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NATIVE AND RECOMBINANT *Fel dI* AS PROBES INTO
THE RELATIONSHIP OF ALLERGEN STRUCTURE TO HUMAN
IgE IMMUNOREACTIVITYJULIAN F. BOND,* ANDREW W. BRAUER, DEBRA B. SEGAL, ANNELIESE K. NAULT,
BRUCE L. ROGERS and MEI-CHANG KUO

ImmuLogic Pharmaceutical Corporation, 610 Lincoln Street, Waltham, MA 02154, U.S.A.

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Abstract—To delineate the relationship between the structural conformation and the stability of an allergen and its antigenicity, we have chosen the major allergen from cat dander, *Fel dI*. From protein sequence analysis data we have examined the structure of the naturally occurring *Fel dI* and we have found it to exist as an anti-parallel heterodimer. We have used ELISA, RAST, Western blot and histamine release techniques to compare the IgE reactivity of a set of cat allergic patient samples to purified, native *Fel dI* and the *E. coli* expressed chains 1 and 2. Results from these studies demonstrate a significant level of IgE reactivity to all forms when examined for direct binding. However, both blot and ELISA competition assays show a much higher reactivity to *Fel dI* in solution compared to the separate recombinant chains and this is supported by the histamine release data. Although native *Fel dI* chain 2 contains an N-linked carbohydrate moiety, this does not seem to play a role in the reactivity of IgE to chain 2. Denaturation of *Fel dI* with alkali conditions leads to a dramatic decrease in IgE reactivity, even though measurable changes to the backbone structure of the protein are minimal. One proposed explanation is that both chains possess a core region determined by their primary structures and that the major IgE epitopes are dependent upon them. The relative reactivity amongst these allergen forms varied with the method of analysis, implying that the conformational requirements for IgE antibody binding are best studied by the application of more than one experimental protocol. Results from these qualitative analyses afford insight into the allergenicity of this exceptionally stable cat pelt protein.

INTRODUCTION

A central question in the examination of the immunological response to a protein allergen is are there inherent properties of the allergen that promote the allergic response? There are numerous examples which can be cited for or against this premise (King, 1976; Aas, 1978; Baldo and Donovan, 1988; Platts-Mills, 1982). We have used the major allergen derived from the domestic cat, *Fel dI*, (Leitermann and Ohman, 1984; De Groot *et al.*, 1988) as a model system to begin to address the question of distinct immunochemical properties of allergenic proteins. *Fel dI* is noted to be quite stable and its presence

can be detected in households long after cat removal and a thorough cleaning (Wood *et al.*, 1989). Another property that may relate to *Fel dI*'s allergenic potential is that it can remain airborne for long periods of time (Swanson *et al.*, 1989; Luczynska *et al.*, 1990). Our interest is in determining whether structural features of this protein relate to both its allergenicity and its *ex vivo* stability.

The *Fel dI* protein has been determined to be a heterodimer of two covalently linked small polypeptides that, under non-reducing conditions, shows an apparent molecular weight of 35,000 (Leitermann and Ohman, 1984). The recent cloning and sequencing of the cDNAs for the protein chains which comprise *Fel dI* (Morgenstern *et al.*, 1991) has allowed a detailed description of these structures. Chain 1, the smaller of the two, is 70 amino acids in length and migrates, under reducing conditions, with an approximate molecular weight of 5000. Chain 2 exists as either a 90 or 92 amino acid isoform in length, with the 92 residue form being the most prevalent (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992). Chain 2 has also been shown to contain an N-linked carbohydrate such that upon chemical reduction and SDS-PAGE it migrates with an approximate molecular weight of 14,000 (Morgenstern *et al.*, 1991; Duffort *et al.*, 1991). The mRNAs coding for these two chains are the products of separate genes and are expressed in the salivary glands and also the skin of the domestic cat (Griffith *et al.*, 1992). Both of these chains

*Address correspondence and request for reprints to Dr Julian F. Bond, ImmuLogic Pharmaceutical Corporation, 610 Lincoln Street, Waltham, MA 02154, U.S.A.

Abbreviations: *Fel dI*, the major allergen from the domestic cat (*Felis domesticus*) named according to the IUIS approved nomenclature [*Annals Allergy* 60, 499-504 (1988)]; HRPO, horseradish peroxidase; rchain 1, recombinant chain 1 of *Fel dI*; rchain 2, recombinant chain 2 of *Fel dI*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-MeOH, 2-mercaptoethanol; IgE, immunoglobulin E; RAST, radioallergosorbent test; ELISA, enzyme linked immunoassay; KOH, potassium hydroxide; PHP, pooled human plasma; TMB, 3,3',5,5'-tetramethylbenzidine; IPTG, isopropyl-D-thiogalactopyranoside; PACM, pipes-albumin-calcium-magnesium buffer.

have been expressed as recombinant proteins in *E. coli* and were used in comparative studies with *Fel dI* (Rogers *et al.*, 1993).

In the present study, we have expanded the analysis of *Fel dI* and the expressed recombinant chains through the use of different analytical methods for IgE reactivity and combined these data with that of protein sequence results. Both rchain 1 (recombinant chain 1) and rchain 2 (recombinant chain 2) of the bacterially expressed polypeptides are reactive with human IgE and can account for at least 50% of the total *Fel dI* reactive IgE in a direct binding RAST assay. The largest variation in results is seen when competition methods are used and this demonstrates differences in the reactivity of IgE to chain 1 or chain 2. The application of more than one method of analysis presents a clearer picture of the extent and type of the IgE reactivity to these allergen preparations.

MATERIALS AND METHODS

Protein sequence analysis

Fel dI was affinity purified from a 10% (wt/vol.) aqueous extract of dust collected from a house with pet cats using a monoclonal immunoaffinity column (Chapman *et al.*, 1988). The purified protein was digested with trypsin (Worthington, Freehold) at 20:1 (wt/wt) protein-to-enzyme ratio in 2 M guanidium hydrochloride without the addition of reducing agent. The tryptic peptide fragments were separated on an LKB 2150 HPLC system with a Waters mbond C18 column (Waters, Milford). The peptides were subjected to amino acid sequence analysis with an Applied Biosystem 477A protein sequenator connected to an on-line phenylthiohydantoin analyzer. A fast screening cycle and a fast OPA (ophthalaldehyde) filter conditioning (Brauer *et al.*, 1984) which was modified from the standard ABI sequencing cycle were used to accelerate the sequence speed and to verify the individual sequences when more than one peptide sequence appeared in the analysis.

Recombinant Fel dI chain 1 and chain 2 expression

Expression vectors, pETII chain 1 and pETII chain 2, were used for transformation of the *E. coli* host strain BL21 (DE3) and selected on plates containing 150 µg/ml ampicillin (Studier *et al.*, 1990). Recombinant expression was induced by the addition of IPTG to a final concentration of 1 mM (Rogers *et al.*, 1991). The guanidine HCl lysate containing rchain 1 and rchain 2 was subjected to Ni²⁺ NTA metal-ion affinity chromatography under denaturing conditions in 8 M urea (Hochuli *et al.*, 1988). After elution from the Ni²⁺ chelating support, Qiagen NTA-Agarose (Diagen GmbH, Dusseldorf, Germany), the rchain 1 and rchain 2 protein preparations were dialyzed three times against PBS to remove urea prior to analysis. Both of the expressed chains have 30 amino acids of extra N-terminal sequence to facilitate bacterial translation [MASMTGGGQMGRISMGGHHHHHEFLVGS] detailed previously (Rogers *et al.*, 1993).

