

Allergens, IgE, mediators, inflammatory mechanisms

Definition of the human T-cell epitopes of Fel d 1, the major allergen of the domestic cat

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Background: A heterodimeric acidic glycoprotein (Fel d 1) has been defined as the major allergen of the domestic cat. Because T-cell help is required for the initiation and maintenance of allergic responses, it is of importance to determine the T-cell-reactive regions of the Fel d 1 molecule.

Methods: Overlapping peptides corresponding to the two chains of Fel d 1 were tested in proliferation assays on polyclonal T-cell lines and for the ability to bind Fel d 1-specific IgE in ELISA and histamine release assays.

Results: Assay of T-cell lines derived from 53 subjects allergic to cats demonstrated that the majority of T-cell reactivity is found in chain 1 of Fel d 1. Two peptides (Fel-1 and Fel-2) containing major epitopes, alone or as a mixture, efficiently activated T cells and exhibited minimal detectable reactivity with IgE by ELISA or histamine release assay.

Conclusions: Two Fel d 1 peptides containing major T-cell epitopes have been identified, have been shown to bind minimal Fel d 1-specific IgE, and are now being tested for the ability to decrease T-cell responses in patients with cat allergy as a new form of immunotherapy. (*J Allergy Clin Immunol* 1996;98:884-94.)

Key words: Cat allergen, T-cell epitopes, synthetic peptides, immunotherapy, IgE antibodies, histamine release

Allergy to the domestic cat (*Felis domesticus*) is a significant problem in many countries, including the United States, where the frequency of cat allergy is estimated to be on the order of 10% of the population.¹ Patients with cat allergy can exhibit a variety of symptoms, ranging from a mild rhinitis to more debilitating allergic reactions. For example, 20% to 30% of patients with asthma have been shown to have cat-specific serum IgE, and it is known that hypersensitivity to cat proteins is a significant cause of extrinsic asthma.^{2,3} Serum IgE from patients allergic to cats has been shown to

Abbreviations used

EBV:	Epstein-Barr virus
MHC:	Major histocompatibility complex
PBLs:	Peripheral blood leukocytes
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PI:	Positivity index
SI:	Stimulation index

bind to a wide spectrum of proteins derived from cat pelt extracts.⁴⁻⁶ However, studies have demonstrated that the dominant cat allergen is a protein called Fel d 1, because Fel d 1-reactive IgE can be detected in serum from a majority of patients allergic to cats, and most cat-specific IgE in these patients binds to Fel d 1.^{7,8}

Fel d 1 has been found in the saliva, pelts, and sebaceous glands of cats and has been purified by immunoaffinity chromatography and biochemically characterized. Fel d 1 is an acidic heterodimeric

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glycoprotein with a molecular mass of approximately 38 kd. Analysis by reducing sodium dodecylsulfate–polyacrylamide gel electrophoresis has shown that the native Fel d 1 molecule is comprised of two chains with apparent molecular weights of 5 to 6 kd and 16 kd.^{9,10} Cloning and sequencing of complementary DNAs encoding these two chains has recently been reported,¹¹ demonstrating that chain 1 (70 amino acids) and chain 2 (92 amino acids) are encoded by distinct genes.¹² Chain 2 has been shown to contain 30% N-linked oligosaccharides and to exhibit a minor polymorphism in amino acid sequence.^{10,12,13}

T cells are intimately involved in allergic reactions by providing signals for IgE switching and for the growth and differentiation of the effector cells of allergic responses (e.g., mast cells, basophils, and eosinophils). T-cell–derived cytokines may also be involved in the maintenance of IgE production by B cells.¹⁴ Thus it is of interest to define the T-cell responses of patients with cat allergy to Fel d 1. To this end, we have used knowledge of the primary structure of this protein to design an overlapping set of synthetic peptides corresponding to the entire structure of chain 1 and chain 2. Using Fel d 1–reactive polyclonal T-cell lines derived from subjects allergic to cats, we tested synthetic peptides in standard T-cell proliferation assays to determine the major areas of the Fel d 1 molecule recognized by T cells. Regions of major T-cell reactivity were identified in a panel of subjects with a great diversity of major histocompatibility complex (MHC) class II types.

Until now, cat pelt extracts containing Fel d 1 have been used for immunotherapy.^{2,15} The major disadvantages of such an approach are IgE-induced side effects, including the risk of systemic anaphylaxis on injection of proteins capable of binding IgE. In addition, traditional immunotherapy requires the repeated injection of extracts over a very long period. With the identification of areas of the molecule strongly reactive with T cells, desensitization protocols can now be designed to use these major T-cell epitope–containing regions to tolerize or diminish the T-cell responses that initiate and maintain the allergic response to Fel d 1. Thus a new class of immunotherapy can now be tested, and experiments have been initiated to determine the mechanism of action of immunotherapy for allergies.

METHODS

Protein purification

Native Fel d 1 protein was purified from an extract of cat hair as described by Chapman et al.,⁹ with modifica-

tions. Briefly, the cat hair was submerged in phosphate-buffered saline (PBS), 0.1% Tween-20, (pH 7.2) containing a mixture of protease inhibitors for 48 hours at 4° C with agitation. The extract was then filtered and applied to an immunoaffinity column coupled with the 1G9 monoclonal anti-Fel d 1 antibody¹³ (provided by Dr. M. Chapman, University of Virginia). After the column was washed extensively, the Fel d 1 bound to the column was eluted with 100 mmol/L glycine, pH 2.5, and immediately dialyzed extensively against PBS, pH 7.2. All preparations were gamma-irradiated (25,000 rad) before use in T-cell assays.

