

Monoclonal antibodies to recombinant Der f 2 and development of a two-site ELISA sensitive to major Der f 2 isoallergen in Korea

Background: Der f 2 is a major sensitizing allergen in patients allergic to house dust mites worldwide. Isoforms of Der f 2 have been reported and are known to have different antigenicities. The aim of this study was to facilitate antigenic analysis and to develop an improved method for the detection of Der f 2 isoallergen, which is prevalent in Korea.

Methods: A two-site ELISA was developed with monoclonal antibodies (mAbs) which were produced against recombinant Der f 2 (rDer f 2) and applied to assess Der f 2 in bedding samples.

Results: A major isoform of Der f 2, found in Korea, was found to have amino acid variations especially at position 100 from lysine to glutamic acid, which is known to reduce significantly the binding affinity of mAbs when used to assess group 2 allergens. The detection limit of the developed two-site ELISA was determined to be about 8 ng/ml with rDer f 2 and 1 µg/ml with *Dermatophagoides farinae* crude extract. The average amount of Der f 2 in dust obtained from bedding samples from 89 homes in Seoul was estimated to be 25.61 ± 10.70 µg/g dust.

Conclusions: Assays using mAbs for rDer f 2 could be useful for the assessment of environmental allergen exposure and mAbs could be used to further characterize the isoallergens of Der f 2.

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Key words: Der f 2; *Dermatophagoides farinae*; ELISA; house dust mite.

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Accepted for publication 7 August 2001

House dust mites are the most important source of aeroallergens in people's homes (1). Antigens generated by house dust mites elicit allergic inflammation from individuals who are genetically predisposed to produce large amount of allergen specific IgE. Group 1 and 2 allergens are known to be the most significant causative agents of such sensitization (2, 3). mAb-based immunoassays have been used to measure, monitor, and assess allergen contamination in the indoor environment (4). Two-site ELISA is one of the most widely used methods to quantify group 1 and group 2 allergens in the environment, but the method for detection of group 2 allergens in Korea requires improvement (unpublished data). *Dermatophagoides farinae* was shown to be a predominant species of house dust mite in Korea (5), and polymorphism of this group 2 allergen has been suggested to prevent precise quantification.

It is of great importance to be able to quantify the

allergen level accurately, as this is directly related to the development of the allergy. The present study describes the development of a mAb-based ELISA to measure Der f 2 allergen levels in Korea.

Material and methods

Production of rDer f 2

Messenger RNA was extracted from cultured *D. farinae*, which were obtained from a Korean domestic environment. RT-PCR was performed to isolate the Der f 2 cDNA with the primers designed by Trudinger et al. (6). Amplified cDNA was ligated with Topo-T-vector and subcloned into pQE30 expression vector (QIAGEN, Valencia, CA, USA). rDer f 2 overexpression was induced by adding 1 mM of isopropyl-D-thiogalactoside (Sigma, St. Louis, MO, USA) and purified by affinity column chromatography using Ni-nitrilotriacetic acid (NTA) agarose (QIAGEN) (7).

mAbs to rDer f 2

Mouse mAbs to rDer f 2 were produced by the fusion of myeloma cells Sp 2.0-Ag 14 and spleen cells from BALB/c mice immunized with rDer f 2 mixed with alum adjuvant three times at 4-week intervals. The hybridomas producing antibodies against rDer f 2 were screened by ELISA and cloned three times by limiting dilution. Positive clones were expanded and mAbs were purified by protein G affinity column chromatography (Pharmacia, Uppsala, Sweden) according to the manufacturer's manual.

Abbreviations. mAb: monoclonal antibody; rDer f 2: recombinant Der f 2; RT-PCR: reverse transcriptase-polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; PBS: phosphate buffered saline; PBST: phosphate buffered saline containing 0.05% Tween 20; BSA: bovine serum albumin.

Immunoblot analysis

Immunoblot analysis was performed according to the procedures described by Gillespie et al. (8). The nitrocellulose membranes were reacted with hybridoma culture fluid and then incubated with 1:1000 diluted goat alkaline phosphatase conjugated antimouse IgG (Sigma). Colour was developed with a substrate for alkaline phosphatase (66 µl of 50 mg/ml of 3-bromo-4-chloro-5-indolyl-phosphate and 33 µl of 50 mg/ml of nitro blue tetrazolium in 100 mM Tris-Cl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂).