Immunoblot analysis

The gel electrophoresis and Western blot transfer procedures were essentially as described elsewhere (Towbin *et al.*, 1979). Briefly, the *Fel dI* antigens were quantitated by the BCA method (Pierce, Rockford) and SDS-PAGE was performed using 15% acrylamide gels under reducing conditions, with 80 mM DTT (dithiothreitol), and at constant current of 40 mA. The transfer to nitrocellulose (0.1 µm, Schleicher & Schuell, Keene) was performed in a Hoeffer apparatus according to the protocol of Towbin *et al.* (1979). After transfer, the blots were rinsed in blot solution (25 mM Tris-HCl, pH 7.5, 0.17 M NaCl and 0.05% Tween 20) and stained for 1 hr with 0.1% India ink. All subsequent incubations with antibodies and washes were performed in blot solution at room temperature. The first antibody incubations were incubated overnight then rinsed and performed with the appropriate second antibody (KPL, Kirkegaard Perry Laboratories, Gaithersburg). The final incubation was performed using [¹²⁵I]-streptavidin (0.1 µCi/ml in 25 ml blot solution) for 1 hr followed by removal of unbound label and autoradiography at -80°C with an intensifying screen. The competition blot assays consisted of an incubation with PHP (pooled patient plasma, *n* = 18) at 25%, with the competing antigens (at a final concentration of 0.1 mg/ml) in blot solution in a total volume of 4 ml, for 8 hr at 4°C. Following this preincubation the solutions were then added to the *Fel dI* blot sections and the incubations were completed as outlined above.

Direct and competition ELISA

Corning assay plates (Corning, NY) were coated with 10 µg/ml of each coating antigen; affinity purified *Fel dI* (Chapman *et al.*, 1988), rchain 1 and/or rchain 2 in PBS at 50 µl/well and incubated overnight at 4°C. After blocking 100 µl/well of the human plasma samples were added to duplicate wells and incubated overnight at 4°C. Second antibody (biotinylated goat anti-human IgE, 1:1000), (KPL) was added at 100 µl/well for 1 hr at room temperature. Streptavidin-HRPO, 1:10,000 was added at 100 µl/well for 1 hr at room temperature (all wells were washed three times with PBS-Tween between each incubation step). TMB Membrane Peroxidase Substrate System (KPL) was freshly mixed, and added at 100 µl/well. The reaction was stopped by the addition of 100 µl/well of 1 M phosphoric acid. Plates were read on a Microplate EL Autoreader (Bio Tek, Winooski) with a 450 nm filter. The absorbance levels of duplicate wells were averaged. The graphed results (log of the Ag concentration vs absorbance) of the ELISA are shown in the figures. For the competition ELISA assays the pooled patient plasma sample was mixed with an equal volume of serially diluted antigens (*Fel dI*, rchain 1 and rchain 2) or with PBS-T (phosphate buffered saline with Tween 20, 0.05%) and allowed to "preincubate" overnight at 4°C. The remaining steps of the ELISA were performed as outlined above.

QuantAffinity bead RAST

Using the QuantAffinity epoxide glass beads supplied by Rainin Instrument Co. (Emeryville) a RAST assay was developed for the detection of IgE binding to *Fel d I* and the recombinant chains. Briefly, beads were coated in 0.5 M sodium phosphate buffer pH 6.8 at an antigen concentration of 0.5 μ g/bead, overnight at room temperature. The coating reactions were stopped by the removal of the coating solution and then by the addition of blot solution with 1% milk to block unreacted sites. The incubations with the PHP sample, depleted of IgG by preincubation with protein G agarose (Pharmacia), were carried out in a volume of 0.75 ml overnight, at room temperature. The same second antibody dilution as for the blot assays was used and IgE reactivity was measured as counts/minute of [^{125} I]-streptavidin using a Packard Cobra 5005 gamma-counter (Packard, Downers Grove).

Histamine release

The histamine release assay used for this study was based on the detection of an acylated derivative of histamine using a specific monoclonal antibody (Morel and Delaage, 1988). The reagents and protocol for this histamine radioimmunoassay are supplied commercially as a kit by Amac Inc. (Westbrook). Briefly, each individual assay consisted of 0.2 ml of whole heparinized blood from the cat allergic patient added to an equal volume of antigen in PACM buffer pH 7.3 (Pipes 25 mM, human serum albumin 0.003%, CaCl_2 4 mM, MgCl_2 1 mM, NaCl 100 mM, KCl 5 mM). The samples were mixed by tapping, incubated for 30 min at 37°C and then the cells were gently pelleted at 1500 rev/min for 3 min. The supernates are removed and processed for the RIA. The control for background spontaneous histamine release was patient blood incubated with only PACM buffer and for the total histamine values 0.1 ml of whole blood was boiled for 3 min in a final volume of 1.0 ml PACM. The

results were graphed as an expression of percent total release.

Antigen treatments

The glycosidase treatment was essentially as described previously (Morgenstern *et al.*, 1991). Briefly, 2 mg of affinity purified *Fel d I* was boiled for 2 min in 0.2% SDS, 5% (v/v) 2-ME in a final volume of 0.5 ml, cooled to room temperature, then diluted with PBS to 1.0 ml with 1% octyl glucoside, 50 mM KH_2PO_4 (pH 7.1) plus six units N-Glycosidase F (Boehringer Mannheim, Indianapolis). This solution was incubated overnight at 37°C, dialyzed against PBS and diluted to a final volume of 2 ml. A mock reaction was performed wherein a *Fel d I* sample was denatured and incubated overnight under the same conditions but in the absence of enzyme.

The treatment with KOH (potassium hydroxide) was as follows; affinity purified *Fel d I* or chains 1 and 2 were incubated at a concentration of 1 mg/ml in 50 mM KOH, pH 12.5 for 16 hr at room temperature. This incubation was stopped by the addition of potassium phosphate buffer to 75 mM, pH 7.1 followed by dialysis against PBS. A control sample underwent a similar incubation at neutral pH.

Antipeptide rabbit sera

As a means of analyzing antibody reactivity to the protein backbone of *Fel d I* a number of peptides were produced and separately used to generate rabbit polyclonal antisera (Morgenstern *et al.*, 1991). In this study we have applied two of these antipeptide antisera, anti-Fel 2 and anti-Fel 18 which were raised against regions from chain 1 and chain 2, respectively. The positions of the amino acids against which these affinity purified antibodies were generated are shown in Fig. 1.