Peptide synthesis

An overlapping set of peptides derived from the sequence of Fel d 1¹¹ was synthesized with Applied Biosystems 430 (Perkin-Elmer Corp., Foster City, Calif.) and Milligen 9050 (Perceptive Biosystems, Framingham, Mass.) peptide synthesizers by using conventional Merrifield solid-phase chemistry with t-Boc or Fmoc amino acids. The peptides were cleaved from the synthesis substrate with hydrofluoric acid (for t-Boc amino acids) or with trifluoroacetic acid (for Fmoc amino acids), desalted by gel filtration, lyophilized, and purified by reverse-phase high-performance liquid chromatography in 0.1% trifluoroacetic acid. The purified peptides were then subjected to amino acid analysis. Amino acid analysis correlations were 0.8 or higher for all peptides. The sequences of the peptides used in this study are shown in Fig. 1. For testing in T-cell proliferation assays, the lyophilized peptides were resuspended at a concentration of 1 mg/ml in PBS (pH 7.2), sonicated, and sterilized by gamma-irradiation (25,000 rad).

Isolation of antigen-reactive human T-cell lines

Subjects were chosen from visitors to the Tufts New England Medical Center Allergy Clinic on the basis of history of allergy to cats and a positive skin test reaction to commercially available cat pelt extract. These subjects had not undergone immunotherapy and were not taking any medications that would significantly alter the immune response. Informed consent, in accordance with New England Medical Center Investigational Review Board regulations, was obtained from all subjects before enrollment. Heparinized peripheral blood specimens were drawn, and the peripheral blood leukocyte (PBL) fraction was purified within 24 hours of the sampling. Briefly, PBLs were separated from the plasma by centrifugation at 150 g for 15 minutes, the pelleted cells were restored to their original volume with heat-inactivated fetal calf serum, and were then diluted 1:1 in incomplete medium (RPMI-1640 [JRH Biosciences, Lenexa, Kan.] supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin/streptomycin, 5×10^{-5} mol/L 2-mercaptoethanol, and 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [pH 7.4]). The PBLs were then purified by centrifugation with lymphocyte separa-

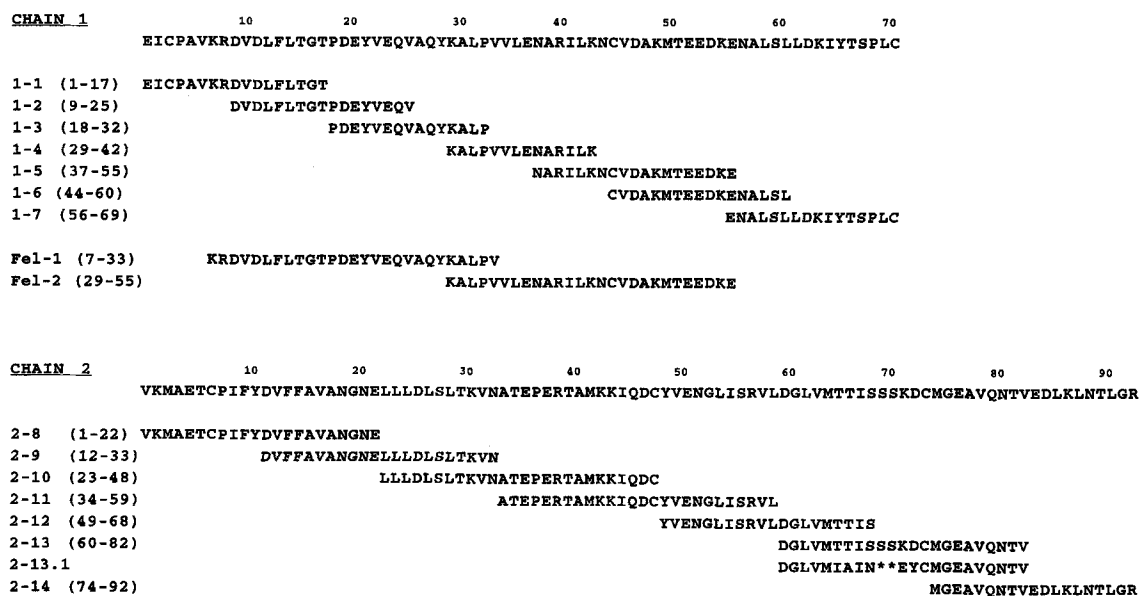


FIG. 1. Complete primary structure of Fel d 1 chain 1 and Fel d 1 chain 2 and overlapping Fel d 1 synthetic peptides used for T-cell epitope mapping. Peptides 2-13 and 2-13.1 represent the known polymorphism (long and short forms, respectively) in chain 2 of Fel d 1. Asterisks indicate amino acid deletions in the short form of Fel d 1 relative to the long form.¹² Fel-1 and Fel-2 have previously been referred to as IPC-1 and IPC-2, respectively, in a previous publication.²⁴

tion medium (Organon Teknika, Durham, N.C.) according to the manufacturer's protocol. The PBLs were washed three times with PBS (pH 7.4) containing 0.1% bovine serum albumin and then resuspended in complete medium (incomplete medium supplemented with 5% heat-inactivated pooled human AB serum).

T-cell lines were established essentially as described previously¹⁶ by stimulation of 1×10^6 PBLs per milliliter in complete medium with 10 μ g of affinity-purified native Fel d 1 per milliliter for 7 days at 37°C in a humidified 5% CO₂ incubator. This antigen concentration was determined to be optimal for most patients in pilot experiments. The viable nonadherent cells were isolated by using lymphocyte separation medium and were cultured in the presence of 5 U/ml recombinant IL-2 (Boehringer/Mannheim, Indianapolis, Ind.) and 5 U/ml recombinant IL-4 (Genzyme, Cambridge, Mass.) until the time of assay.

