifications of previously described assays, in which hybrid supernatants were used to inhibit binding of ^{125}I-labeled MoAb Fd1A or Fd1B to solid-phase *Fel d* I or to inhibit the binding of ^{125}I-*Fel d* I to the solid-phase MoAb.[33, 34] Thirteen hybrids were cloned by limiting dilution: two clones had specificities similar to Fd1A (3E4, 5E3); three clones had specificities similar to Fd1B (6F9, 1G9, and 8F3); and eight clones showed good reactivity in antigen binding radioimmunoassay,

but no significant inhibition of Fd1A or Fd1B binding, and were assumed to be directed against different sites on *Fel d* I (2H4, 10F7, 8B4, 8E4, 7D11, 3F11, 10E6, and 1E8). The MoAbs were produced as ascites, and all were isotype IgG_1, as determined by immunodiffusion with monospecific antisera.[33] Selected MoAbs (3E4, 6F9, 1G9, 8F3, 2H4, and 10G7) were purified by preparative isoelectric focusing for use in epitope mapping experiments.[34] The use of animals for this study was approved by the Animal Research Committee of the University of Virginia, under guidelines for the use and care of animals formulated by the National Council for Medical Research.

Human antibodies

A human serum pool was prepared with sera from six patients with cat allergy who had high levels of IgG and IgE antibodies to *Fel d* I (463 to 3660 U/ml and 102 to 440 U/ml, respectively). The antibody units were established from an IgG antibody control curve with serum from a patient with cat allergy, which was arbitrarily designated to contain 5000 U of IgG antibody per milliliter. This serum had an IgG antibody titer of 1:5000 in antigen binding radioimmunoassay. Control sera were obtained from three nonatopic individuals, with negative RAST results to cat dander extract and no detectable IgG or IgE antibodies to *Fel d* I. Collection of human sera for use in these studies was approved by the Human Investigation Committee of the University of Virginia.

Epitope mapping

These experiments were carried out with modifications of techniques described previously for MoAbs to mite allergens.[34] Briefly, plastic microtiter wells (Immulon 2 Removawells, Dynatech, Alexandria, Va.) were coated with an anti-*Fel d* I MoAb at 1 μg/well and incubated with house dust extract containing 50 U/ml *Fel d* I for 2 hours (1 U *Fel d* I is ~4 μg protein[25]). The wells were then incubated with 50 μl of serial dilutions of cold MoAb together with 50 μl of ^{125}I-anti-*Fel d* I MoAb for 2 hours, washed five times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), and then counted in a gamma counter. Inhibition curves were plotted by comparing inhibition of ^{125}I-labeled MoAb binding to *Fel d* I by cold MoAb. Uninhibited values were the mean of eight wells incubated with 1% bovine serum albumin PBS-T.

Binding of selected MoAbs to hair and dander extracts of other cat species was compared by solid-phase radioimmunoassay with extracts coupled to CNBr-activated Sepharose. The Sepharose-coupled allergen (250 μl) had been titered to contain the equivalent of 1.8 U *Fel d* I. The immunosorbent was incubated with anti-*Fel d* I MoAb ascites (1:9000 to 1:27,000 dilution of ascites containing 1 to 5 mg/ml MoAb), followed by ^{125}I-labeled goat anti-mouse IgG as described previously.[30]

Carbohydrate analyses and deglycosylation experiments

Fel d I (20 μg) was hydrolyzed in 400 μl of 2 mol/L trifluoroacetic acid (Sequanal grade, Pierce Chemical Co., Rockford, Ill.) in Teflon-lined capped tubes at 100° C for 4 hours. The hydrolysates were dried in vacuo and reconstituted in 200 μl 40 μmol/L 2-deoxy-D-glucose (grade III, Sigma Chemical Co., St Louis, Mo.), which served as an internal standard. A 50 μl sample was injected onto a 4.6 × 250 mm Carbopac PA-1 anion exchange column linked to a PAD pulsed amperometric detector with a gold working electrode (Dionex Corp., Sunnyville, Calif.). Monosaccharides were eluted with 18 mmol/L NaOH at 1.0 ml/min and quantitated relative to 2-deoxyglucose. To compensate for incomplete hydrolysis and other possible losses, the final quantitation of each monosaccharide was obtained by correction relative to glycoproteins of known monosaccharide composition (fetuin, asialofetuin, and interferrin), which had undergone the same treatment.

Deglycosylation experiments were carried out with 200 μg aliquots of *Fel d* I at a concentration of 20 μg/μl in distilled water. Four different deglycosylation procedures were compared, as follows: (1) endoglycosidase F/N-glycosidase F treatment—3 μl enzyme (0.15 U, Boehringer Mannheim, Indianapolis, Ind.) was incubated with *Fel d* I overnight at 37° C in 50 mmol/L sodium phosphate buffer/50 mmol/L ethylenediaminetetraacetic acid/0.5% octylglucoside, pH 7.0, and then incubated with 2 μl enzyme for an additional 24 hours; (2) periodate oxidation—3 μl 80 mmol/L periodic acid in 200 mmol/L acetate buffer, pH 4.5, was incubated with *Fel d* I overnight in the dark and neutralized with 1.3 μl of 10% glycine[35]; (3) β-elimination—*Fel d* I was incubated with 3 μl 200 mmol/L KOH overnight at room temperature; and (4) trifluoromethanesulfonic acid (TFMS) treatment—200 μg lyophilized *Fel d* I was incubated on ice with 200 μl dry anisole and 300 μl TFMS (Aldrich Chemical Company, Milwaukee, Wis.) for 4 hours in capped tubes under nitrogen.[36] The reaction was stopped with 800 μl of 60% ice-cold pyridine and 50 μl 1 mol/L NaOH, dialyzed against two changes of 2 L of 10 mmol/L NH_4HCO_3, lyophilized, and resuspended in 15 μl PBS. The deglycosylated samples and PBS-treated controls were stored at −20° C.

