IgE antibodies to recombinant forms of *Fel d* I: Dichotomy between fluid-phase and solid-phase binding studies

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Background: *The major cat allergen* Fel d *I consists of two polypeptide chains linked by disulfide bonds, each of which has been expressed in bacteria. To investigate the antigenic structure of* Fel d *I, antibody binding to the native molecule and to each recombinant chain were compared.*

Methods: *Polyclonal human IgE and IgG antibodies and monoclonal antibodies (mAbs) to* Fel d *I were compared for binding to* Fel d *I, chain 1, or chain 2 by fluid-phase inhibition radioimmunoassay, RAST, and immunoabsorption.*

Results: *In the fluid-phase assay, neither recombinant chain significantly inhibited the binding of antibody to native* Fel d *I at concentrations of up to 10 μg/ml. Partial inhibition was observed when chain 1 was used, which inhibited the binding of two mAbs by 40% and 75%. In contrast, when the solid-phase RAST assay was used, IgE antibodies bound both chains with high specificity, and there was a good quantitative correlation between IgE antibody binding to* Fel d *I and both chain 1 ($r = 0.58$, $p < 0.01$) and chain 2 ($r = 0.47$, $p < 0.01$). Up to 70% of IgG or IgE anti-*Fel d *I antibodies could be absorbed by either chain 1 or chain 2, and both chains in combination produced similar absorption values in response to native* Fel d *I. Four mAbs were fully absorbed by chain 1, but not chain 2, and three mAbs were not absorbed by either chain.*

Conclusions: *The results demonstrate a dichotomy between antibody binding to recombinant* Fel d *I chains, which may be explained by confirmational differences between the chains in the fluid phase or on solid supports. The results also suggest that chain 1 is an important site for mAb-defined B-cell epitopes on* Fel d *I. (J ALLERGY CLIN IMMUNOL 1995;95:1221-8.)*

Key words: *Recombinant allergen, cat allergen, IgE antibodies, monoclonal antibodies*

Immediate hypersensitivity to the domestic cat has been extensively studied with *Fel d* I, a major glycoprotein allergen that induces IgE (and IgG) antibody responses in more than 80% of patients allergic to cats.[1-6] *Fel d* I is produced in cat salivary and lacrimal glands and in skin sebaceous glands, and it is secreted in copius amounts.[7-10] The allergen accumulates in house dust at levels as high as 1.5 mg/gm dust and becomes airborne on particles of 2 to 10 μm in diameter, which can cause acute attacks of asthma.[11-15]

Fel d I has a complex structure when compared with other inhaled allergens, most of which are single polypeptide chains.[16] It is a 36 kd heterodimer, consisting of two chains linked by disulfide bonds; chain 1 consists of 70 amino acids, and chain 2 exists as a 90- or 92-amino acid isomer.[17-19]

Abbreviations used

HSA:	Human serum albumin
mAb:	Monoclonal antibody
rCh1:	Recombinant chain 1 of *Fel d* I
rCh2:	Recombinant chain 2 of *Fel d* I
RIA:	Radioimmunoassay

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The chains are encoded by separate genes, each of which has been sequenced.[18, 19] In addition, 20% of the molecular weight of *Fel d* I is fucose-rich carbohydrate linked to a single asparagine residue on chain 2.[17, 18, 20] Deglycosylation under nondenaturing conditions has little effect on antibody binding to *Fel d* I, whereas reduction and alkylation eliminate both monoclonal and IgE antibody binding.[17, 20, 21] These studies show that the main antigenic determinants are located on the protein chains and suggest that the epitopes are conformational. Monoclonal antibodies (mAbs) define four nonoverlapping B-cell epitopes on *Fel d* I, one of which is conserved among other species of the cat family *Felidae*.[5, 6, 20, 22-24]

In this study we have compared binding of *Fel d* I and the recombinant *Fel d* I chains to murine mAbs and human IgE antibodies by fluid-phase and solid-phase immunoassay and by immunoabsorption. Polyclonal IgE antibodies bind to each recombinant chain, and the epitopes recognized by four of the mAbs were located on chain 1. However, significant differences were obtained by using the chains to bind in the fluid phase or the solid phase, suggesting that the conformation of the chains is an important determinant of antibody binding. The results suggest that it may be possible to express both recombinant chains in a form that retains the B-cell epitope repertoire of native *Fel d* I.