T-cell proliferation assays

T-cell lines were used for assay after the rest cycle of their growth. T cells (2×10^4) were cultured in duplicate in 200 μ l of complete medium with 5×10^4 gamma-irradiated (3500 rad) PBLs as antigen-presenting cells in the presence of various concentrations of peptide or protein antigens in round-bottom, 96-well dishes (Costar, Cambridge, Mass.). After 3 days of culture at 37°C in a humidified 5% CO₂ incubator, each well was pulsed with 1 μ Ci tritiated thymidine (ICN Biochemicals, Costa Mesa, Calif.) for an additional 16 hours. The wells were then harvested, and incorporation of tritium was measured by liquid scintillation counting. The stan-

dard deviation of the duplicate wells was generally less than 20% of the mean. The results were converted to stimulation indices (SIs) (counts per minute of antigen-stimulated cultures divided by counts per minute of cultures in complete medium only). For presentation, the five highest SIs to peptides of the overlapping set for each T-cell assay were noted and given a rank from 1 to 5 (5 = highest, 1 = lowest). The ranks given to each peptide in each of the 53 assays were summed, resulting in a sum of the ranks as a measure of the relative strength of each response in the population. The data are also expressed as a positivity index (PI), which is the product of the mean SI and the frequency in the population of an SI greater than or equal to 2.0. Ranked sum is used to directly determine the relative values of the peptide responses when compared with each other. The PI is used as a measure of the strength and frequency of responses to peptides relative to the responses to the Fel d 1 protein. In these analyses, the highest SI obtained, regardless of peptide dose, was used in the calculations, because high-dose inhibition was observed in some experiments.

Epstein-Barr virus transformation of B-cell lines and HLA typing

Epstein-Barr virus (EBV) transformation of PBLs was performed by incubating 3×10^6 PBLs with 1 ml of B-95/8 marmoset cell (ATCC CRL1612) conditioned medium in the presence of 1 μ g/ml phorbol myristate acetate at 37°C for 30 minutes. The cells were then diluted to 5×10^5 cells/ml in complete medium contain-

ing 5% fetal calf serum and were cultured in 96-well culture dishes (Costar) until colonies were visible. The EBV lines were then expanded in complete medium containing 5% fetal calf serum. EBV-transformed lines were typed for DR, DRw, and DQ by using polymerase chain reaction (PCR) typing technology. This typing was carried out by Dr. Lee-Ann Baxter-Lowe of the Blood Center of Southeastern Wisconsin, Milwaukee.

IgE ELISA

Measurements of Fel d 1-specific IgE were performed essentially as described previously.¹⁷ Briefly, a solid-phase ELISA for the detection of IgE reactive with purified native Fel d 1 or synthetic peptides was performed by coating Corning plates (Corning Glassworks, Corning, N.Y.) with purified native Fel d 1, Fel-1, or Fel-2. IgE, depleted of most of the IgG by extraction with Protein G-agarose (GammaBind G-Agarose; Genex Corp., Gaithersburg, Md.), in pooled plasma from 20 subjects allergic to cats was then tested for its ability to bind to Fel d 1 or its peptides.

Histamine release assay

The procedure for the detection of histamine was carried out according to the manufacturer's protocol. Briefly, 200 μ l of serial dilutions of Fel-1, Fel-2, or native purified Fel d 1 in PACM buffer (25 mmol/L piperazine-N,N'-bis[2-ethanesulfonic acid], 0.003% human serum albumin, 4 mmol/L CaCl₂, 1 mmol/L MgCl₂, 100 mmol/L NaCl, 5 mmol/L KCl [pH 7.3]) were gently mixed with 200 μ l of heparinized whole blood drawn from subjects allergic to cats. After incubation at 37° C for 30 minutes, the samples were centrifuged at 1500 rpm for 3 minutes, and the supernatant fluids were removed and processed for the detection of histamine by radioimmunoassay (Immunotech, Marseille, France). The control for background spontaneous histamine release was heparinized blood from the same subject incubated in PACM buffer only. The results are presented as percentages of histamine released in the presence of antigen relative to the histamine released from blood boiled for 3 minutes in a final volume of 1.0 ml of PACM buffer.

RESULTS

Subject profiles

Subjects in this study were selected from visitors to the Tufts New England Medical Center Allergy Clinic. Clinical histories were taken, and the subjects were skin prick tested with a commercially available cat skin test extract containing Fel d 1, 20 to 40 μ g/ml (ALK, Copenhagen, Denmark). All subjects included in the study had strongly positive skin test results (3 mm or greater wheal compared with saline as a negative control), had not received immunotherapy, and were not taking any medication that would affect the immune response. EBV-transformed B-cell lines were derived from each

TABLE I. MHC class II types and Fel d 1 peptide responses

Subject No.	Peptide responses	DR†	DRw	DQ
583	9, 2, 11, 3, 6*	4, 7	53	7, 9
584	4, 3, 8, 2	2, 6	52	1, 7
585	7, 3, 4, 1, 2	2, 5	52	1, 7
587	4, 5, 6, 9, 8	2, 5	52	1, 2
591	4, 2, 7, 9, 14	1, 6	52	1
605	4, 3, 1, 9, 2	4, 5	52, 53	7, 8
606	12, 7, 14, 1, 3	3, 7	52, 53	2, 9
607	4, 2, 3, 7, 9	3, 12	52	2, 7
612	11, 3, 12, 4, 4, 14	1, 12	52	1, 7
614	3, 7, 4, 13, 2	2	52, 53	1
615	4, 6, 1, 2, 13	3, 4	52, 53	2, 8
621	4, 5, 7, 10, 3	3, 6	52	1, 2
635	10, 12, 3, 1, 4	1, 5	52	1, 7
641	4, 14, 8, 13, 9	4, 6	52, 53	1, 7
647	4, 7, 10, 1, 6	6, 7	52, 53	1, 2
650	5, 3, 1, 2, 14	4	53	4, 8
685	2, 14, 3, 5, 13	2, 7	53	1, 2
687	5, 12, 7, 10, 1	7	52, 53	2, 7
688	7, 14, 13, 5, 2	4, 5	52, 53	7, 8
689	2, 5, 4, 7	4, 6	52, 53	1, 8
690	6, 5, 7	3, 7	52, 53	2
695	7, 5, 10, 1, 2	3, 7	52, 53	2
696	4, 3, 14, 2, 7	3, 4	52, 53	2, 7
697	1, 14, 13, 2	3, 7	52, 53	2
703	7, 10, 6, 3, 5	2, 5	52	1, 7
725	7, 3, 1, 2, 11	2	nd‡	1
727	12, 7, 3, 9	5	52	7
728	3, 4, 13, 5, 6	2, 4	53	1, 8
736	4, 12, 7, 2, 3	1, 5	52	1, 7
737	6, 7, 4, 1, 2	3, 8	52	2, 7
738	13, 8, 10, 5, 6	4, 6	52, 53	1, 8
739	3, 4, 7, 2, 1	6, 8	52	1, 4
741	14, 8, 9, 7, 4	4, 7	53	2, 8
742	8, 14, 9, 3, 7	7	53	2
743	9, 14, 8, 10, 5	3, 4	52, 53	2, 7
745	10, 14, 3, 2, 5	6, 7	52, 53	1, 2
897	5	4, 6	52, 53	1, 8
902	9, 13, 5, 10, 2	3, 4	52, 53	2, 8
908	3	2, 6	52	1
944	3, 2, 1, 5, 4	4, 7	53	2, 8
951	9, 2, 10	3, 7	52, 53	2, 9
956	4, 3, 2, 5, 1	2, 6	52	1