The immune reactivity of the samples was assessed by two-site MoAb immunoassay or by inhibition radioimmunoassay with human IgG or IgE antibodies. Serial twofold dilutions of samples (10^{-3} to 4×10^{-6}) were compared for binding in a two-site MoAb assay for *Fel d* I, with MoAb 6F9 on the solid phase and biotinylated MoAb 3E4 to detect bound allergen.[24] For the inhibition radioimmunoassay, dilutions of the deglycosylated *Fel d* I samples were used to inhibit the binding of ^{125}I-*Fel d* I to either human IgG antibodies (1:20 dilution of cat allergic serum pool) or IgE antibodies (1:4 dilution of cat allergic serum pool). Sample dilutions (0.1 ml) were incubated with 0.1 ml serum

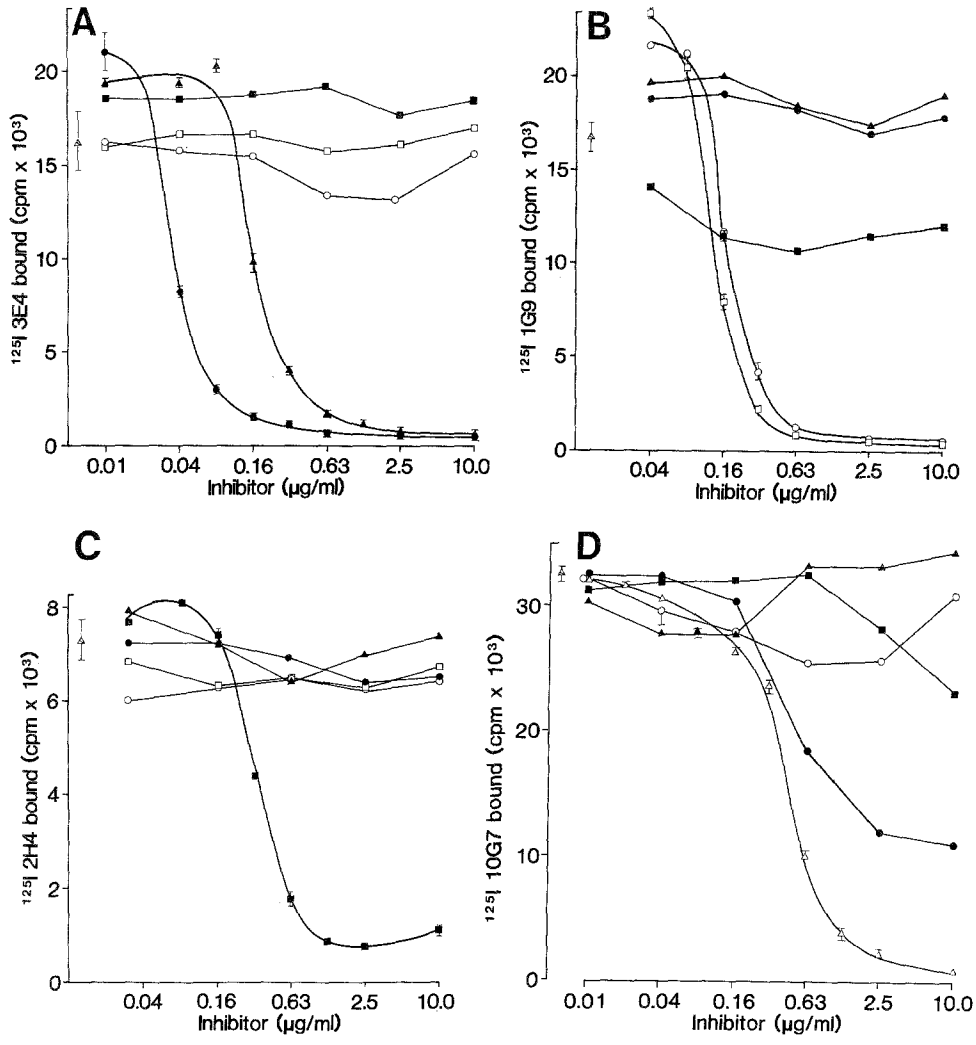

FIG. 1. Epitope mapping of *Fel d* I with MoAbs. **Panels A** to **D** show selected cross-inhibition radioimmunoassay curves with MoAbs that define epitopes Fd1A to D, respectively. ^{125}I-labeled MoAbs were inhibited with cold MoAb 3E4 (▲), Fd1A (●), 1G9 (□), 6F9 (○), 2H4 (■), or 10G7 (△). The mean ± SD of eight control wells incubated with 1% bovine serum albumin PBS-T is indicated (◬). The epitope specificity of all the MoAbs tested, including clones not shown on these curves, was as follows: Fd1A - Fd1A, 3E4, 5E3; Fd1B - Fd1B, 1G9, 6F9, 8F4; Fd1C - 2H4, 8B4, 8E4, 7D11, 3F11, 10E6, 1E8; Fd1D - 10G7.