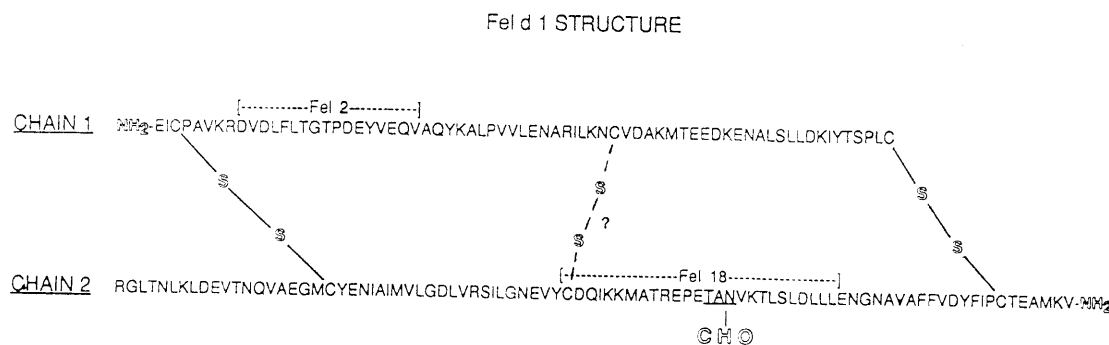


Fig. 1. The proposed structure of *Fel d I*. This structure is based on data generated from protein and cDNA sequencing. The two chains, 1 and 2, are 70 and 92 amino acids in length, respectively. The positioning of the chains as an anti-parallel heterodimer with the indicated disulfide linkages comes from data generated by protein sequencing following trypsin digestion of *Fel d I*. The middle disulfide linkage, indicated with a question mark, has not been unambiguously observed. The N-linked carbohydrate structure at residue 33 on chain 2 was localized by the lack of a signal at this cycle during protein sequencing and flanking consensus amino acids. The bracketed regions indicate the stretch of amino acids, from the two chains that were used for generation of rabbit antisera and were applied to the studies that follow. Each expressed recombinant chains including an N-terminal 30 amino acid leader sequence (see Materials and Methods).

RESULTS

Fel dI is an antiparallel heterodimer

The sequence of both chains of the *Fel dI* protein has been determined at the protein and cDNA level (Morgenstern *et al.*, 1991). Further protein sequence analysis, following trypsin digestion, suggests that the native structure of *Fel dI* is a heterodimer formed by the two chains. Figure 1 shows the position assignment of the disulfide cross-linkages of *Fel dI* as deduced from the peptide fragments obtained from tryptic digestion under non-reducing conditions with peptide sequence analysis. The tryptic cleavage map yielded more than 40 peaks while only 14 fragments were predicted from the primary sequence. Amino acid sequence analysis revealed that the abundance of tryptic peptides was at least partly due to various proteolytic cleavages of the affinity purified protein prior to the specific trypsin treatment. The complexity of the tryptic map made it impossible to compare reduced and non-reduced tryptic maps to identify disulfide cross-linked peptides. Instead, peptide sequence analysis was used to determine the disulfide cross-linkage only on the non-reduced set. The HPLC fractions containing the separate peptide sequences were identified and then *o*-phthaldehyde treatment was applied in a second analysis at the step before proline residues (Brauer *et al.*, 1984) to delineate and quantitate the individual peptide sequences. Using this approach, peptides containing Cys3 of chain 1 (EICPAVK) were always associated with peptides containing Cys73 of chain 2 (various forms of VLDGLVMIAINEYCMG) in approximately equal quantities; and the peptides containing Cys70 of chain 1 (IYTSPCL) were always associated with peptides containing Cys7 of chain 2 (MAETCPIFYDVF or AETCPIFYDVF or CPIFYDVF) at an approximately equal molar ratio. Both of these correlations were derived from peptide sequence analyses of multiple fractions, in which three clean pairs of Cys3/Cys73 (chain 1/chain 2) and one clean pair of Cys70/Cys7 were identified. The remaining two central cysteines, Cys44 of chain 1 and Cys48 of chain 2, were identified in equivalent yields in several HPLC peaks, however, other peptide sequences were also present and these could not be removed by OPA methods. Only the most predominant of the two forms of chain 2 was detected in this analysis (Griffith *et al.*, 1992). From these data the pairing of the central cysteines is suggestive but not definitive. These results therefore indicated that at least two pairs of interchain disulfide linkages are present in native *Fel dI*, and as depicted in Fig. 1 this structure is an antiparallel heterodimer of the two chains.

Direct IgE binding to Fel dI versus recombinant Fel dI chains 1 and 2

Western blot assays were performed on affinity purified native *Fel dI* and bacterially expressed recombinant chain 1 and chain 2 derived from *Fel dI* cDNA, lanes 1, 2, and 3 respectively (Fig. 2A). The first two antibody preparations that we used in this analysis were the two anti-peptide antisera described above (Fig. 1)

and were applied separately to demonstrate their specificity for each of the chains. Anti-*Fel 2*, reactive with the chain 1 peptide, shows the relative molecular weight of natural chain 1 to be approximately 5000 with the expressed recombinant slightly larger (Fig. 2A, the first panel). Both of the expressed recombinant chains have bacterial leader sequences comprised of 30 residues in addition to the native sequence (Materials and Methods, also Rogers *et al.*, 1993). By this analysis the signal from anti-*Fel 2* is much stronger to the recombinant chain 1 than to the polypeptide chain 1 derived from native *Fel dI*. The next section (Fig. 2A) shows the pattern of binding with the anti-chain 2 specific sera, anti-*Fel 18*. With any of the chain 2 specific antibodies, including human IgE, the Western blot signal from native chain 2 always appears as a diffuse signal due to its carbohydrate modification. The lower molecular weight limit of this chain 2 blot pattern is approximately 14,000. About 50% of the apparent molecular weight of native chain 2 is comprised of N-linked carbohydrate, as shown previously (Morgenstern *et al.*, 1991; Duffort *et al.*, 1991). A combination of two monoclonal antibodies, designated 3E4 and 2H4, that are specific for chain 1 and 2, respectively, were used as probes in this assay to demonstrate the pattern given by antibodies raised against native, affinity purified *Fel dI*. The subsequent panels give the binding profiles of IgE from two skin test positive cat-allergic patients, 417 and 288, with the last panel as the non-allergic negative control (Fig. 2A). The same two positive plasma samples (417 and 288) were also used in a direct ELISA on the same antigen preparations (Fig. 2B). With patient 288 the IgG binding is much stronger to native *Fel dI* than to rchain 1 or rchain 2. However, IgE from patient 417 shows an almost identical ELISA reactivity profile all three antigens. The IgE reactivity for the three different antigen forms has been further extended to 15 other allergic patient plasma samples with similar variation in results (data not shown). To date all samples positive for native *Fel dI* binding by these methods are also positive to both recombinants.