Epitope analysis of mAbs

For epitope analysis, rDer f 2, which was composed of 129 amino acids, was dissected into three fragments with a small number of overlapping amino acids, A (residues 1–47), B (residues 44–94), and C (residues 85–129). Three cDNA fragments were amplified by PCR and ligated into the prokaryotic expression vector pGEX 4T (Pharmacia) to obtain peptide fragments. Each recombinant peptide was expressed in *E. coli* and purified with a glutathione-S-transferase (GST) affinity column. Immunoblotting and ELISA were performed with each GST-fusion peptide against mAbs.

Labelling of horseradish peroxidase to mAb

mAb 1F06 was labeled with horseradish peroxidase (HRP) according to the procedures described by Tijssen and Kurstak (9) with slight modification. Five mg of HRP dissolved in 1.2 ml of water was mixed with 0.3 ml of 0.1 M sodium periodate in 10 mM of sodium phosphate buffer, pH 7.0. The mixture was incubated at room temperature for 20 min and dialyzed overnight against 1 mM of sodium acetate buffer, pH 4.0. Then, 5 mg of anti-Der f 2 mAb (1F06) in 20 mM carbonate buffer, pH 9.5 was added and incubated for 2 h at room temperature. After adding 100 µl of sodium borohydride in water (4 mg/ml), the solution was allowed to stand for 2 h at 4°C, and then dialyzed against phosphate buffered saline (PBS) overnight. HRP conjugated mAb was titrated to determine the optimal dilution for ELISA.

House dust samples

Sampling visits were made between September and October 1999 over which time 89 dust samples were obtained by vacuuming the bedding with household electric vacuum cleaners. Samples of 5 mg were extracted overnight in 0.2 ml of PBS, pH 7.4 at room temperature with vigorous shaking and after centrifugation 0.1 ml of the supernatant was used for ELISA.

Measurement of Der f 2 by a two-site ELISA

Microtiter plates (Costar, Cambridge, MA, USA) were coated overnight at 4°C with 10 µg/ml of an anti-rDer f 2 mAb 2F38 in 0.1 M sodium bicarbonate buffer, pH 9.6. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and incubated with 3% skimmed milk in PBS for 1 h at 37°C. After washing three times with PBST, 100 µl of two-fold dilutions of mite extracts, or rDer f 2, or house dust extracts in PBS, were added to the wells and incubated at 37°C for 1 h. The plates were washed three times and incubated with 100 µl of HRP conjugated anti-rDer f 2 (1F06) diluted 1:1000 in PBST containing 1% bovine serum albumin (BSA) for 1 h at 37°C. The wells were developed with 0.05% orthophenylenediamine and 0.006% hydrogen peroxide in 0.1 M phosphate-citrate buffer, pH 5.0 after washing three times. The enzyme reaction was stopped with 50 µl of 2 M H₂SO₄ and the absorbance at 490 nm was measured with an ELISA reader (Dynatech MR 5000, Alexandria, CA, USA).

Results

Der f 2 isoforms in Korea

RT-PCR was performed to investigate the polymorphism of Der f 2 in Korea and seven different isoforms were found (manuscript in preparation). The isoform which has an amino acid variation at position 100 from lysine to glutamic acid was used in the following studies because it appeared in seven of 15 Der f 2 cDNA sequences. The deduced amino acid sequences of PCR-amplified cDNA showed some differences (three to five out of 129 amino acids) from the previously reported Der f 2 sequences (Fig. 1). This fact motivated us to generate a new mAb sensitive to isoallergens with an amino acid variation at position 100. rDer f 2 was purified by affinity column chromatography and used to immunize mice.

Specificities of mAbs

In order to analyze epitopes of the allergen and to develop a method for quantifying Der f 2, mAbs against rDer f 2 were produced. Highly *D. farinae*-specific mAbs (1F06, 1F56, 1F70) were obtained, although Der p 2 and Der f 2 were known to have high sequence homology, and three mAbs (2F12, 2F38, 2F53) were cross-reactive with the *Dermatophagoides pteronyssinus* and *D. farinae* extracts. All were found to be IgG1 using mouse mAb isotyping reagents (Sigma). As shown in Fig. 2, one clone (2F12) reacted with peptide A, two clones (2F38, 2F53) only with a full-length fragment, and three clones (1F06, 1F56, 1F70) to peptide C. Reactivities were confirmed by immunoblot analysis with crude mite extracts (Fig. 3). Two mAbs (2F38, capture antibody; and 1F06, detection antibody) were selected for the two-site ELISA.