dilution for 2 hours, followed by 2 ng ^{125}I-*Fel d* I (2 hours), and immune complexes were precipitated overnight with monospecific sheep anti-human IgG (The Binding Site, La Jolla, Calif.) or goat anti-IgE, as described previously.[37] IgE myeloma protein (P.S.), kindly provided by Dr. K. Ishizaka, was added to the IgE assays (0.1 ml, 1:200 dilution) as carrier protein. The precipitates were washed three times in borate-buffered saline, pH 8.0, and counted in a gamma counter.

^{125}I labeling

Native reduced and alkylated and deglycosylated (TFMS-treated) *Fel d* I were labeled with ^{125}I by the chloramine T technique.[37] Ten micrograms of each preparation were labeled with 0.5 mCi ^{125}I (IMS 30, Amersham International, Arlington Heights, Ill.), and specific activities of 30 to 50 μCi/μg protein were obtained. Purified anti-*Fel d* I MoAbs (15 μg) were also labeled with ^{125}I by the chloramine T technique.

Reduction and alkylation

Fel d I (50 to 100 μg) was reduced and alkylated with 0.5% 2-mercaptoethanol and 4% 4-vinyl pyridine in 250 mmol/L Tris-HCl/1 mmol/L ethylenediaminetetraacetic acid, pH 8.5, as described previously.[37]

TABLE I. Binding of anti *Fel d* I MoAb to antigen derived from different cat species

Species (common name)	Percent ^{125}I anti-IgG bound to MoAbs				
	3E4 Fd1A	6F9 Fd1B	1G9 FdIB	2H4 Fd1C	10G7 Fd1D
Felis domesticus (Domestic cat)	23	34	27	13	35
Panthera onca (Jaguar)	<0.5	2	4	0.6	1.3
Panthera tigris longipilis (Siberian tiger, n = 2)	<0.5	25,17	29,21	0.5	1.2
*Panthera leo** (Lion, n = 5)	<0.5	12 ± 4	18 ± 5	0.5	1.3
Panthera pardus kotiya (Ceylonese panther)	<0.5	24	31	<0.5	ND
Panthera uncia (Snow leopard)	<0.5	12	27	<0.5	ND
Leptailurus serval (Serval)	<0.5	7	<0.5	<0.5	0.9
Leopardus pardalus (Ocelot)	<0.5	7	29	<0.5	1.1

Hair or dander extracts from different species were coupled to CNBr-activated Sepharose and incubated with dilutions of MoAbs, followed by ^{125}I-labeled anti-mouse IgG. Results show the percent of ^{125}I anti-IgG bound with MoAbs directed against different epitopes on *Fel d* I (Fd1A-D).

ND, Not done.

*Mean ± SD of results obtained with five dander extracts from different cages containing eight animals in all.

Immunoprecipitation and sodium dodecylsulfate–polyacrylamide gel electrophoresis

^{125}I-labeled proteins (2 ng, in 0.1 ml 1% BSA PBS-T) were incubated with 1 μg anti-*Fel d* I MoAb or dilutions of polyclonal mouse or human anti-*Fel d* I antibodies for 4 hours and immunoprecipitated overnight with goat anti-mouse IgG (Chemicon, El Segundo, Calif.), sheep anti-human IgG, or goat anti-IgE. The precipitates were washed, counted in a gamma counter, and boiled in sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (6% SDS, 6% glycerol, 0.03% bromophenol blue, in 220 mmol/L Tris HCl, pH 7.0). Samples (~30,000 cpm) were applied to a 20% acrylamide gel with a 5% stacking gel and electrophoresed at 60 mA for 4 hours. Gels were stained with Coomassie Brilliant Blue R-250, (Imperial Chemical Industries, Macclesfield, Cheshire, U.K.), dried on Whatman 3MM filter paper (Whatman, Inc., Clifton, N.J.), and autoradiographed with Kodak XR 250 film (Eastman Kodak Co., Rochester, N.Y.) at −70° C. Proteins were also analyzed by SDS-PAGE on silver-stained, high-density gels with the Pharmacia Phastsystem (Pharmacia, Piscataway, N.J.), with low molecular weight (MW) markers (2.5 to 20 kd; Diversified Biotech, Newton Centre, Mass.).