RAST assay

We also applied the RAST direct binding assay to the examination of IgE binding to the *Fel dI* forms. This RAST assay was one in which the antigen was covalently attached to epoxide-glass beads (Rainin QuantAffinity Beads) and IgE binding is detected with ¹²⁵I-labeled streptavidin binding to biotinylated anti-IgE second antibody. For this analysis we used PHP (pooled human plasma from eighteen cat allergic patients) that for these experiments had been depleted of IgG with protein G-Agarose to eliminate any interference from IgG binding. Figure 2(C) shows the results of this analysis with the negative control being IgE binding to beads coated with human serum albumin (HSA). Results obtained by the RAST assay suggest that a significant proportion of the anti-*Fel dI* reactivity can be accounted for by the sum of the binding to the recombinant chains at least in this direct binding format. A separate positive control

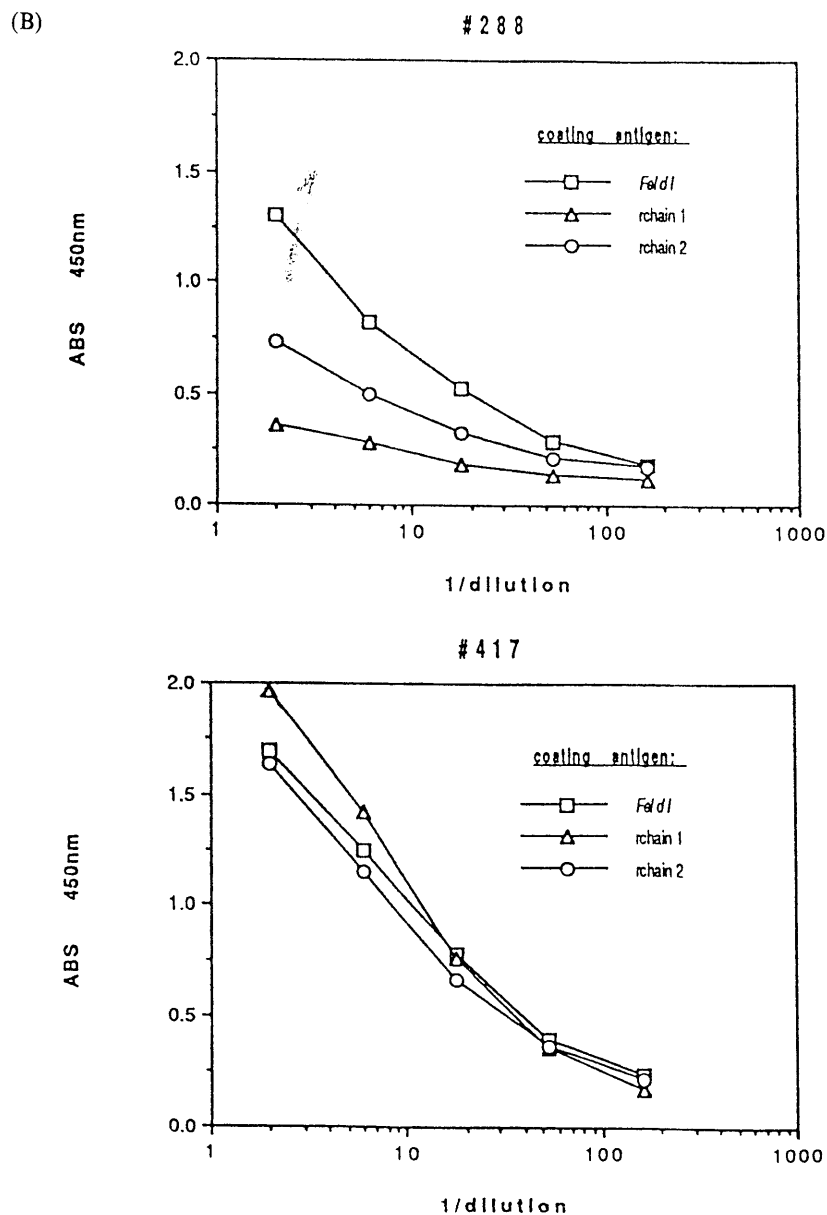
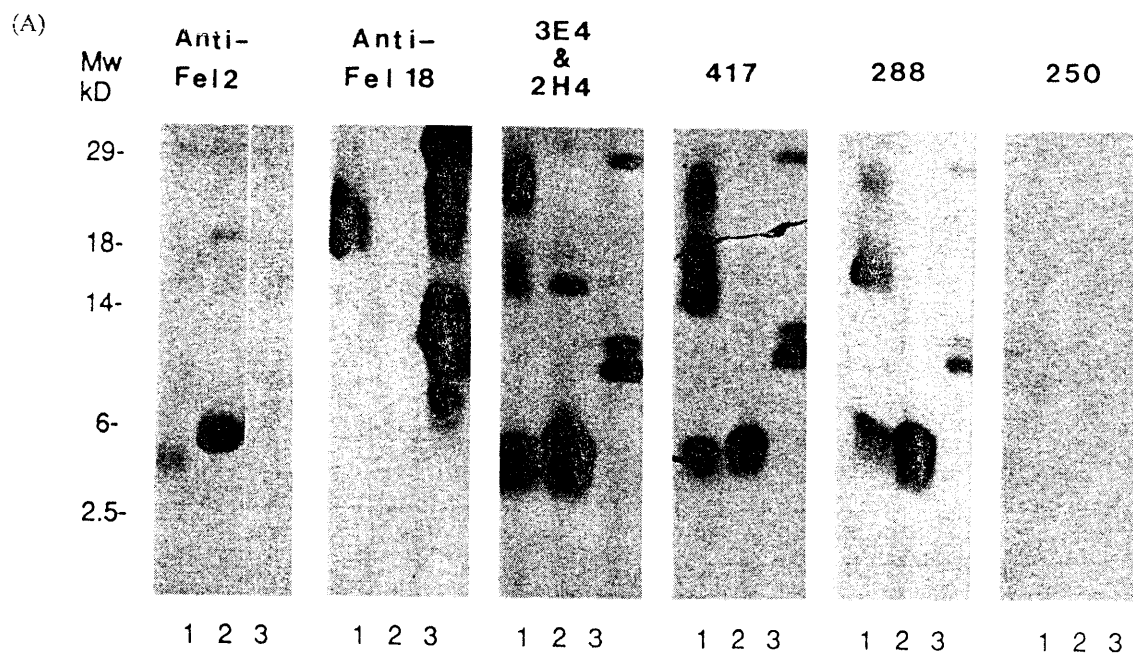


Fig. 2(A,B)

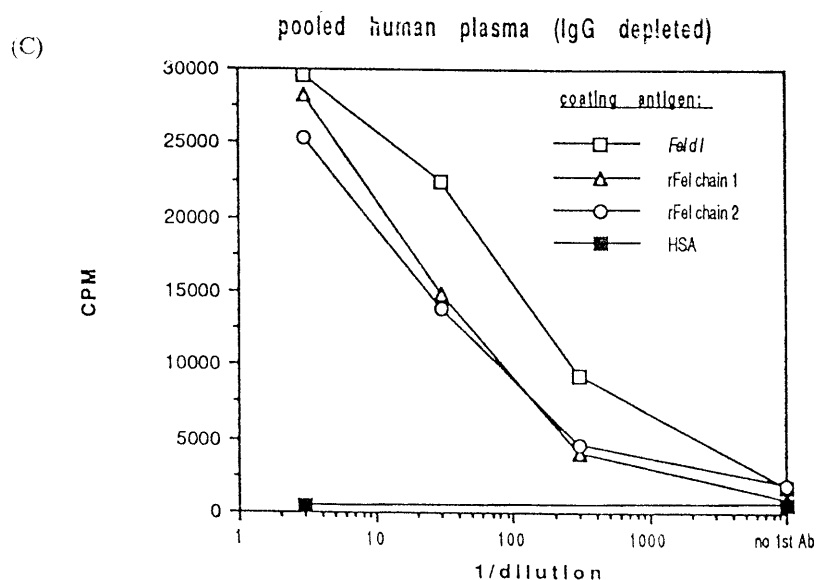


Fig. 2(C)