METHODS

Native and recombinant *Fel d* I

Fel d I was purified from house dust extract with mAb-based affinity chromatography as described previously.[6] Complementary DNA's encoding chain 1 and chain 2 were obtained by polymerase chain reaction from mRNA isolated from cat mandibular and parotid glands.[18] Each chain was constructed with an additional 30 amino acids at the amino terminus including the gn10 leader sequence, six histidine residues, and a thrombin cleavage site and was subcloned into the T7 polymerase–based pET-11d expression vector.[25] The recombinant chains were expressed in *Escherichia coli* and isolated by nickel-ion affinity chromatography: chain 1 (rCh1) had an apparent molecular weight of 10 kd, whereas chain 2 (rCh2) had a molecular weight of 12 kd.[25]

Iodine 125 labeling

Proteins were radiolabeled with ^{125}I by the chloramine-T technique as described previously.[20] Fifteen micrograms of protein was labeled with 0.5 mCi of ^{125}I. Reduced and alkylated *Fel d* I was labeled by the same technique.

mAbs

A panel of six IgG_1 anti-*Fel d* I mAbs was used in this study (clones 6F9, 1G9, 8F3, 3E4, 10G7, and Fd1A). The epitope specificity of these mAbs has been previously described in detail.[6, 20] The mAbs defined four nonoverlapping epitopes, designated Fd1A to D. Four mAbs directed against reduced and alkylated *Fel d* I were obtained from Dr. Robert Aalberse, University of Amsterdam (8F6 and 11F3), or from Dr. Manuel Lombardero Abello S.A., Madrid, Spain (RA-1 and RA-2). All mAbs were used as 50% saturated ammonium sulfate fractions of ascites.

Human sera

Serum was obtained from 43 patients allergic to cats who had either positive skin test responses to cat allergen extract (wheal $> 5 \times 5$ mm) or a positive RAST response.[15] A previously described serum pool comprised of sera from six donors with cat allergy was also used.[20] Serum samples were also obtained from six nonallergic control subjects who had negative RAST responses.

Inhibition radioimmunoassay

Serial two- to fourfold dilutions of native *Fel d* I, rCh1, and rCh2 (10 μg/ml to 0.03 ng/ml) were used to inhibit the binding of ^{125}I-*Fel d* I to human IgG (1:50 dilution of serum pool), human IgE (1:10 dilution of serum pool), or mAb (0.4 to 5.0 μg/ml). *Fel d* I, rCh1, or rCh2 dilutions (0.1 ml) were incubated with 0.1 ml antibody dilution for 2 hours, followed by incubation with ^{125}I-*Fel d* I for 2 hours. Immune complexes were precipitated with monospecific sheep anti-human IgG, goat anti-human IgE, or goat anti-mouse IgG. Precipitates were washed and counted in a gamma counter.[20]

RAST

Cat hair and pelt extract, rCh1, rCh2 (5 μg/disk) were coupled to CNBr-activated paper disks. Human serum was diluted 1:2 or 1:10 in horse serum to a volume of 0.1 ml and incubated with the disks overnight. Disks were washed and incubated with ^{125}I goat anti-human IgE for 6 hours. The amount of radioactivity bound was determined with a gamma counter.[26, 27]

Immunoabsorption experiments

Fel d I, rCh1, and rCh2 were bound to CNBr-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) (2 mg protein/gm Sepharose) as described previously.[26, 27] Sera (1:2 dilution) or mAbs (4 to 50 μg/ml), 0.2 ml, were incubated with 0.05 ml of a 40% suspension of beads overnight at 4°C. Supernatants were removed and assayed for IgG and IgE antibody to *Fel d* I by antigen-binding radioimmunoassay (RIA). Nine human sera, the "cat allergic" serum pool, and six mAbs were assayed after separate absorptions with *Fel d* I, rCh1, rCh2, rCh1 plus rCh2, or with human serum albumin (HSA)–Sepha-