Circular dichroism spectroscopy

The circular dichroism (CD) spectra of native reduced and alkylated and deglycosylated *Fel d* I were compared with the use of a JASCO J600C spectropolarimeter *(JASCO, Easton, Md.),* as described previously, and estimates of secondary structure were made according to the method of Chen et al.[37, 38]

RESULTS

Topographic mapping of *Fel d* I and its homologues with MoAbs

Previously, nonoverlapping epitopes had been identified on *Fel d* I with two MoAbs, Fd1A and Fd1B.[10, 25] In the present study the repertoire of B-cell epitopes on *Fel d* I was investigated with 13 additional MoAbs. Using cold MoAb to inhibit binding of ^{125}I-*Fel d* I to solid-phase MoAbs, we found that five MoAbs had the same specificity as either Fd1A (clones 3E4 and 5E3) or Fd1B (clones 1G9, 6F9, and 8F3). Two other MoAbs (clones 2H4 and 10G7) did not inhibit binding and appeared to be directed against different sites (data not shown). On the basis of these studies, four MoAbs (3E4, 1G9, 2H4, and 10G7) were labeled with ^{125}I and used in cross-inhibition radioimmunoassays to compare the epitope specificity of the entire panel of anti-*Fel d* I MoAbs. Cold MoAbs were used to inhibit binding of each ^{125}I-labeled MoAb to *Fel d* I, which was coupled to the solid phase with a capture MoAb (2H4). The cross-inhibition radioimmunoassay results showed that the ^{125}I-labeled MoAbs defined four essentially nonoverlapping epitopes, designated Fd1A to D (Fig. 1). These epitopes, Fd1A, B, C, and D were defined by MoAbs that produced more than 80% cross-inhibition of the binding of ^{125}I-labeled 3E4, 1G9, 2H4, and 10G7 epitopes to *Fel d* I, respectively (Fig. 1). The level of "nonspecific" inhibition by MoAbs directed against different epitopes was generally less than 25%. Partial

TABLE II. Chemical modification of *Fel d* I: Effect on antibody binding

Antibody†	Epitope	^{125}I allergen bound (cpm)*		
		Native	Reduced & alkylated	Deglycosylated
MoAb				
Fd1A	A	32,532	1,125	5,790
3E4	A	35,144	4,747	17,703
6F9	B	32,706	728	1,880
1G9	B	33,846	1,051	2,287
8F3	B	19,457	746	1,688
2H4	C	20,808	672	2,131
10G7	D	19,208	752	1,450
Anti-mite MoAb (n = 3)		1,797	718	1,313
Polyclonal antibody				
Mouse IgG anti-*Fel d* I		42,890	11,104	15,735
Human IgG antibody‡		39,343 (931)	988 (520)	4,503 (659)
IgE antibody		14,425 (350)	298 (239)	2,201 (569)

Binding of MoAb or polyclonal antibody to ^{125}I-*Fel d* I or the reduced and alkylated or deglycosylated allergen was compared by means of antigen binding radioimmunoassay.

*Two nanograms of ^{125}I allergen (~120,000 cpm) was used in each assay. Values are expressed in counts per minute precipitated by monospecific anti-mouse IgG, anti-human IgG, or anti-human IgE.

†MoAb used at 10 μg/ml; polyclonal mouse anti-*Fel d* I at 1:100 dilution; human IgE antibody at 1:12 dilution; and human IgE at 1/2 dilution.

‡Serum pool from six patients with cat allergy. Control values with serum from a donor with negative skin test results are shown in parentheses.

overlap was observed with MoAb Fd1A, which inhibited binding of MoAb 10G7 (epitope Fd1D) by 65% (Fig. 1). These results suggest that the observed topographic differences are not absolute, but they nonetheless provide a useful framework for distinguishing among MoAbs directed against different determinants on *Fel d* I.

Comparison of the binding of anti-*Fel d* I MoAbs to hair and dander extracts from seven other cat species provided further evidence that distinguished epitope Fd1B from other epitopes. The MoAbs directed against epitope Fd1B (1G9 and 6F9) showed significant binding to most of the *Panthera* spp. extracts, and to *L. serval,* and *L. pardalus* extracts, when tested by solid-phase radioimmunoassay (up to 30% ^{125}I-anti-mouse IgG bound) (Table I). Similar data were also obtained with MoAb 8F3 (not shown). In contrast, MoAbs directed against epitopes Fd1A, C, and D, which bound strongly to *Fel d* I, showed undetectable or weak (< 1%) binding to other cat species (Tables I and II). These results suggest that epitope Fd1B is a cross-reactive determinant that is expressed by domestic cats and by other cat lineages.

Experiments were carried out to determine whether the anti-*Fel d* I MoAb could inhibit human IgE antibody binding to *Fel d* I in solid-phase radioimmunoassays. When used singly, at concentrations of 50 μg/ml, MoAbs directed against epitopes Fd1A-D produced less than 30% inhibition of *Fel d* I as determined by RAST. When combinations of MoAbs directed against Fd1A or B (clones Fd1A, 3E4, and 6F9) or against Fd1A or D (clones 3E4 and 10G7) were used, up to 80% inhibition was obtained. Mean values for 20 sera from patients with cat allergy, inhibited by anti-Fd1A and anti-Fd1B MoAbs, were 60% ± 14%, as compared with 8% ± 10% with control MoAbs directed against mite group I allergens.