Fig. 2. Direct binding to *Fel dI* and recombinant chain 1 and 2. Western blots sections, (A), were generated with the *Fel dI* antigens as follows: lane 1, affinity purified *Fel dI* 10 µg/lane; lane 2, rchain 1 5 µg/lane; lane 3, rchain 2 5 µg/lane. The two peptide specific rabbit antisera anti-*Fel* 2 and anti-*Fel* 18 were used at 1:2000 dilution. The mix of monoclonal antibodies, 3E4 and 2H4, were both used at a 1:1000 dilution. The human plasma samples, cat allergic patients 417, 288 and non-cat allergic 250 were all used at 1:10 dilution. (B) Direct ELISA with the same antigen set used for the Western blots at a coating concentration of 10 µg/ml. The IgE binding of serially diluted plasma samples from patient 288 (upper panel) and patient 417 (lower panel) are shown. (C) RAST with covalently coupled antigen on glass beads (QuantAffinity Beads) analyzed with serially diluted PHP (plasma from a pool of 18 cat allergic patient samples). The binding of the IgE to the three allergen preparations is compared to human serum albumin (HSA) as the negative control.

assay was run using monoclonal antibodies against *Fel dI* to demonstrate that the coated antigen was not limiting in this assay (data not shown). Although these

results are based on pooled allergic human plasma it represents an average reactivity, by comparison to a small set of individual patients (not shown), and does

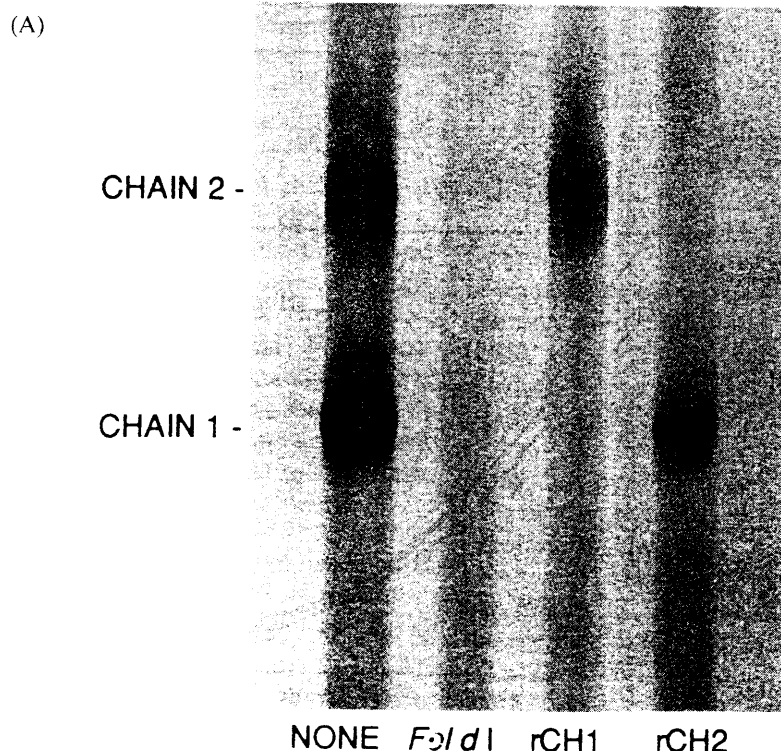


Fig. 3(A)

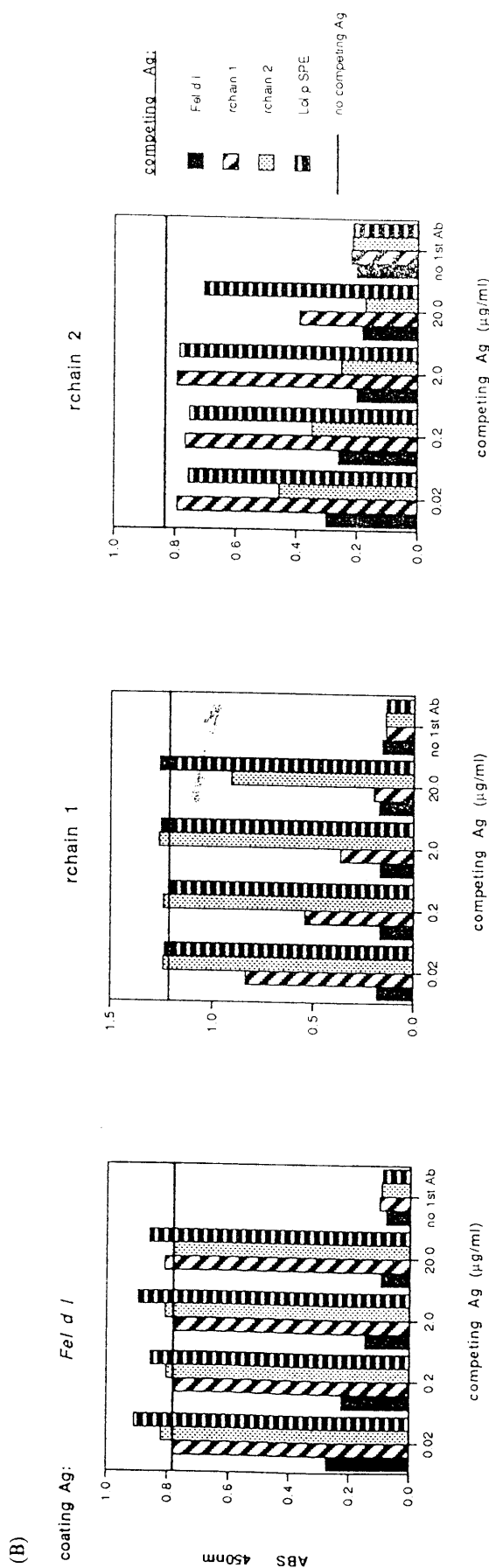


Fig. 3(B)

Fig. 3. Competitive assays for IgE binding. (A) The binding of PHP IgE to *Fel d*1 on Western blot sections was tested in the presence of the following antigens (at 0.1 mg/ml): lane 1, no competing antigen; lane 2, *Fel d*1; lane 3, rCH 2 is rchain 1; lane 4, rCH 2 is rchain 2. (B) PHP IgE binding to the *Fel d*1 antigens was analyzed by ELISA in the presence of no competing antigen, indicated by the line in each panel, or in the presence competing antigens at the indicated concentrations. The *Lol p* SPE is soluble pollen extract from ryegrass, *Lolium perenne*, used as the negative allergen control. A background control, with no first antibody shows the lack of second antibody binding to coating antigen.

not seem to be biased towards the highest anti-*Fel dI* IgE containing plasma samples.