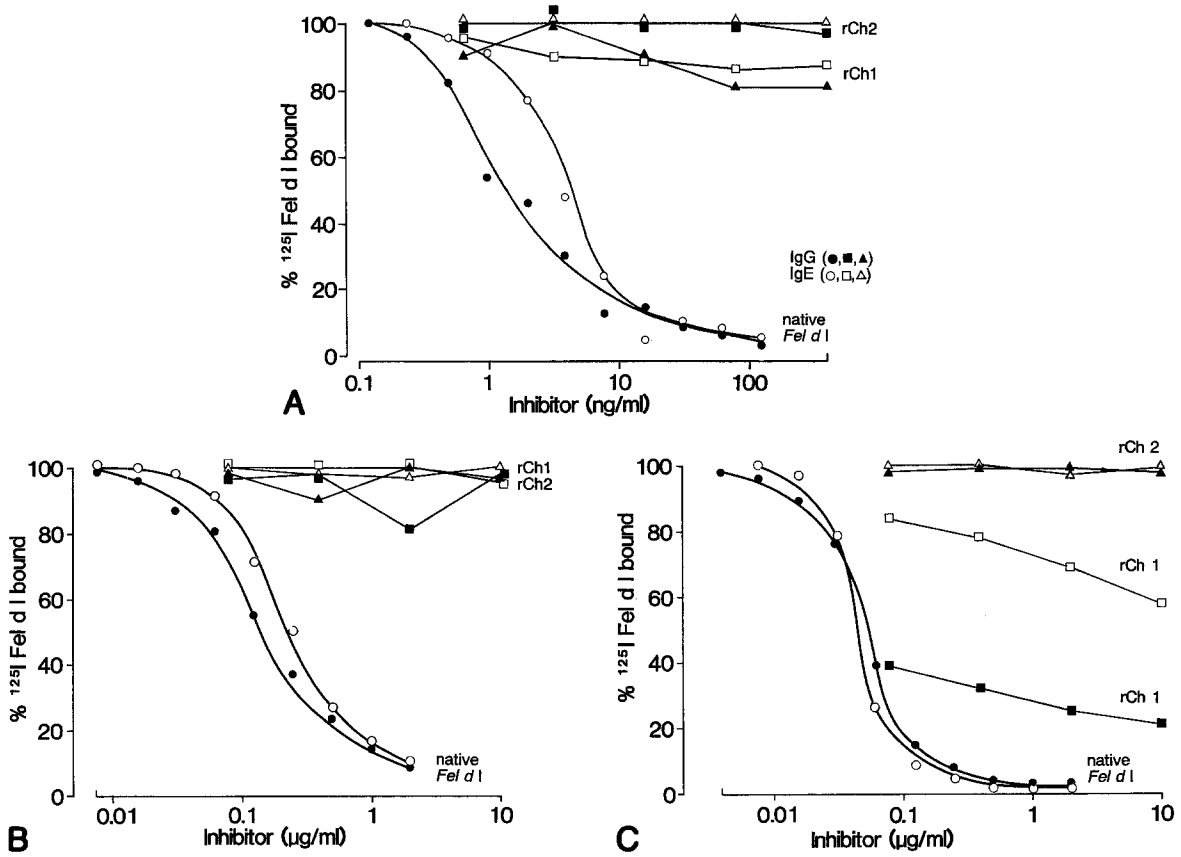

FIG. 1. Inhibition RIA. **A,** Recombinant *Fel d* I chains do not inhibit IgG (■, ●, ▲) or IgE (□, ○, △) antibody binding to native ^{125}I-*Fel d* I. **B,** Recombinant *Fel d* I chains do not inhibit binding of mAb 8F3 (□, ○, △) or 10G7 (■, ●, ▲). **C,** rCh1 partially inhibits binding of mAbs 3E4 (■, ●, ▲) and 6F9 (□, ○, △).

rose as a control. Results were expressed as percent absorption relative to HSA absorption.[26, 27]