Effects of reduction and alkylation and deglycosylation on antibody binding and on *Fel d* I structure

Selected MoAbs, polyclonal mouse IgG, and human IgG and IgE anti-*Fel d* I antibodies were compared for binding to native ^{125}-*Fel d* I or to ^{125}I-labeled reduced and alkylated or deglycosylated allergen by antigen binding radioimmunoassay. For most MoAbs and for human IgG and IgE antibodies, binding to either reduced and alkylated or deglycosylated (TFMS-treated) *Fel d* I was less than 10% of the binding to native allergen (Table II). Polyclonal mouse anti-*Fel d* I and MoAb 3E4 retained binding activity for the treated allergens, but the levels of binding were lower than those for native *Fel d* I. Analysis of the

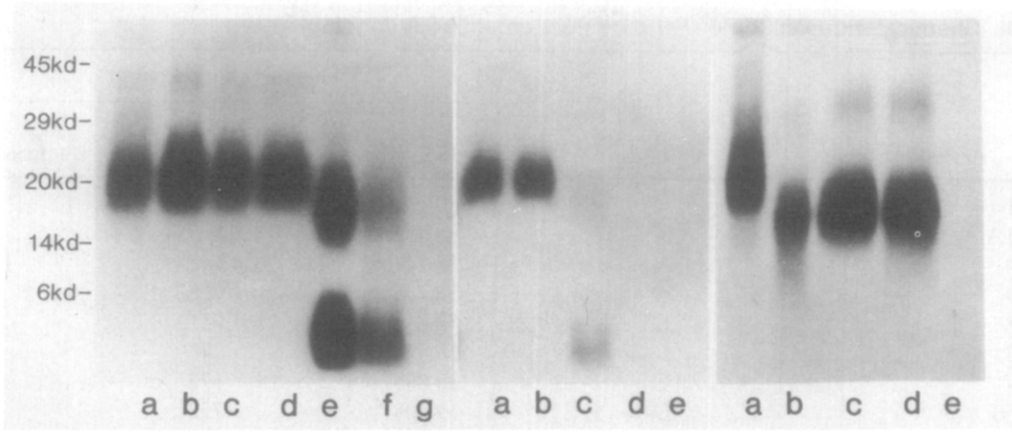

FIG. 2. SDS-PAGE analysis of *Fel d* I, *Fel d* I peptides, or immunoprecipitates. **Left panel:** *(a)* ^{125}I-labeled native *Fel d* I; *(b-d)* ^{125}I-*Fel d* I immunoprecipitated with polyclonal mouse IgG antibody *(b),* MoAb 3E4 *(c),* or MoAb 1G9 *(d); (e)* ^{125}I-labeled reduced and alkylated *Fel d* I; *(f)* ^{125}I-labeled native *Fel d* I immunoprecipitated with MoAb 1G9 and analyzed under reducing conditions (2-mercaptoethanol); *(g)* control anti *Der f* I MoAb, 4C1.[34] **Center panel:** ^{125}I-*Fel d* I immunoprecipitated with human IgG antibody *(a)* or IgE antibody *(b); (c)* immunoprecipitate of human IgG antibody analyzed under reducing conditions; *(d, e)* control immunoprecipitates for IgG and IgE antibodies, respectively, with serum from a nonatopic donor. **Right panel:** *(a)* ^{125}I-*Fel d* I; *(b)* ^{125}I-labeled TFMS-treated *Fel d* I; *(c, d, e)* TFMS-treated *Fel d* I immunoprecipitated with MoAb 3E4 *(c),* polyclonal mouse IgG antibody *(d)* or normal mouse serum *(e).*

TABLE III. Carbohydrate composition of *Fel d* I

	Experiment (i)		Experiment (ii)	
Monosaccharide	**Mole/mole of protein**	**% of total carbohydrate**	**Mole/mole of protein**	**% of total carbohydrate**
Fucose	9.3	12.4	12.7	12.0
Galactosamine	1.3	2.1	<1.0	<1.0
Glucosamine	24.0	43.4	23.4	42.9
Galactose	19.5	28.7	17.7	26.5
Mannose	10.4	15.3	8.9	13.3

Quantitative monosaccharide analysis of two preparations of *Fel d* I (i and ii, containing 21.0 μg and 19.3 μg *Fel d* I, respectively, as determined by amino acid analysis). Results are expressed as moles monosaccharide per mole protein and as percent of carbohydrate, assuming an MW of 36,000 for *Fel d* I.

^{125}I-labeled allergens by autoradiography or immuneprecipitation showed an ~17 kd band for native *Fel d* I, two polypeptide chains of ~3 kd and 14 kd for reduced and alkylated *Fel d* I, and a single polypeptide chain of 12 to 14 kd for the deglycosylated allergen (Fig. 2).