*Fel d 1-reactive T-cell lines established from the subjects listed, as described in Methods, were tested for proliferation in the presence of Fel d 1 synthetic peptides at 3, 15, or 75 μ g/ml. The five highest peptide responses, regardless of concentration, are shown in order of magnitude, from the highest to the lowest. Some of the T-cell lines responded to fewer than 5 peptides. The peptides are numbered as shown in Fig. 1, except the designation of the chain from which the peptides are derived is deleted for brevity.

†HLA class II types as determined by PCR.

‡Unable to determine by PCR.

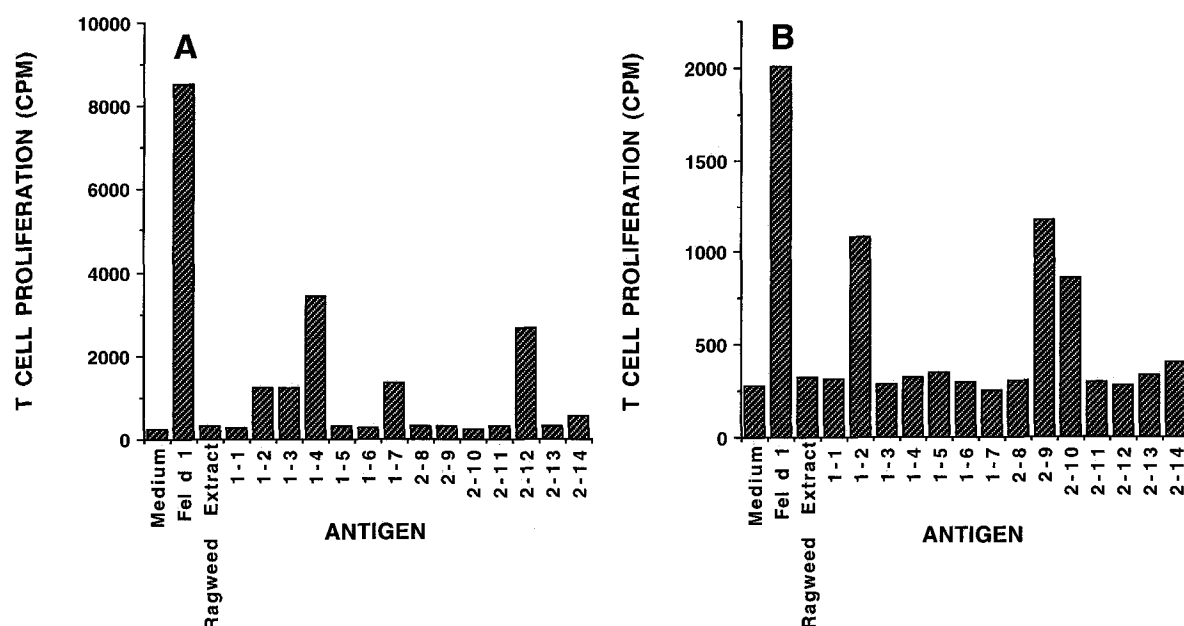


FIG. 2. Proliferative responses of two Fel d 1-reactive T-cell lines to overlapping Fel d 1 synthetic peptides and whole Fel d 1 protein. Short-term polyclonal T-cell lines were derived from the PBLs of two cat-allergic subjects, 736 (**A**) and 951 (**B**), as described in Methods, and were assayed for proliferation in response to the antigens indicated with irradiated autologous PBLs as antigen-presenting cells. Each antigen was titrated (3, 15, or 75 $\mu\text{g/ml}$), and the highest level of proliferation, regardless of dose, is presented.

subject and were MHC class II typed by using PCR technology. The results of this examination are shown in Table I and indicate that this panel of subjects exhibits a diversity of class II types.

T-cell responses to Fel d 1 peptides

Fel d 1-reactive T-cell lines were established by stimulating PBLs from patients allergic to cats with affinity-purified native Fel d 1, as described in Methods. These T-cell lines were then tested for reactivity in proliferation assays to Fel d 1 protein and an overlapping set of synthetic peptides corresponding to both chains of the Fel d 1 molecule. The sequence of both of these chains is shown in Fig. 1, and the set of peptides used for analysis is outlined. Because of a minor polymorphism in chain 2,¹² two peptides were synthesized to cover amino acids 60-82. The amino acid sequence of the 2-13 peptide is derived from the long form of Fel d 1 chain 2, which is preferentially expressed in the salivary gland of the cat. The amino acid sequence of the 2-13.1 peptide corresponds to the short form of Fel d 1 chain 2, which is preferentially expressed in the skin of the cat. The long form of Fel d 1 is the major form detected by amino acid sequencing of purified native Fel d 1.^{11, 12} Peptides were added to proliferation assays at three doses (3, 15, or 75

$\mu\text{g/ml}$), and the highest response to each peptide, regardless of dose, was noted for each patient.