Competition assays indicate a distinction between solid phase and solution binding of Fel dI

Another method of demonstrating antigen antibody specific interaction is by competition binding analysis. We have taken two approaches for these analyses: we preincubate PHP with the specific antigen in solution and then assay the IgE reactivity of this solution on: (1) SDS-PAGE Western blot sections of the various *Fel dI* antigens or (2) ELISA plates coated with the same set of antigens. Figure 3(A) shows the results of four preincubation experiments using PHP again as the source of anti-*Fel dI* IgE and Western blotted native *Fel dI* as the source of the solid phase antigen. Lane 1, Fig. 3(A), shows the pattern obtained with blotted *Fel dI* using plasma in the absence of added antigen. Lanes 2, 3, and 4 in Fig. 3(A), show the residual IgE binding after preincubating the plasma with 0.1 mg/ml of added biochemically purified *Fel dI*, rchain 1 and rchain 2, respectively. Preincubation with native purified *Fel dI* completely removes IgE binding to both chains (lane 2, Fig. 3A). When rchain 1 is used as the competing solution phase antigen, the signal for chain 1 is markedly reduced while there is little or no effect on the chain 2 signal (lane 3, Fig. 3A). Preincubation of IgE with rchain 2 removes all reactivity to the blotted chain 2 from native *Fel dI* (Fig. 3A, lane 4). These same assays, with individual patient plasma samples show a similar overall binding inhibition pattern to that observed with PHP.

The other assay that we have used to examine these solution phase interactions was through competition ELISA assays. For this assay the same plasma pool used in the blot competition study was analyzed with the three different antigens; affinity purified *Fel dI*, rchain 1 and 2 which were used for both solution and solid phases. Figure 3(B) shows the graphed results of these competition ELISA assays where panels 1, 2, and 3 refer to plates coated with *Fel dI*, rchain 1 and 2, respectively. The PHP dilution for all the competitive ELISAs was kept at 1:30. A grass pollen extract (*Lol p* SPE) was used as the non-competing control and this gives a signal equivalent to the non-competing antigen binding signal. Only *Fel dI* in solution is capable of competing IgE binding to itself on the plate with no competitive reactivity shown by either of the two rchains. The rchain 1 in solution effectively competes IgE binding to itself on the plate, however, its inhibitory capacity is much lower than that of native *Fel dI*. Using rchain 2 as the solution phase competitor on rchain 2-coated wells there is strong inhibition of IgE binding but again the native *Fel dI* is clearly more effective as a competitor. The major difference between the results with this ELISA antigen configuration (Fig. 3B) versus the blot method (Fig. 3A) is the lack of inhibition of IgE binding to *Fel dI* by the two separate recombinant chains.

Changes in IgE reactivity to Fel dI following different treatment protocols

The N-linked carbohydrate found on chain 2 of affinity purified *Fel dI* can be enzymatically removed using N-Glycosidase F, as shown previously (Morgens-tern *et al.*, 1991; Duffort *et al.*, 1991). For efficient enzymatic digestion the antigen sample is denatured by boiling and reduction with 2-MeOH such that all subsequent examination of the digested preparation is performed on denatured material. The enzyme cleavage products were examined by Western blotting. A mixture of monoclonal antibodies against chains 1 and 2, including 1G9 the antibody used for the affinity purification of *Fel dI* (Chapman *et al.*, 1988), gives the binding pattern shown in the first panel of Fig. 4(A). The molecular weight of chain 2, after digestion, shown in lane 4, decreases from approximately 14,000 down to 7000. The apparent molecular weight of chain 1 remains unchanged following this enzymatic digestion. A carbohydrate staining protocol using a similar blot section showed only chain 2 staining before digestion and a complete loss of staining after the enzymatic digestion (data not shown). The second panel, Fig. 4, shows the results of the antisera anti-*Fel 2* and anti-*Fel 18*, binding to a similar blot section emphasizing the shift in chain 2 following enzyme digestion. The position of chain 2 on the blot section shifts with carbohydrate removal but the binding of human IgE, PHP, [third panel, Fig. 4(A)] does not seem to be reduced. This confirms reports by others applying RAST analysis following carbohydrate removal (Duffort *et al.*, 1991). The results obtained by Western blotting were confirmed by binding analysis using ELISA. As shown in Fig. 4(B), even after *Fel dI* has been completely denatured and the carbohydrate removed, the IgE reactivity with PHP is not changed. Using the anti-peptide antisera in a direct binding ELISA format we could show that these antibodies recognize only denatured and not native *Fel dI* (data not shown).

Although carbohydrate staining was negative following the removal of the N-linked carbohydrate moiety we nevertheless examined the possibility of O-linked carbohydrate modifications on chain 1 and 2. This was done by alkali treatment, as a non-specific method for O-linked carbohydrate removal (see Materials and Methods), and followed by direct antibody binding analysis. Figure 5 shows the blotting results of this analysis. The KOH-treated sample demonstrates no obvious molecular weight shift and little difference in peptide specific antisera reactivity when the treated sample is compared to the two control samples, lane 2 vs 1 and 3 in the first panel (Fig. 5). However, when the same antigen preparations are probed with PHP IgE, as in the second panel, a marked decrease in signal is observed. With alkali treated *Fel dI* this loss of IgE signal is seen with both chain 1 and 2 with only slight reactivity retained by the chain 1. In light of the experiments demonstrating IgE reactivity to the recombinant forms of *Fel dI*, which are non-glycosylated, it was unlikely that this finding was due to a mammalian