The results of deglycosylation with TFMS were in keeping with previous studies, suggesting that *Fel d* I was a glycoprotein.[27, 29] To further investigate the structural and antigenic effects of glycosylation, the monosaccharide composition of *Fel d* I was determined, and the effect of MoAbs and human IgG and IgE antibody binding to deglycosylated *Fel d* I was analyzed. Several deglycosylation procedures were compared (β-elimination, periodate oxidation, endoglycosidase digestion, and TFMS), and the products were analyzed by SDS-PAGE and compared with native allergen for antibody binding by immunoassay. Quantitative monosaccharide analysis by high-performance liquid chromatography showed that *Fel d* I was a fucose-rich glycoprotein with a monosaccharide composition of 12% fucose, 43% glucosamine, 27% galactose, and 14% mannose (Table III). Sialic acid was not detected. SDS-PAGE analysis with silver stain showed different polypeptide profiles, depending on the method used for deglycosylation (Fig. 3). Removal of N-linked sugars by digestion with endoglycosidase F/N-glycosidase F generated a major band at 9 kd, presumed to be

Fel d I chain 2, which contains a concensus site for N-glycosylation (Fig. 3, *F*).[29] Treatment with TFMS resulted in a single broad band of 12 to 14 kd, consistent with the results obtained on immunoprecipitation (Fig. 3, *D*). Unlike reduced and alkylated *Fel d* I, the single 12 to 14 kd polypeptide generated by TFMS treatment stained strongly with silver and was assumed from its MW to contain elements of chains 1 and 2. Periodate oxidation (Fig. 3, *E*) appeared to have little effect on the 17 kd *Fel d* I band but generated a smear of higher MW components, whereas β-elimination with 30 mmol/L KOH resulted in major polypeptide bands at 14.5 kd, 22 kd, and ~30 kd (Fig. 3, *C*). Fig. 3 also shows that under reducing conditions, the major *Fel d* I polypeptide chain is a dense 3.2 kd band, whereas only diffuse staining is seen at higher MW ranges (8 to 17 kd) (Fig. 3, *A* and *B*). This contrasts with the intense signal of the ^{125}I-labeled 14 kd polypeptide on autoradiography (Fig. 2).

The immunoreactivity of deglycosylated *Fel d* I was compared with native allergen by two-site MoAb ELISA and by inhibition radioimmunoassay, with the different deglycosylated allergens to inhibit the binding of ^{125}I-*Fel d* I to human IgG or IgE antibody. Treatment of *Fel d* I with TFMS or KOH resulted in almost complete loss of either MoAb or human antibody binding (>100-fold reduction) (Fig. 4, *A-C*). In contrast, *Fel d* I showed only an ~twofold reduction in antibody binding after endoglycosidase F/N-glycosidase F treatment and ~sixfold reduction in binding after periodate oxidation.

Secondary structural analyses by CD spectroscopy provided estimates of 30% alpha helix, 20% β-sheet, 25% β-turn, and 25% random coil for native *Fel d* I. On either reduction and alkylation or deglycosylation with TFMS, *Fel d* I showed significant changes in secondary structure, losing up to 60% of the alpha helix (Fig. 5). These structural changes could in part explain why the treated allergens bind poorly to mouse and human antibodies.

DISCUSSION

In the present study we analyzed the repertoire of B-cell sites on *Fel d* I with MoAbs raised against native allergen and investigated the antibody binding activity of *Fel d* I peptides generated by reduction and alkylation or deglycosylation. The topographic mapping studies show that MoAbs define at least four nonoverlapping epitopes on *Fel d* I, and of these, epitope Fd1C appeared to

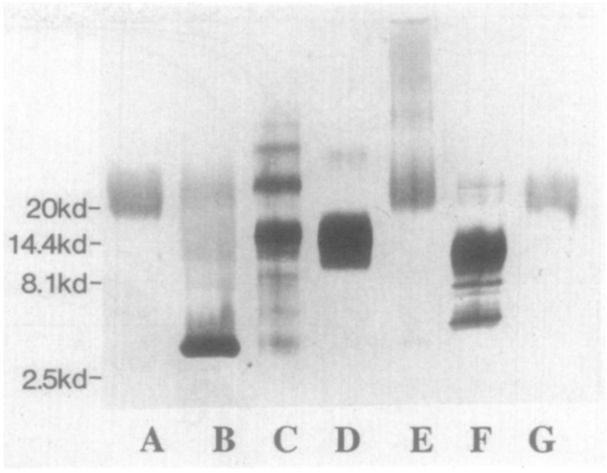

FIG. 3. Silver-stained SDS-PAGE gel of native reduced and alkylated or deglycosylated *Fel d* I. Lanes show native *Fel d* I *(A);* or *Fel d* I that was reduced and alkylated *(B);* or treated with KOH *(C),* TFMS *(D),* periodic acid *(E),* or endoglycosidase F/N-glycanase *(F). Lane G* was a PBS control for the endo F/N-glycanase reaction.

be "immunodominant" in that it was recognized by 7 of 15 MoAbs. Distinctions between these epitopes were further demonstrated by fine specificity analyses, that is, comparisons of MoAb binding to *Fel d* I homologues from other cat species. The results showed that of the four MoAbs defined sites present on *Fel d* I from domestic cats; only one (epitope Fd1B) was present on *Fel d* I homologues from different species. Cross-reactivity between this family of *Fel d* I proteins has been demonstrated with polyclonal human IgE and IgG_4 antibodies, so that it is unlikely that there is a single common epitope on these molecules. Our results indicate that the murine anti-*Fel d* I antibody response could be predominantly directed against species-specific sites (as with mite group I allergens[34]) and also suggest sequence polymorphisms, and possibly other structural differences, among the various cat proteins, which could explain the selectivity of MoAb binding. Although combinations of MoAbs directed against epitopes Fd1A, B, or D inhibited IgE antibody binding to *Fel d* I, it is difficult to determine whether this reflects MoAb binding to epitopes recognized by IgE or stearic hindrance effects of multiple MoAbs binding to *Fel d* I. Our results for both mite group I allergens and *Fel d* I suggest that mapping studies with MoAbs define different antigenic sites and are essential for designing immunoassays, whereas MoAbs are of limited value in defining epitopes recognized by IgE antibodies.[34]