Der f 2 allergen levels in bedding samples

A two-site ELISA for the quantification of Der f 2 was developed using two different anti-rDer f 2 mAbs (1F06, 2F38). This two-site ELISA can detect only Der f 2 because antibody detection is species-specific. The detection limit was determined to be approximately 8 ng/ml by doubling dilutions of rDer f 2 (Fig. 4). The dose-dependence curve of *D. farinae* extract was quantified to examine its sensitivity with respect to natural Der f 2 (Fig. 5), and the detection limit was determined to be approximately 1 µg/ml with mite crude extract. Test sensitivity was compared to the widely used assay (Indoor Biotechnologies Inc., Charlottesville, VA) with rDer f 2 (Fig. 6). Anti-Der p 2 mAbs 1D8 and 7A1 (Indoor biotechnologies), which have been widely used to detect group 2 allergens, were able to detect rDer f 2 only when its concentration was above 30 ng/ml. This developed ELISA was applied to assess Der f 2 in bedding samples from 89 homes in Seoul. The concentration of the allergen was estimated to be

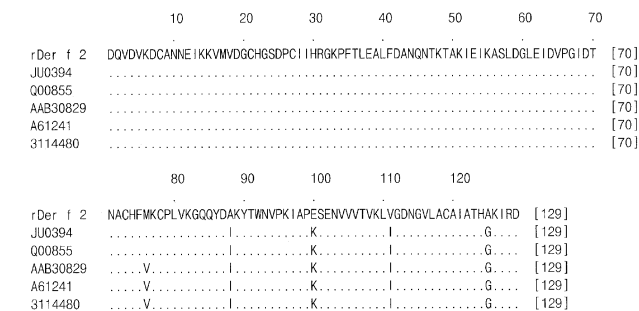


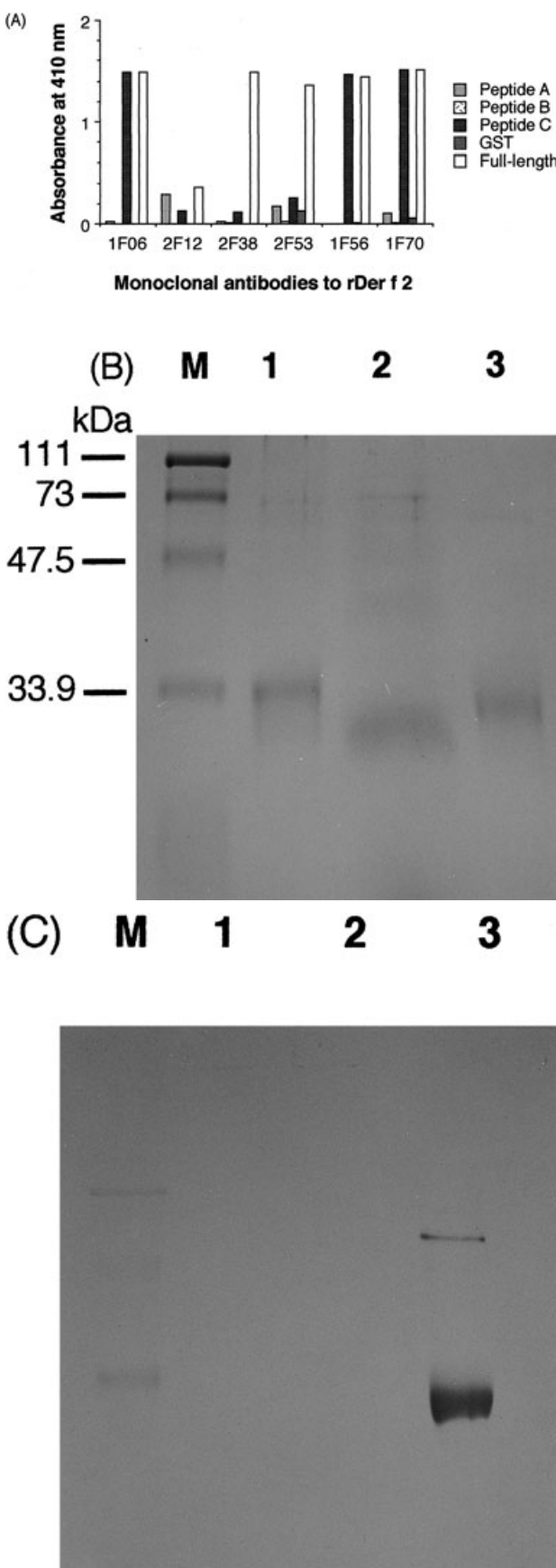
Figure 1. Amino acid sequence alignment of rDer f 2 and previously known Der f 2. Accession numbers of each sequence retrieved from the EMBL database are denoted on the left.