RESULTS

Inhibition of antibody binding to native *Fel d* I by rCh1 and rCh2

The immunoreactivity of rCh1 and rCh2 was first investigated by fluid-phase inhibition RIA by using rCh1, rCh2, or *Fel d* I to inhibit antibody binding to ^{125}I-*Fel d* I. Dose-dependent inhibition curves were obtained by using native *Fel d* I to inhibit human IgG or IgE antibody binding, with greater than 95% inhibition when 100 ng/ml *Fel d* I was used (Fig. 1, *A*). In contrast, neither rCh1 nor rCh2 showed significant inhibition of IgG or IgE antibody binding at concentrations of up to 10 µg/ml. Similar results were obtained by using *Fel d* I, rCh1, and rCh2 to inhibit the binding of murine mAbs 8F3 and 10G7 to *Fel d* I (Fig. 1, *B*). However, binding of mAbs 3E4 and 6F9 to *Fel d* I was partially inhibited by rCh1 (up to 40% and 75%, respectively) (Fig. 1, *C*). The inhibition curves obtained with rCh1 were much flatter and required higher antigen doses than those with the native allergen, indicating that rCh1 did not retain the complete antigenic structure of the native allergen. Monoclonal antibody binding was not inhibited by rCh2 when it was added at the same concentrations (Fig. 1, *C*).

Binding of IgE antibodies to solid-phase *Fel d* I, rCh1, or rCh2

Although the inhibition RIA results suggested that rCh1 and rCh2 could not compete with *Fel d* I for antibody binding, this assay compares relatively high-affinity antigen-antibody interactions, and it is possible that the recombinant chains did not retain a suitable conformation in the fluid phase to effectively compete in the assay. To investigate this possibility and to compare the specificity of IgE antibody from a large number of patients allergic to cats, the reactivity of *Fel d* I,