The T-cell responses of two representative subjects allergic to cats (subjects 736 and 951) are shown in Fig. 2. The T-cell line from subject 736 (Fig. 2, *A*) showed a vigorous proliferative response to native Fel d 1 but no response to ragweed pollen extract as a specificity control. This T-cell line responded to several of the Fel d 1 peptides, with major responses to the N-terminal and C-terminal portions of chain 1 and to the middle of chain 2. The majority of the "clustered" T-cell epitopes for subject 736 appear to be located in the N-terminal half of chain 1. In contrast, the T-cell line derived from subject 951 (Fig. 2, *B*) showed a slightly different profile of major responses. Assay of this T-cell line revealed major reactivity to peptides in the N-terminal portion of chain 2 but also showed a major response to a peptide of chain 1. Assay of several T-cell lines derived from the same individuals showed very similar patterns of reactivity to the overlapping set of Fel d 1 peptides, although the absolute magnitude of the proliferative responses varied from T-cell line to T-cell line. These results demonstrate that short-term polyclonal T-cell lines can be very useful in determining the major areas of reactivity

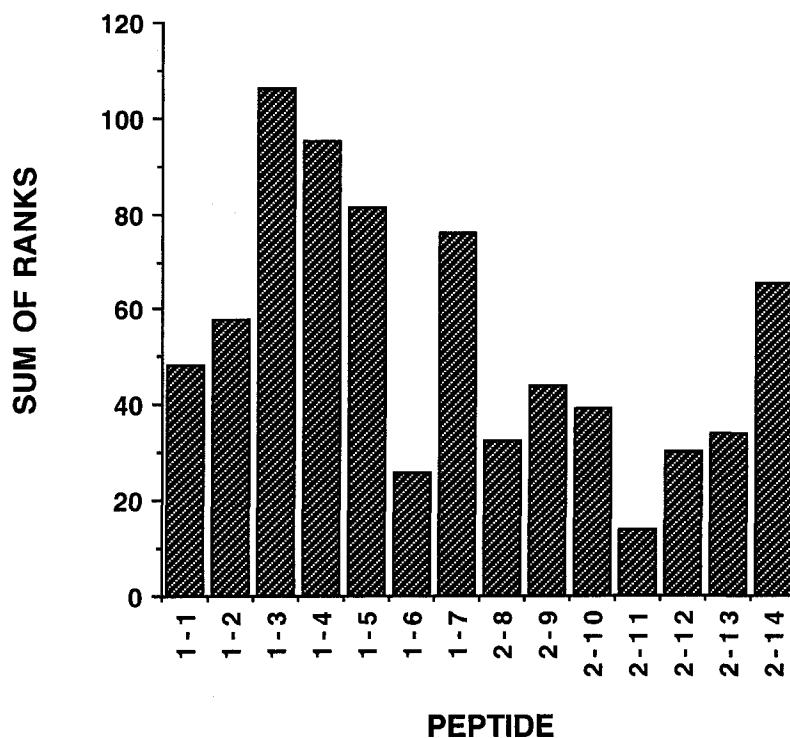


FIG. 3. T-cell reactivity profile of responses to Fel d 1 peptides by short-term polyclonal Fel d 1-reactive T-cell lines derived from 53 subjects allergic to cats. Three concentrations (3, 15, or 75 $\mu\text{g/ml}$) of synthetic Fel d 1 peptides were tested for ability to stimulate proliferation of 53 Fel d 1-reactive polyclonal T-cell lines. The highest SI for each peptide for each T-cell line was noted, regardless of dose. SIs for responses to each peptide were summed and provide an estimation of relative reactivity of regions of the Fel d 1 molecule in this population of subjects allergic to cats. The average medium control response was 717 cpm. As an example of positive responses ($\text{SI} \geq 2.0$), the average response to Fel 1-3 was 5058 cpm.

in the Fel d 1 molecule. The major advantages of such an approach, compared with primary proliferative responses to peptides or to a large panel of clones reactive with peptides, are that the native protein is processed naturally and polyclonal responses are generated for analysis without deriving and propagating large numbers of clones from many patients. Therefore the chances of preserving and identifying the major reactive regions for each patient are quite high. Furthermore, primary *in vitro* T-cell responses to Fel d 1 peptides are generally weak, presumably because of low precursor frequency (less than 1/40,000, unpublished observations).

With this approach, responses to Fel d 1 peptides in a panel of 53 subjects allergic to cats were examined. A summary of the results of these experiments is shown in Fig. 3. Because of the relatively large variation in the magnitudes of responses between subjects, the data were analyzed by assigning a rank (5 = highest, 1 = lowest) to the five highest responses obtained in each

subject. The ranks assigned to each peptide in the panel of 53 subjects were then summed. The results of this analysis (Fig. 3) indicate that several regions of the Fel d 1 molecule contain major T-cell epitopes in this population of subjects. Specifically, the N-terminal half of chain 1 appears to contain the majority of the epitopes in Fel d 1-primed polyclonal T-cell responses. Other areas of the molecule also contain epitopes of lower reactivity. The peptides encompassing residues 9-55 and 56-69 of chain 1 and residues 74-92 of chain 2 contain the major epitopes of the Fel d 1 molecule. The minor amino acid polymorphism in chain 2 did not affect the polyclonal T-cell responses to this region. T-cell lines activated with the purified native Fel d 1 used in this study, containing mostly the long form of chain 2, reacted similarly to the peptides 2-13 and 2-13.1 (data not shown).