25.61 ± 10.70 µg/g dust using mAbs 1F06 and 2F38, whereas it was estimated to be 15.46 ± 47.37 µg/g dust using mAbs 1D8 and 7A1 (Fig. 7).

Discussion

The biological function of group 2 allergens is poorly understood, but the level of group 2 allergens in house dust mite has been reported to be as high as that of the group 1 allergens (10). mAb-Based immunoassays have been applied to monitor and assess group 2 allergen contents in the indoor environment (11, 12), and we have previously described the development of a two-site ELISA specific to Der p 2 (13) and localized the Der p 2 in the digestive tract and faecal pellets (14). Some sequence variations have been reported from groups 1, 2, 3, and 5 (15–18). In addition, variations were clearly shown among different areas and in different commercial extracts (19). The effect of amino acid variation in Der f 2 has also been examined (20). Major Der f 2 isoforms in Korea were found to have an amino acid variation of lysine to glutamic acid at position 100, while previous studies have shown that a substitution of lysine at position 100 significantly reduced the affinity of Der p 2 for mAb 7A1, which is widely used to assess group 2 allergens (21). The variation in antigenicity may be a reflection of the differences in the deduced amino acid sequence of PCR-amplified cDNA, and previously reported Der f 2 cDNA clones (Fig. 1). Isoallergenic rDer p 2 has been shown to endow different allergenicities and mAb IgG binding (Park et al., in submission). The variations in isoform content have important implications both for the standardization of extracts and the use of recombinant allergens (22). Therefore, the

Figure 2. Epitope analysis of monoclonal antibodies. GST-fused rDer f 2 was divided into three (1: 1–47 amino acids; 2: 44–94 amino acids; 3: 85–129 amino acids). The reactivity of the monoclonal antibodies to the three dissected fragments and full-length rDer f 2 was examined by ELISA (A). 2 µg of each peptide were electrophoresed onto 15% SDS-polyacrylamide gel (B) and immunoblotting was performed with monoclonal antibody 1F06 (C).