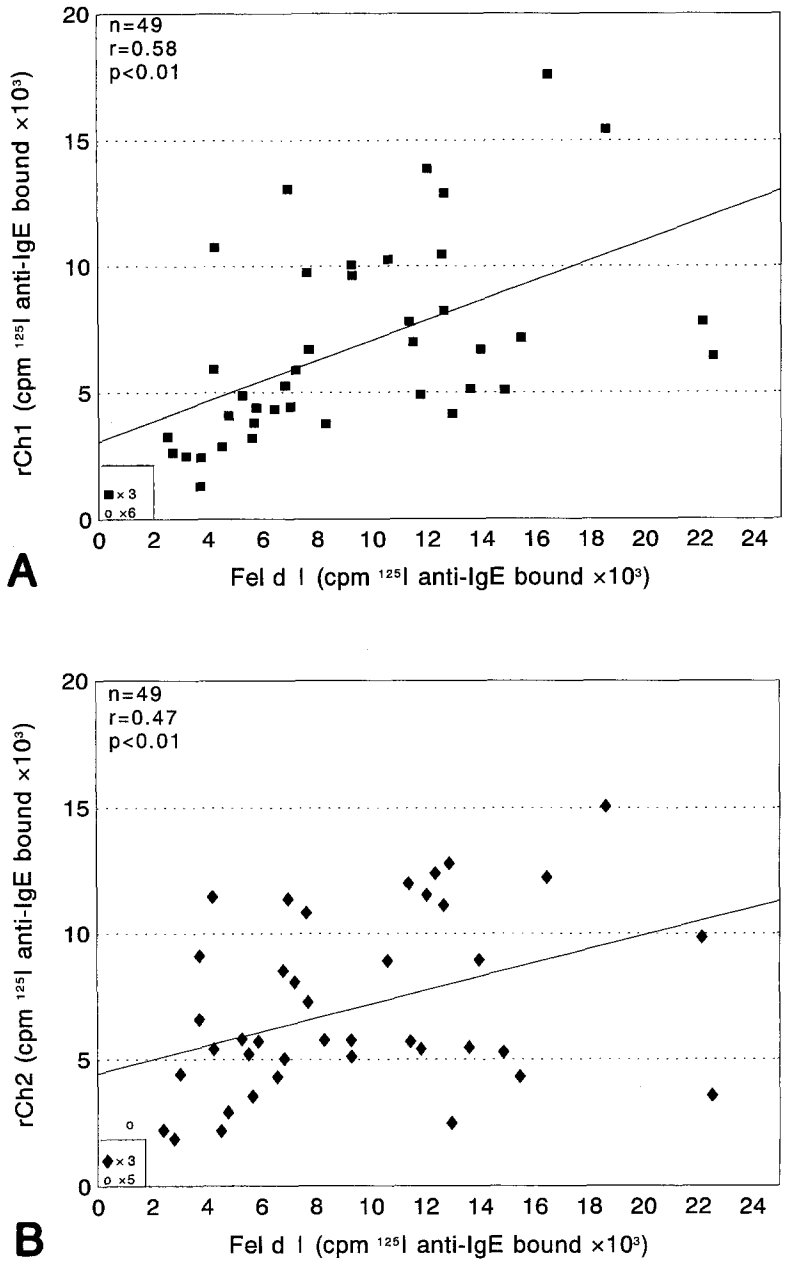

FIG. 2. Comparison of IgE antibody binding to rCh1 **(A)** or rCh2 **(B)** by RAST. Sera from patients allergic to cats (■, ◆) or nonallergic control subjects (○) are indicated.

rCh1, and rCh2 were compared by RAST. The results showed that the majority of sera contained IgE antibody to both rCh1 and rCh2 and that there was a good quantitative correlation between IgE antibody to native *Fel d* I and to the recombinant allergens (Fig. 2, *A* and *B*). Some sera showed two- to fourfold greater binding to *Fel d* I as compared with the recombinant chains. However, in other cases the quantities of anti-IgE bound by *Fel d* I, rCh1, or rCh2 were very similar.

Absorption of antibody by Sepharose-coupled *Fel d* I, rCh1, or rCh2

The RAST results suggested that recombinant *Fel d* I chains were able to be recognized by human IgE antibodies. To determine the proportion of IgG and IgE antibody directed against the recombinant chains, sera from selected patients with cat allergy were absorbed with *Fel d* I, rCh1, rCh2, or a combination of rCh1 and rCh2. Antibody binding to ^{125}I-*Fel d* I was compared by antigen-binding

TABLE I. Immunoabsorption of IgE antibodies by *Fel d* I or recombinant *Fel d* I chains

Serum	Antibody to *Fel d* I*		Percent absorption relative to HSA-Sepharose†							
			Fel d I		rCh1		rCh2		rCh1+2	
	IgG BA (U/ml)	IgE BA (U/ml)	IgG	IgE	IgG	IgE	IgG	IgE	IgG	IgE
Cat allergic serum pool	1920	220	89	92	5	17	<1	14	82	82
VL	5000	700	90	91	13	42	<1	12	21	54
JW	1920	620	93	97	30	44	30	30	75	77
AS	699	255	94	96	64	60	50	56	93	93
MK	463	199	96	98	38	40	38	42	92	92
KB	494	119	96	97	27	31	40	48	89	89
BB	174	16	88	88	60	51	49	38	76	57
LM	940	275	95	97	43	41	38	40	92	92
CD	4800	45	96	95	5	12	6	27	71	75
RK	139	44	81	84	68	71	49	58	82	81
Geometric mean	893	148	92	93	25	36	16	33	72	78

*As determined by antigen binding RIA with ^{125}I-*Fel d* I. Results are expressed in arbitrary units of IgG or IgE binding activity (BA) per milliliter relative to serum from patient VL.[20]

†Sera were absorbed with Sepharose-coupled allergens and assayed for IgG and IgE antibody to native *Fel d* I by RIA. Absorption values were calculated relative to HSA-Sepharose controls.

RIA before and after absorption, relative to the control immunosorbent (HSA-Sepharose). The results showed that in the majority of the sera tested (8 of 10), IgG and IgE antibodies were more than 90% absorbed by *Fel d* I as compared with absorption with HSA (Table I). Used alone, rCh1 absorbed up to 70% of the anti-*Fel d* I antibody, with geometric mean absorption values of 25% and 36% for IgG and IgE antibodies, respectively. Similar absorption values were obtained with rCh2. For each of the sera tested, the absorption values when rCh1 or rCh2 was used alone were lower than those obtained when *Fel d* I was used. However, when sera were absorbed with pooled rCh1 and rCh2 Sepharose, the degree of absorption was comparable to that obtained with *Fel d* I. Eight of the 10 sera tested showed greater than 70% absorption of IgG or IgE antibody, with geometric mean values of 72% and 78%, respectively (Table I). In two cases this level of absorption exceeded the sum of the absorption with the individual chains (serum pool and patient CD, Table I). A comparison of absorption values from four sera is shown in Fig. 3. Patient VL showed significantly lower absorption when rCh1 and rCh2 were used than that obtained when *Fel d* I was used. For individual sera, there was a good correlation between the amount of IgG or IgE antibody depleted by immunosorption (Table I). As controls for these studies, four sera were absorbed with native or recombinant *Der p* II, a major house dust mite allergen. Less than 5% of the IgG or IgE directed to *Fel d* I was absorbed by either allergen, thus confirming the specificity of the absorption experiments. Additionally, ryegrass-specific IgE in two sera from patients with grass pollen allergy showed only 11% absorption by *Fel d* I, rCh1, rCh2, or a combination of rCh1 and rCh2, as measured by RAST (data not shown).