Table I lists the five highest peptide responses obtained in T-cell proliferation assays of the polyclonal T-cell lines derived from 42 of the 53

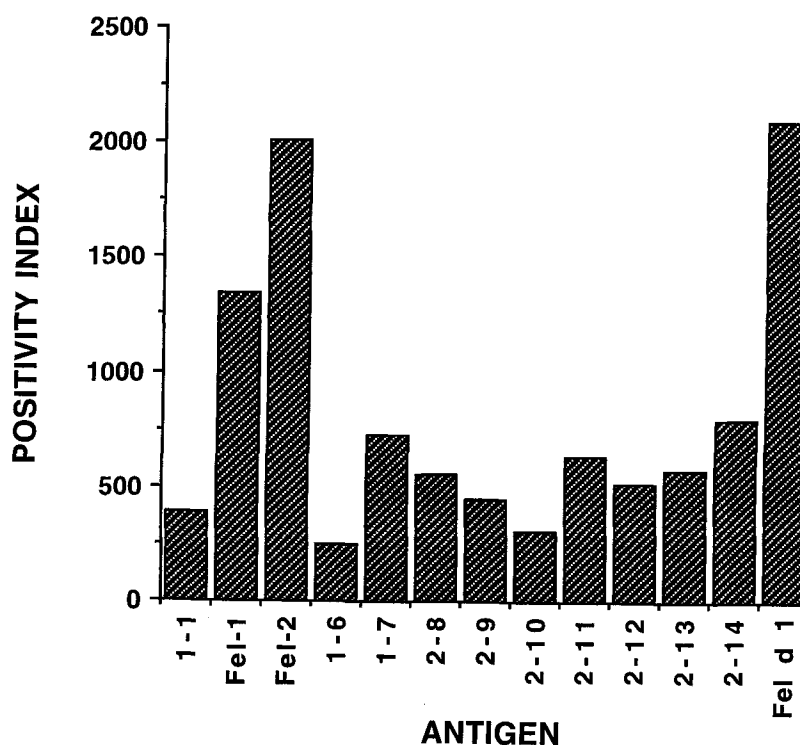


FIG. 4. A comparison of Fel d 1 protein, Fel d 1 peptide Fel-1, and Fel d 1 peptide Fel-2 in the activation of short-term polyclonal Fel d 1-reactive polyclonal T-cell lines. Fel d 1-reactive T-cell lines were established from 53 subjects allergic to cats, as described in Methods, and were tested for proliferation in response to three concentrations (3, 15, or 75 μ g/ml) of synthetic Fel d 1 peptides. The highest SI for each peptide for each T-cell line was noted, regardless of dose. Results are presented as PI for each antigen response, which is the product of mean SI and frequency of response of at least twofold over medium control.

individuals tested, along with their MHC class II types. No strong associations of peptide responses to MHC types are apparent from these data. As shown in Fig. 3, chain 1 peptides scored strongly positive in these assays more frequently than chain 2 peptides. Thus as in other systems, peptides are capable of binding to a wide spectrum of class II molecules and thus could be used to treat a diverse population of allergic subjects.

T-cell responses to composite peptides containing the major epitopes

Because allergen peptides of less than 30 amino acids generally bind little IgE,¹⁸ two areas of the Fel d 1 molecule with the highest density of T-cell epitopes (Fig. 3) in contiguous stretches of up to 27 amino acids were chosen for the design of peptides containing major T-cell epitopes. The two resulting peptides, Fel-1 (chain 1, amino acids 7-33) and Fel-2 (chain 1, amino acids 29-55) were synthesized and tested for reactivity with the panel of 53 T-cell lines. These particular peptides were designed to contain

an overlap in order to maximize the inclusion of potential T-cell epitopes in this major area of T-cell reactivity. The structures of these two peptides are shown in Fig. 1. Fig. 4 shows the result of the tabulation of the relative reactivities of Fel-1 and Fel-2 in comparison with other peptides covering the complete sequence of both chains and in comparison with native Fel d 1. The data are expressed as a PI, which is a measure of the frequency of a positive response, as well as the magnitude of the positive responses. In this way the responses to each peptide can be compared with the responses to Fel d 1 protein. The responses to peptides were also analyzed by the method of the sum of ranks, which resulted in a similar profile (data not shown). The results of this experiment demonstrate that these two peptides are very potent stimulators of Fel d 1-reactive T cells. Similar assays, including one with a mixture of Fel-1 and Fel-2, were performed on 10 of the T-cell lines, and the results are presented in Table II as mean SIs of the highest response at any of the three doses tested. The results indicate that the

mixture of these two peptides is a very potent stimulator of Fel d 1-reactive T cells. Furthermore, 98% of the T-cell lines derived from the panel of 53 patients with cat allergy demonstrated positive responses to one or both of these peptides.

Fel-1 and Fel-2 do not bind detectable levels of Fel d 1-reactive IgE and demonstrate minimal histamine release from sensitized basophils

To determine whether the Fel-1 and Fel-2 peptides are capable of binding IgE, these two peptides were tested by ELISA and histamine release assay in comparison with Fel d 1. As shown in Fig. 5, *A*, direct binding of IgE in pooled plasma from 20 patients with cat allergy to the Fel-1 peptide or the Fel-2 peptide was not detectable. In control experiments anti-peptide antisera as positive controls confirmed that the peptide had coated the ELISA wells. Furthermore, IgE from individual patients demonstrated no detectable binding to either of the peptides or to peptides conjugated to human serum albumin (data not shown). Fig. 5, *B*, shows the results of histamine release assay of 10 individual subjects allergic to cats. No histamine release greater than 20% of the maximum was detected over a very wide range of peptide concentrations (0.5 to 2000 nmol/L). These results confirm and extend the finding that Fel-1 and Fel-2 bind minimal levels of Fel d 1-specific IgE and predict that these two peptides could be injected into human beings with minimal immediate hypersensitivity type reactions, compared with extracts containing native Fel d 1.