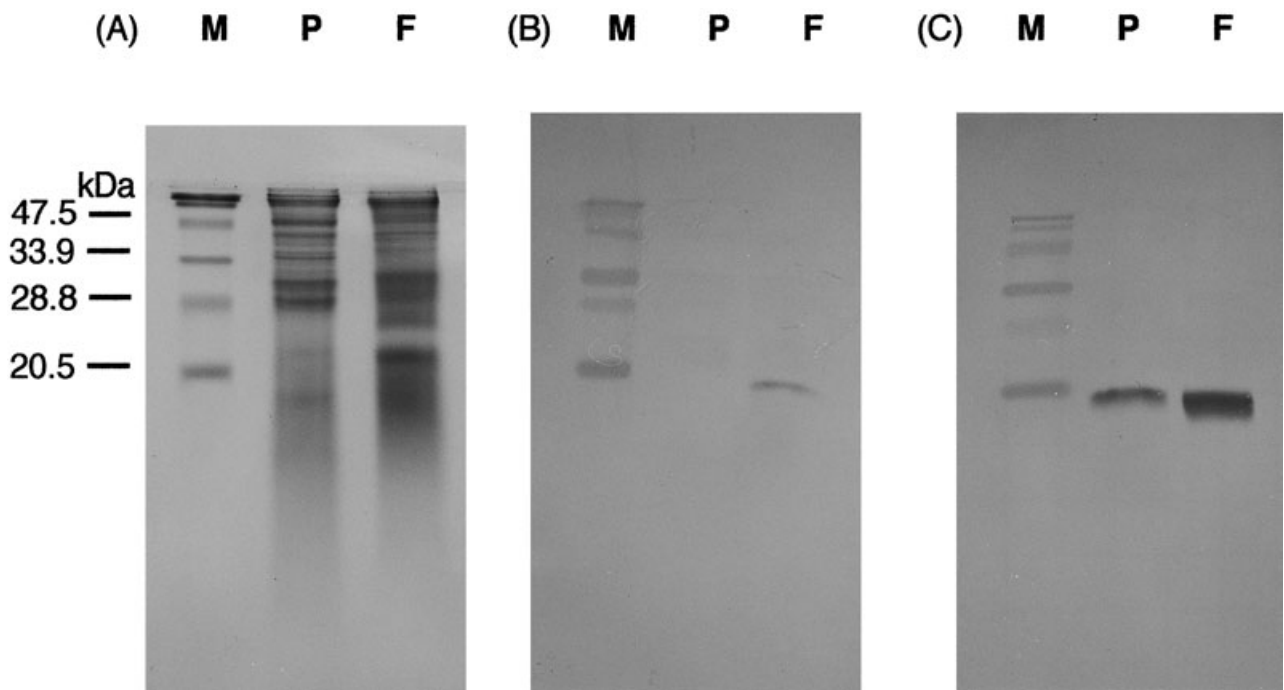


Figure 3. Immunoreactivities of monoclonal antibodies against native Der f 2. 30 μ g of mite crude extracts (*D. pteronyssinus*: lane P and *D. farinae*: lane F) were run onto 15% SDS-polyacrylamide gel (A) and immunoblotted with monoclonal antibodies ((B); 1F06 (C); 2F38).

binding ability of mouse mAbs to isoallergens must be considered. In this study, a two-site ELISA was developed using rDer f 2 to detect the predominant isoallergen in Korea, and its sensitivity proved to be comparable to the widely used assay previously reported (11, 12).

It has been proposed that exposure at levels of more than 2 μ g of Der p 1 per 1 g of dust is a risk factor for

sensitization in mite-allergic subjects, while the comparable level for non-atopic children was approximately 50 μ g/g dust (23, 24). Almost all bedding samples (86 of 89) collected in homes in Seoul were found to have levels of Der f 2 between 2 μ g/g and 50 μ g/g of dust, which is a proposed sensitization risk factor in patients allergic to mites.

A comparison of ELISA sensitivity to rDer f 2

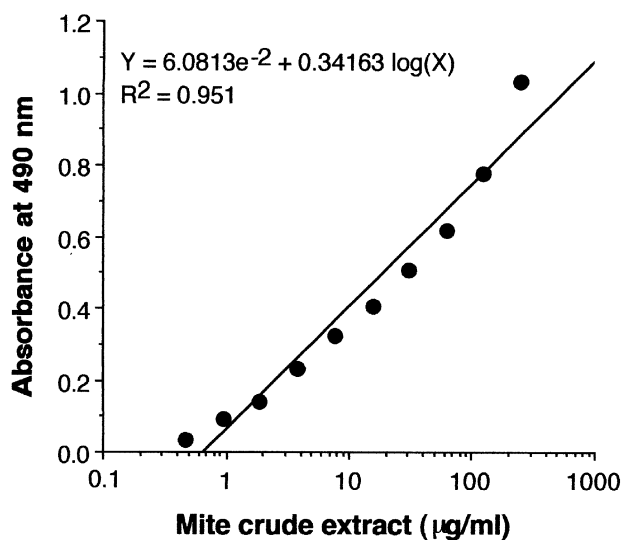


Figure 4. Dose-dependent curve of the rDer f 2 by a two-site ELISA. Two-site ELISA was quantified with doubling dilutions of rDer f 2 from 250 ng/ml. The detection limit was determined to be 8 ng/ml.