Similar immunoabsorption experiments were carried out to determine whether murine mAbs generated against native *Fel d* I could bind the recombinant chains and to investigate the location of the B-cell epitopes. A total of seven mAbs, which recognize four distinct B-cell epitopes, were compared (Table II, *A*). The results showed that four mAbs (Fd1A, 3E4, 6F9, and 1G9) directed against either epitope Fd1A or Fd1B were significantly absorbed by rCh1 (>70%), but not by rCh2 (<5%). Three other mAbs (8F3, 2H4, and 10G7) were not absorbed by rCh1 or rCh2, either alone or in combination. None of the seven mAbs were significantly absorbed when rCh2 was used, whereas the geometric mean absorption with native *Fel d* I was 78%.

We further investigated the recombinant chains by using four mAbs directed against reduced and alkylated *Fel d* I. Two mAbs, RA-2 and 11F5, were significantly absorbed by rCh1, 73% and 78%, respectively (Table II, *B*), but none of the four mAbs were absorbed by rCh2. Native *Fel d* I, which is not recognized by any of these mAbs in antigen-

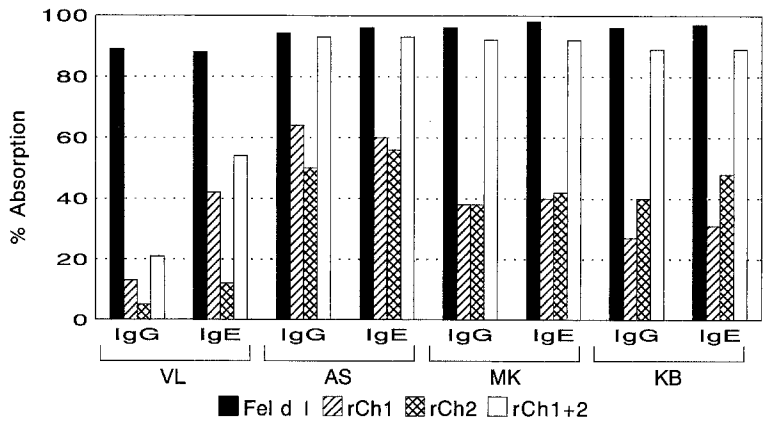

FIG. 3. Immunoabsorption of IgG and IgE antibodies by allergens coupled to Sepharose from four patients allergic to cats *(VL, AS, MK, KB).* Sera were absorbed with Sepharose-coupled allergens and assayed for IgG and IgE antibody to native *Fel d* I by RIA. Absorption values were calculated relative to HSA-Sepharose controls.

TABLE II. Immunoabsorption of anti-*Fel d* I mAb by native *Fel d* I, rCh1, or rCh2*

	Epitope†	Percent absorption by			
		Fel d I	rCh1	rCh2	rCh1+rCh2
A: mAb anti-*Fel d* I					
mAb					
Fd1A	A	77	84	<5	80
3E4	A	70	96	<5	92
6F9	B	77	96	<5	93
1G9	B	78	73	<5	84
8F3	B	95	<5	7	<5
2H4	C	67	<5	33	<5
10G7	D	87	30	<5	6
B: mAb anti-R&A *Fel d* I					
RA-1	R&A	18	<5	<5	<5
RA-2	R&A	59	73	<5	<5
11F5	R&A	56	78	<5	70
8H6	R&A	44	5	<5	<5

R&A, Reduced and alkylated.
*Monoclonal antibodies to native *Fel d* I *(A)* or R&A *Fel d* I *(B)* were absorbed with Fel d I or recombinant *Fel d* I chains. The mAbs were assayed before- and after absorption by antigen-binding RIA with ^{125}I-*Fel d* I or ^{125}I-R&A *Fel d* I.
†Nonoverlapping sites defined with mAbs.[5, 6, 20]

binding RIA, showed moderate levels of absorption (18% to 59%).