DISCUSSION

This study was undertaken to determine the important T-cell-reactive portions of Fel d 1. The strategy employed was to establish polyclonal T-cell lines reactive with Fel d 1 from a large panel of patients allergic to cats. Patient PBLs were activated in vitro with purified native Fel d 1 and expanded in the presence of IL-2 and IL-4 until sufficient numbers of cells were available for assay. These short-term T-cell lines were then tested in secondary proliferation assays for reactivity with a set of overlapping peptides derived from the sequence of the two chains of the Fel d 1 molecule. The intent was to be able to study a wide spectrum of T-cell reactivity without the need for generating a large number of clones from a large number of individuals. In addition, the responses obtained in secondary proliferation assays should more closely represent the complete potential T-cell responses

TABLE II. Secondary T-cell proliferation of Fel d 1-reactive T-cell lines in response to Fel-1, Fel-2, a mixture of Fel-1 and Fel-2 or Fel d 1

Subject No.	SI*			
	Fel-1	Fel-2	Fel-1 + Fel-2	Fel d 1
585	2.7	3.5	4.0	2.5
605	8.3	10.4	10.5	9.2
607	7.3	4.4	4.3	4.9
614	4.7	1.5	3.1	2.8
615	6.6	6.4	3.6	11.2
687	5.3	10.1	8.1	14.2
689	7.5	7.8	42.7	24.1
695	4.4	10.9	5.0	3.3
736	7.0	9.6	20.1	36.4
745	2.4	10.5	9.7	8.2
Mean $\pm$ SEM	5.4 $\pm$ 0.8	7.5 $\pm$ 1.1	11.1 $\pm$ 3.9	11.7 $\pm$ 3.5

*Fel d 1-reactive T-cell lines established from the subjects listed, as described in Methods, were tested for proliferation in the presence of native purified Fel d 1 or the Fel d 1 synthetic peptides Fel-1, Fel-2 or an equimolar mixture of Fel-1 + Fel-2 at 2, 10, or 50 μ g/ml. The highest SI, regardless of dose, is shown.

in vivo than cultures carried for long periods in vitro. Finally, data from primary proliferation assays to peptides were not included in this analysis because of potential cross-reactions on T cells not primed with Fel d 1 and because of the generally very weak primary in vitro responses of T cells from patients allergic to cats challenged with peptides. One round of in vitro stimulation for selection of T-cell lines was used to allow natural processing and presentation of Fel d 1 peptides by antigen-presenting cells, and at the same time provided a large number of relevant cells for assay.

With this approach, several regions of the Fel d 1 molecule containing major T-cell epitopes were identified in a panel of 53 subjects allergic to cats. By examining the relative magnitudes of the responses of individual T-cell lines to an overlapping set of Fel d 1 peptides, it was determined that, on average, T-cell lines from these subjects react most strongly to the N-terminal half of chain 1 of the Fel d 1 molecule. Two overlapping peptides were synthesized, which contain most if not all of the T-cell epitopes of amino acids 8-55 of chain 1 of Fel d 1. These two peptides, when tested on T-cell lines, demonstrated comparable T-cell reactivity when compared with the individual peptides from which they were derived. The epitope maps presented were derived from testing of the complete set of Fel d 1 peptides shown in Fig. 1 on T-cell