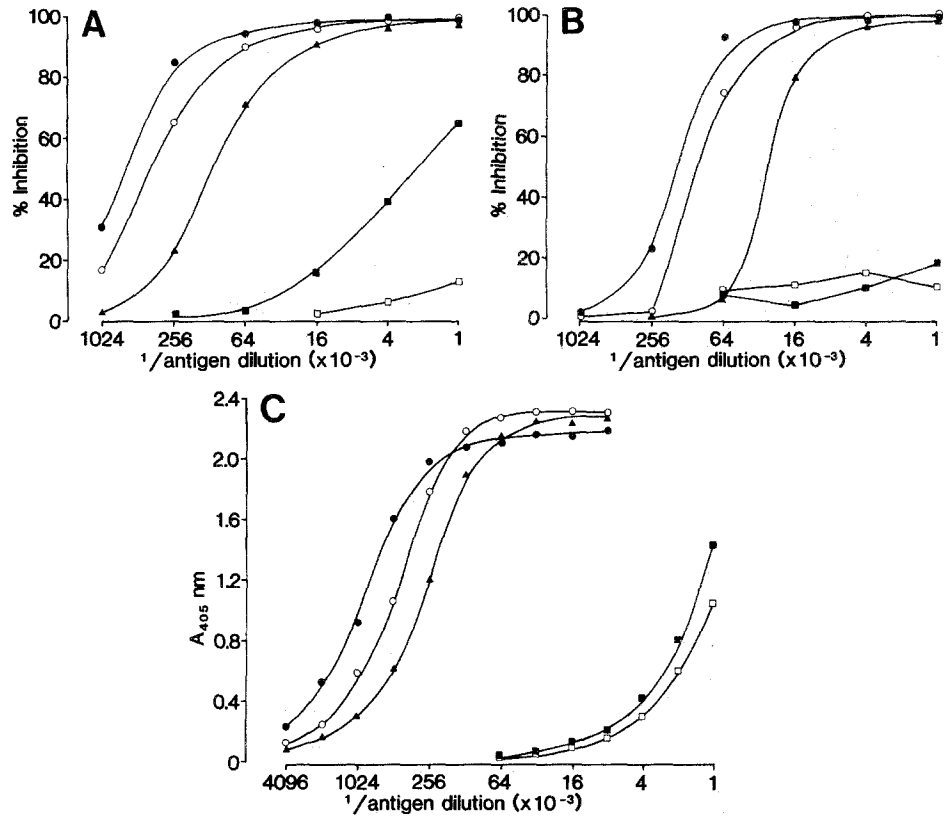

FIG. 4. The effects of different methods of deglycosylation of *Fel d* I on antibody binding. Binding of human IgG **(panel A)** or IgE **(panel B)** antibodies to ^{125}I-*Fel d* I was inhibited with serial dilutions of native allergen (●) or serial dilutions of allergen that had been treated with periodic acid (▲), TFMS (□), KOH (■), or endo F/N-glycanase (○). Approximately 110,000 cpm ^{125}I-*Fel d* I was added to each assay, and uninhibited binding of human IgG or IgE antibody to the radiolabel was 81,300 ± 754 cpm and 17,784 ± 502 cpm, respectively. The allergen preparations were also compared for binding in a two-site MoAb ELISA for *Fel d* I with MoAb 6F9 on the solid phase and biotinylated MoAb 3E4 for detection **(panel C)**. The ELISA curves show absorbance readings at 405 nm with different dilutions of allergen.

The reduction and alkylation and deglycosylation experiments were carried out to further investigate the nature of the epitopes recognized by MoAbs or human antibodies. It is clear from the results that *Fel d* I is a complex molecule, which can be dissociated by these treatments into several low MW (3 to 14 kd) polypeptides. The generation of 14 kd and 3 kd peptides after reduction and alkylation would not be predicted from the molecular cloning studies, showing two chains of 70 and 92 amino acids (MW 7863 and 10121, respectively), which are thought to be linked by disulfide bonds.[29] Our results are consistent with those of others[27, 29] and suggest that chains 1 and 2 form a 14 kd core, which is not fully dissociated by reducing conditions, and that the 3 kd fragment may be derived by cleavage of "accessible" cysteine residues. Evidence from amino acid sequencing suggests that the 3 kd peptide could be the N-terminal end of chain 1 (residues Glu^{1} to Cys^{44}).[25, 27, 29] The mechanism whereby the 14 kd peptide is "protected" from reducing agents has not been established but may be related to the presence of N-linked oligosaccharides. In support of this view we have found that deglycosylated (TFMS-treated) *Fel d* I is cleaved into two bands of ~3 kd and ~6 kd under reducing conditions (unpublished observations).