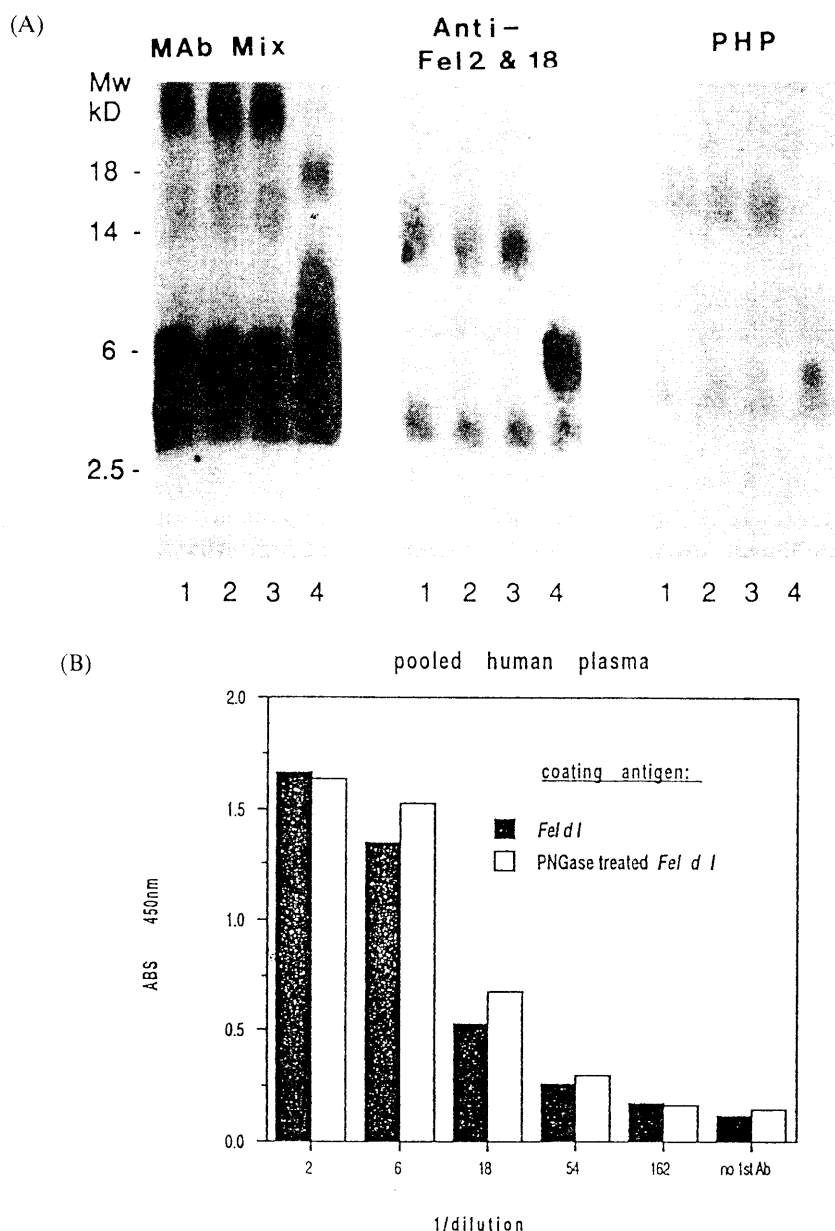


Fig. 4. Direct binding to the *Fel d*I antigens following specific treatments. (A) Western blot sections probed with: MAb mix comprised of monoclonal antibodies 3E4, 2H4 and 1G9 the antibody used for affinity purification (all at 1:2000 dilution), peptide specific antisera anti-*Fel* 2 plus anti-*Fel* 18 (both at 1:2000 dilution); and PHP (at a 1:10 dilution) on lanes of the following antigens: 1, untreated *Fel d*I; 2, *Fel d*I control only concentrated and denatured; 3, *Fel d*I control incubated with no enzyme; 4, PNGase digested *Fel d*I (all antigens at 10 µg/lane). (B) ELISA assay of *Fel d*I control incubated with no enzyme and PNGase digested *Fel d*I with serial dilutions of PHP.

post-translational system of O-linked glycosylation. An analysis of IgE reactivity following alkali treatment of the rchain 1 was therefore done (the next three panels, Fig. 5). The third panel shows the results of this treatment set using the chain 1 peptide specific antisera, anti-*Fel* 2, where the signals for treated and untreated *Fel d*I (lane 1 and 2) are roughly equivalent as are the signals for treated and untreated rchain 1 (lanes 4 and 5). However, when KOH-treated rchain 1 was probed with the patient plasma samples (the last two panels, Fig. 5) a striking loss of IgE reactivity is seen. This loss of reactivity has so far

been observed with all patient samples analyzed on both KOH-treated affinity purified *Fel d*I and the KOH treated rchain 1. These effects are also seen when the binding is assayed by direct ELISA and this property is also exhibited with alkali treated chain 2 examined by both methods (data not shown). The base treated *Fel d*I has been examined for amino acid structural changes by N-terminal protein sequence analysis and the only modification observed has been a low but significant alkali derived deamidation of Asn residues throughout the molecule (up to 25%, data not shown).

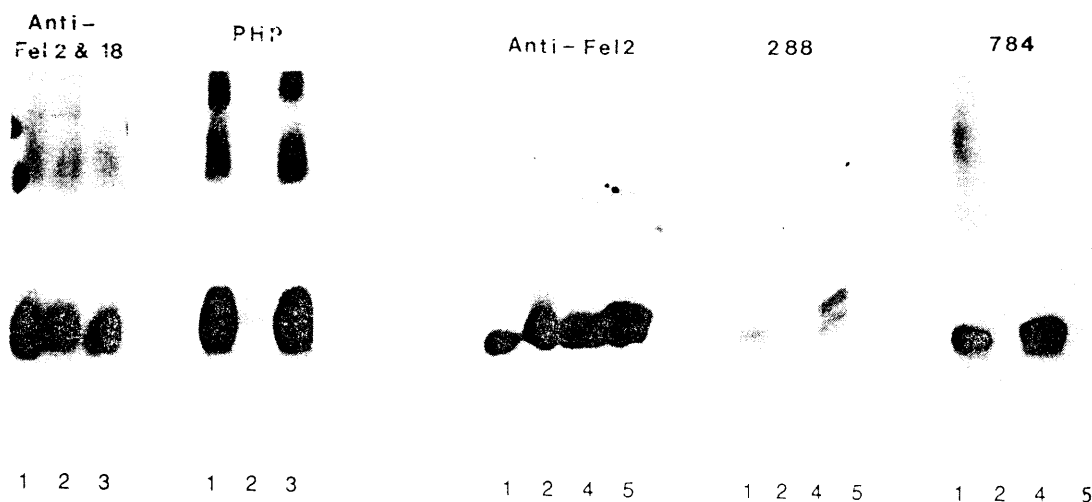


Fig. 5. Western blot of *Fel dI* preparations with KOH treatments with the antigen sets as follows: lane 1, untreated *Fel dI*; lane 2, *Fel dI* treated with 50 mM KOH; lane 3, incubation control *Fel dI*; lane 4, untreated rchain 1; and lane 5, rchain 1 treated with 50 mM KOH. The blot sections were probed with: the mix of anti-peptide antisera (at 1:2000 dilution), PHP at 1:10, anti-Fel 2 at 1:2000, patients 288 and 784 at 1:10.

Histamine release analysis

We have pursued leukocyte histamine release as an *in vitro* model of IgE specific mediator release. In this study blood samples from three different cat allergic patients were analyzed against serial dilutions of the respective treated and untreated antigens described above. Figure 6(A) shows the histamine release profile using whole blood from patient 417 in response to *Fel dI*, recombinant chain 1 and recombinant chain 2. The results are expressed as a percentage of the total histamine released (see Materials and Methods). All three antigens are capable of inducing histamine release, but the native *Fel dI* is much more potent than either of the two rchains using patient 417. The direct binding data with this patient's IgE showed equivalent reactivity with all three antigens (Fig. 2B) whereas the histamine release shows the rchains to be much less active than the native molecule.

Another patient sample, 681, was examined for comparative histamine release to deglycosylated *Fel dI* versus the untreated and the mock treated sample. The results of this analysis (Fig. 6B) reflect the findings of the direct binding assays that the reduction and denaturation of *Fel dI* followed by enzymatic deglycosylation steps do not noticeably alter the IgE binding of the *Fel dI* (Fig. 4A). The triphasic nature of the histamine release curves seen in many patients when using *Fel dI* as the releasing antigen is most likely due to contaminating antigen present in trace amounts in the affinity purified preparation. One contaminant could possibly be cat serum albumin known to be an allergen in some cat allergic patients (Anderson and Baer, 1981; Wallenbeck and Einarsson, 1987; Duffort *et al.*, 1987).

To test the relative reactivity of the alkali treated *Fel dI*, histamine release assays were also performed using this preparation in comparison to untreated *Fel dI*. Figure 6(C) shows the results of the analysis using patient 288 as the source of cat allergic patient whole

blood. The peak of histamine release with the untreated sample is seen at 2 nM while the peak with the KOH-treated *Fel dI* is reached only when the concentration of antigen is at 200 nM.