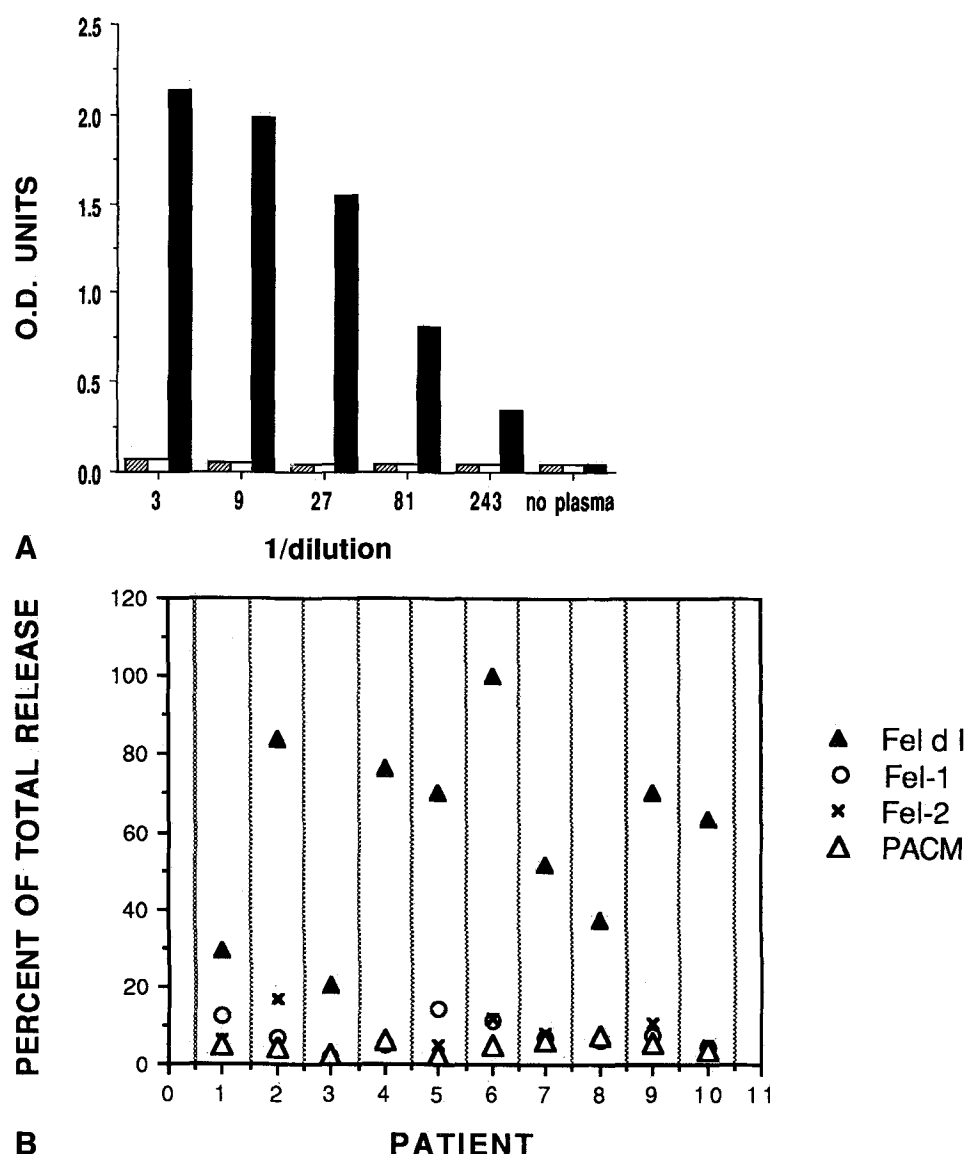


FIG. 5. IgE reactivity of Fel d 1 (solid bars), peptide Fel-1 (hatched bars), and peptide Fel-2 (open bars). **A**, Direct ELISA of specific IgE directed against Fel d 1, Fel-1, and Fel-2 in pooled plasma from 20 subjects allergic to cats. **B**, Release of histamine in response to Fel d 1, Fel-1, and Fel-2 from peripheral blood basophils of 10 individual subjects allergic to cats. These data represent the maximum release for each antigen over a range of 0.5 to 2000 nmol/L. O.D., Optical density.

lines derived from 53 patients allergic to cats. In more recent experiments, the Fel-1 and Fel-2 peptides have been tested on T-cell lines derived from over 300 subjects allergic to cats (data not shown), demonstrating strong T-cell reactivity to one or both peptides in 98% of the population. These later experiments did not include the complete overlapping set of peptides used to define the major regions of reactivity but were used to determine the frequency of response to only the Fel-1 and Fel-2 peptides in a very large population of individuals allergic to cats. Other investigators¹⁹

have recently published an analysis of the epitopes of the Fel d 1 molecule recognized by T-cell clones derived from a small number of subjects allergic to cats. Unlike data from our experiments, their clones recognized chain 2 peptides more frequently than chain 1 peptides. These epitopes were clearly recognized by T-cell lines derived from some individuals in our polyclonal T-cell assays but would be regarded as minor in the population, because they were generally recognized at a lower magnitude and lower frequency than chain 1 peptides. Because the Fel d 1 peptides are presented

to the responding CD4⁺ T cells in the context of MHC class II molecules, it was of interest to examine the MHC class II types of the subjects included in this study. It is very important to ensure that the epitope mapping is carried out on a diverse and representative sample of MHC class II types, so that these particular peptides would be predicted to contain T-cell epitopes in a diverse group of individuals. Forty-two of the panel of 53 subjects who were MHC typed by PCR showed a diversity of MHC class II types. No obvious associations between T-cell responses to certain peptides and MHC class II type can be seen from the data. Thus it appears that Fel d 1 peptides are capable of binding to a variety of MHC class II molecules, as would be predicted from recent studies demonstrating that MHC class II molecules bind peptides in a promiscuous fashion.²⁰ A prediction of these findings is that two peptides of the Fel d 1 molecule containing major T-cell epitopes would be effective treatment for a wide and diverse population of patients with cat allergy. In future studies, cloning of T cells reactive with certain of the peptides will be initiated to determine their restriction specificities and cytokine profiles. Such studies will provide a finer analysis of the T-cell responses to Fel d 1 peptides. In addition, precursor frequency analysis of T-cell response to Fel-1 and Fel-2 in patients with cat allergy is being carried out to support the proliferation data presented in this study.

Several cat immunotherapy studies demonstrating clinical efficacy have appeared in the literature.^{2, 15} The approach has generally been to administer escalating doses of a cat pelt extract containing Fel d 1 subcutaneously over the course of several weeks until a maintenance dose is achieved. The maintenance dose is usually defined as a maximum tolerated dose. After such treatment, symptom and drug use (i.e., antihistamines) scores have improved, and challenge of treated patients with the allergen has resulted in lower responses. The major disadvantage of this approach is that the allergens injected have the capacity to bind IgE on mast cells and basophils and initiate systemic anaphylaxis. In addition, this type of therapy involves weekly or biweekly injections over a long period, usually years. The mechanism of immunotherapy is not clear. It has been suggested by several groups that symptom improvement is due to the production of blocking IgG. However, several groups, including our own,²¹⁻²³ have demonstrated that T cells from treated patients respond less vigorously than T

cells from control patients in standard T-cell proliferation assays or in cytokine production assays in response to the allergen, suggesting that T-cell anergy and diversion of the T-cell immune response are also viable mechanisms to explain successful immunotherapy.

With the identification of the major T-cell epitope-containing regions of the Fel d 1 molecule, it is now possible to test a new type of immunotherapy, based on administration of synthetic peptides that bind minimal levels of Fel d 1-specific IgE, and thus should not cause the major side effects of conventional immunotherapy caused by immediate hypersensitivity. Other investigators¹⁸ have shown that a different set of synthetic peptides of the Fel d 1 molecule can bind significant amounts of Fel d 1-specific IgE, although with very low affinity. Such findings emphasize the importance of screening potential T-cell epitope-containing peptides for IgE binding before use in human beings. The Fel-1 and Fel-2 peptides defined in our studies have been shown to bind minimal levels of IgE in a number of assay systems. With these peptides, it will now be possible to determine in a more defined way the mechanism of action of successful immunotherapy. It has recently been shown that Fel d 1 peptides can tolerize T-cell responses in mice previously immunized with Fel d 1 protein.²⁴ The extension of this work in human immunotherapy clinical trials is currently underway and will provide data concerning the mechanism of action of this new type of allergy immunotherapy.

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