The carbohydrate analyses showed that *Fel d* I is a fucose-rich glycoprotein with little or no galactosamine or sialic acid. The lack of galactosamine suggests that the bulk of the carbohydrate is N-linked through the Asn^{33}-Ala-Thr^{35} glycosylation site on chain 2.[29] Chain 1 shows homology to uteroglobulin, which does not contain carbohydrate.[39, 40] The presence of such a large amount of fucose in complex oligosaccharides is unusual, and although its biologic significance is unclear, it is interesting to note that other fucosylated proteins (e.g., blood group substances) are also found in saliva, which is an important source of *Fel d* I.[2-5, 25] In contrast to the

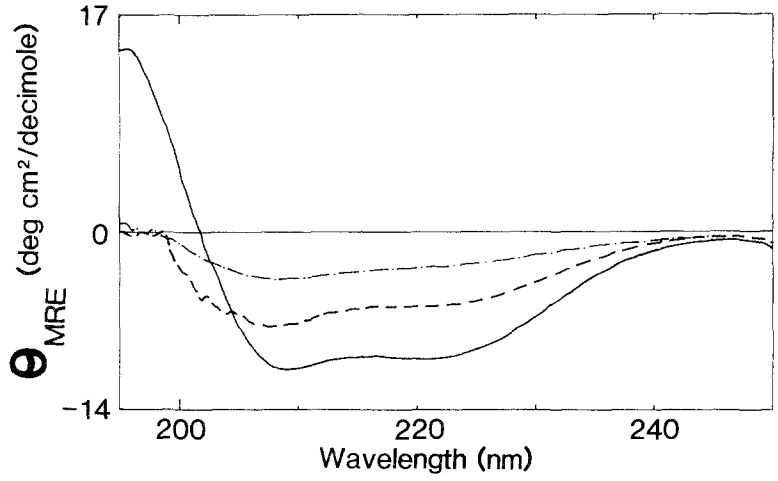

FIG. 5. CD spectra of native (—), reduced and alkylated (- -), or deglycosylated *Fel d* I (—·—·—).

reduction and alkylation experiments, enzymatic deglycosylation generated a 9 kd polypeptide (presumably chain 2), which retained antibody binding activity. This suggests that carbohydrate is not an important part of the epitope(s). Treatment with TFMS or KOH removed almost all the antigenic reactivity of *Fel d* I for either MoAbs or human antibodies. Similar observations on the effect of KOH have recently been made by Bond et al.[41] TFMS is recommended for deglycosylation studies because it is reported to have minimal effect on the structural integrity of the polypeptide chains in glycoproteins.[42] However, TFMS treatment of *Fel d* I caused significant loss of secondary structure, as assessed by CD spectroscopy, and it has also been suggested that KOH causes deamidation of asparagine residues in *Fel d* I.[41] Thus both TFMS and KOH can cause denaturation, and this appears to be the likely explanation of their effect on antibody binding.

Previous studies with skin testing and RAST inhibition showed weaker binding of IgE antibodies to reduced and alkylated *Fel d* I, as compared with native allergen.[2, 27] In the present study only one MoAb (3E4, epitope Fd1A) showed significant binding to reduced and alkylated *Fel d* I, and polyclonal mouse or human antibodies also showed weak binding to the denatured allergen. This was the only example of chemical treatment having a selective effect on antibody binding. Usually, procedures that reduced antibody binding, uniformly affected MoAbs or human IgG or IgE antibodies. The reduction and alkylation results suggest that B-cell epitopes on *Fel d* I are conformational. Murine MoAbs have been raised against denatured *Fel d* I, and they recognize either the 14 kd or the 3 to 4 kd band on immunoblots but do not bind to native allergen.[43] The determinants recognized by these MoAbs have recently been analyzed with overlapping synthetic peptides. As part of those studies, MoAbs directed against epitope Fd1B of the native allergen were found to bind four peptides derived from either chain 1 (1-16 and 60-70) or chain 2 (1-14 and 43-56), providing preliminary evidence that residues in these peptides could be brought together to form one of the conformational sites.[44]

In conclusion, the results demonstrate a diverse repertoire of B-cell epitopes on *Fel d* I and an unusual degree of structural complexity in this glycoprotein allergen. The availability of MoAbs to nonoverlapping epitopes will be extremely useful for identifying the amino acid residues involved at each of the four antigenic sites. Recombinant *Fel d* I chains have been produced and binding of MoAbs and human IgE to each chain is currently being investigated.[45, 46] The evidence that *Fel d* I is conserved among several cat species and is produced in copious amounts by domestic cats suggests that this molecule has an important biologic function. The homology to uteroglobulin and the presence of fucose-rich complex oligosaccharide on chain 2 provide structural clues that may be useful in determining the function of *Fel d* I. Further understanding of the immunobiology of *Fel d* I and its homologues should enable the development of new immunotherapeutic strategies or of biologic methods for controlling the production of this allergen and thereby reduce the prevalence and morbidity of IgE-mediated hypersensitivity to cats.

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