DISCUSSION

Our initial results obtained with a fluid-phase binding assay indicated that neither rCh1 nor rCh2 was able to compete with native *Fel d* I in binding to human IgG or IgE antibody, even when present in molar excess of greater than 50-fold. However, subsequent experiments with solid-phase RAST assays or immunoabsorption clearly showed that both rCh1 and rCh2 were capable of binding both human IgG and IgE antibodies. Similar results have recently been reported by Bond et al.[21] who showed that both recombinant chains could inhibit binding to native *Fel d* I in a solid-phase immunoblot inhibition assay but were unable to competitively inhibit *Fel d* I binding in an ELISA. There are two possible explanations for the apparent dichotomy between the results of the fluid-phase and solid-phase methods for assessing the immunoreactivity of recombinant *Fel d* I. The first is that the fluid-phase assay preferentially selects for high-affinity antibody binding, because it involves use of very low doses of radiolabeled allergen (~2 ng per test). This would suggest that most of the anti-*Fel*

d I antibodies tested (both human and mouse) had higher affinity for the native allergen than the recombinant chains. In support of this view, it has recently been shown that IgE antibody binding to synthetic peptides from *Fel d* I is several orders of magnitude lower than binding to native allergen.[28] An alternative explanation is that the recombinant chains adopt different structural conformations in the fluid phase than in the solid phase and that in solution the recombinant chains have a more random and less stable conformation, which is unable to effectively inhibit binding to the native allergen.

Absorption of mAbs provided strong evidence that the murine B-cell epitopes Fd1A and B are predominantly located on chain 1. Four of five of the mAbs tested, which were directed against these epitopes, were absorbed with rCh1, but not rCh2. Additional evidence comes from the fluid-phase experiments, which demonstrated that rCh1, but not rCh2, was able to partially inhibit the binding of anti Fd1A/B mAb to radiolabeled *Fel d* I, whereas mAbs directed to epitopes Fd1C and Fd1D showed no significant inhibition. Chain 1 also appears to contain at least one epitope on reduced and alkylated *Fel d* I as judged by the immunoabsorption data. In contrast, polyclonal IgG and IgE antibodies were absorbed by either rCh1 or rCh2, in approximately stochiometric amounts, suggesting that both chains contain residues that contribute to the antigenic determinants recognized by sera of patients allergic to cats.

Recent studies have emphasized the value of using recombinant allergens in the diagnosis and treatment of allergic disease. Several monomeric protein allergens retain their immunoreactivity after expression in bacterial (or yeast) vectors and are good candidates for diagnostic purposes (e.g., *Bet v* I, *Bet v* II, *Der p* II).[29-34] Thus for example, use of recombinant *Bet v* I and *Bet v* II would probably diagnose more than 95% of patients with birch pollen allergy. Other recombinant allergens (e.g., mite group I, ragweed groups 1 and 5, and *Fel d* I) have been expressed but show reduced antibody binding compared with natural allergen, because they have multiple disulfide bonds or are multimeric proteins.[21, 34-39] It may be possible to improve the reactivity of recombinant *Fel d* I by establishing optimal conditions for folding of the expressed chains. Alternatively, a dicistronic expression vector could be used to target both chains to the bacteria periplasm, where disulfide bond formation is favored.[40, 41] At present, it is only possible to isolate native *Fel d* I with conventional biochemical techniques or mAb affinity chromatography. The development of improved methods for producing optimally folded recombinant *Fel d* I chains would provide large quantities of allergen for cellular studies. Improved expression systems would also facilitate further structural analyses of *Fel d* I, including determination of the tertiary structure, and would be essential for the recombinant allergen to be used clinically for diagnostic or therapeutic purposes.

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