DISCUSSION

The studies described here constitute an immunochemical examination of a small allergen that has been defined at the primary sequence level (Morgenstern *et al.*, 1991). The availability of *Fel dI* and the two recombinantly expressed polypeptide chains that comprise its structure has allowed us to begin to dissect the biological properties of this common household allergen.

Our analysis of protein sequencing data indicate that the native *Fel dI* chains lie anti-parallel to each other as a heterodimer and at least two of the possible disulfide bonds lead to their covalent interaction. The central two cysteines were not clearly determined to be connected and could still potentially lead to oligomeric *Fel dI* structures which have been implicated previously (Leitermann and Ohman, 1984). The determination of this structure does not exclude the possibility of the existence of other less abundant configurations for the two chains of native *Fel dI*.

We have shown that both *Fel dI* chains, whether native or recombinant, have anomalous migration properties under reducing conditions with a smaller apparent molecular weight than their primary amino acid sequence could indicate. This may imply a core structure that is a distinct property of both chains and that this structure is conferred strictly by the primary amino acid sequence. This proposed structure is either maintained during the denaturation treatment prior to gel electrophoresis or regenerated as electrophoresis is carried out. Antisera generated against linear epitopes of *Fel dI* have poor recognition of the native molecule such that there is increased reactivity of the anti-peptide sera to either

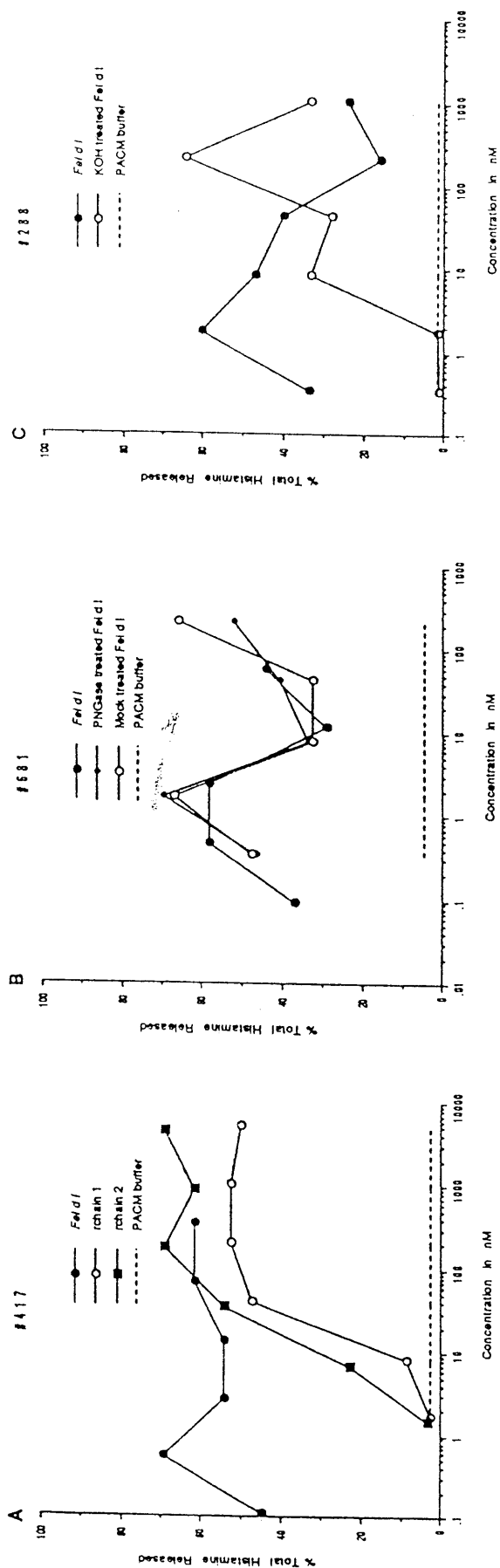


Fig. 6. Histamine release sets. (A) Patient 417 whole blood tested with native *Fel dI*, chain 1, chain 2 or buffer. The histamine quantitation was performed by RIA and graphed as percentage of total histamine released determined from a boiled sample. (B) Patient 681 histamine release comparing PNGase treated *Fel dI* sample with the two control preparations. (C) Comparison of KOH-treated *Fel dI* to untreated with patient 288.

chain following denaturation. Previous studies have demonstrated a marked loss of IgE reactivity upon reduction and alkylation of *Fel d I* (Duffort *et al.*, 1991), however, we find no evidence that cysteine reduction alone causes decreased binding.

The reactivity to the separate recombinant forms demonstrates the fact that IgE epitopes are generated without prior association of the two chains and this is not interfered with by the 30 extra amino acids used for efficient bacterial expression. The direct assays, ELISA and RAST, imply that about 50% of the overall activity against the native molecule can be directed against epitopes in the recombinant molecules. However, these results must be qualified by the data from the competition ELISA and histamine release analysis where the rchains in solution have much lower IgE reactivity than does the native molecule. The use of different methods for the detection of IgE reactivity allows for a more accurate picture of the overall binding capacity of a particular allergen. This certainly holds true for the differences seen when the antigen is recognized on a solid support versus in solution. With the blot inhibition assay, where the inhibiting rchains are at high concentration, a loss of binding to solid phase *Fel d I* is observed but this is not seen with the antigen concentration range used in the competition ELISA. A more detailed analysis to quantitate the comparative levels of IgE binding and the relative affinities of the different forms is underway. Methods are also being designed to associate the two chains *in vitro* to test for differences in reactivity compared to the separate rchains and native *Fel d I*.

The presence of the N-linked carbohydrate has only a minimal influence on IgE recognition so that the explanation of the differences between the native and recombinant forms must lie elsewhere. Why the alkali treated *Fel d I* and recombinant chains lose so much of the reactivity is not known. The most likely explanation is that during the base treatment incubation some structural requirement is disrupted (i.e. through deamidation) or some crucial amino acid residues are altered in a manner that has not yet been detected by direct protein sequencing. The modification with alkali may contribute to a change in the proposed core region of each chain. In agreement with this, linear epitopes appear to constitute a minor fraction of the overall *Fel d I* IgE reactivity. One method of analyzing the presence of linear epitopes is through screening of peptides for IgE binding. From studies that we and others have undertaken (Van Milligen *et al.*, 1992) there does appear to be some IgE binding to specific *Fel d I*-derived peptides. However, this peptide reactivity is too low to account for the binding exhibited by the recombinant chains. This lends further support for the proposition of conformational IgE epitopes determined by the primary amino acid sequence of each chain.

Can this unique reactivity and the implications of a core structure based on primary sequence play a role in the allergenicity of this dander allergen? The properties of *Fel d I* indicated by these studies, in combination with

previous work, show it to be quite a stable molecule (Wood *et al.*, 1989). There are other studies that have demonstrated *Fel d I* to be associated with small particles (<5 µm in diameter) which remain airborne for long periods of time (Luczynska *et al.*, 1990). These properties lead to increased environmental presence and therefore chronic exposure. It is this chronic exposure that may play a crucial role in the development of increased IgE immunoreactivity leading to allergy and asthma.

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