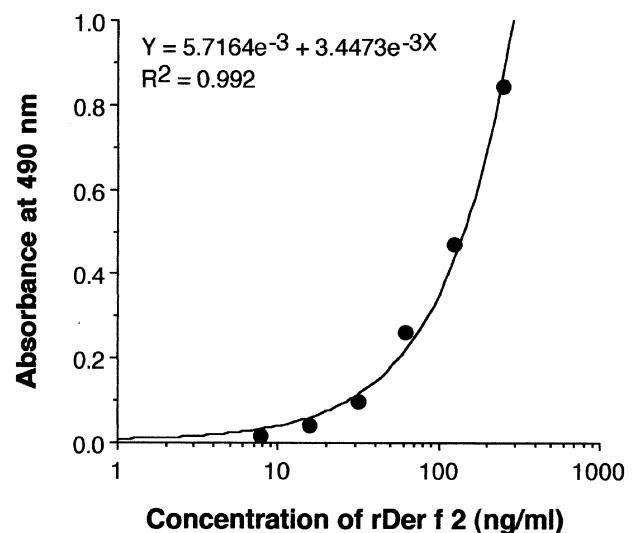


Figure 5. Dose-dependent curve of the *D. farinae* extract by a two-site ELISA. Two-site ELISA was quantified with doubling dilutions of the mite extract from 500 μ g/ml. The detection limit was determined to be 1 μ g/ml.

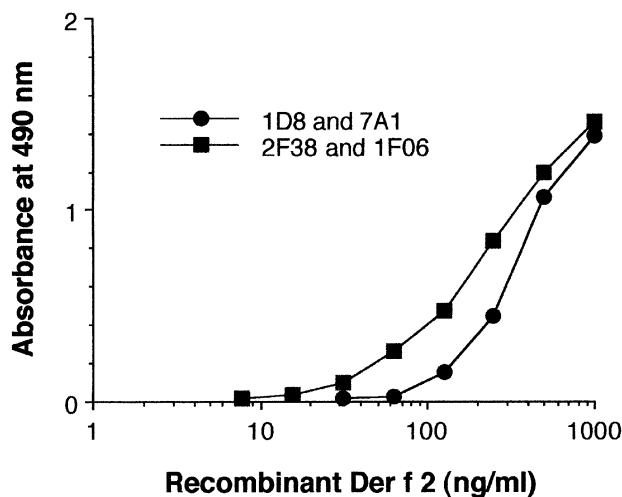


Figure 6. Comparison of sensitivity to rDer f 2 of the previously used monoclonal antibodies (1D8 and 7A1, Indoor biotechnologies) and the newly produced monoclonal antibodies (2F38 and 1F06).

allowed us to conclude that the newly developed two-site ELISA is more sensitive at least for the detection of Der f 2, the major isoallergen in Korea. The assay using mAbs against rDer f 2 could be useful for the assessment of environmental allergen exposure, and mAbs will be used for the further characterization of Der f 2.

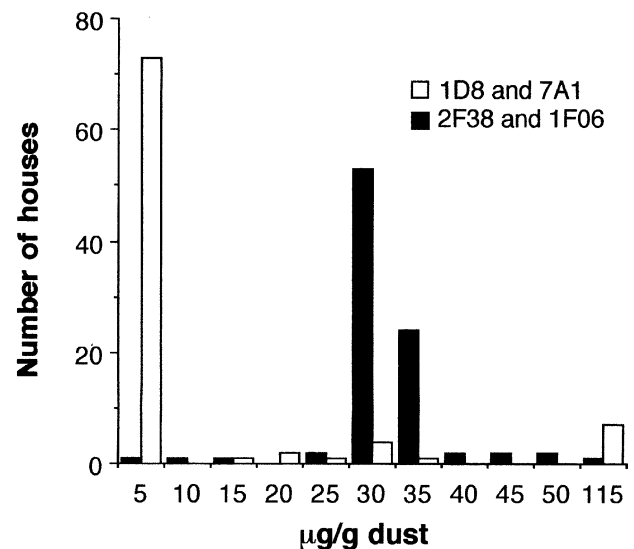


Figure 7. Measurement of Der f 2 in dust obtained from bedding samples from 89 homes in Seoul. The average was estimated to be 25.6 ± 10.70 µg/g dust using mAbs 1F06 and 2F38, and 15.46 ± 47.37 µg/g dust using mAbs 1D8 and 7A1, respectively.

Acknowledgments

Special thanks should be given to Miss Sang-Mi Lee for her skilful technical assistance and Mr Jongweon Lee for his help in preparation of the figures and manuscript. This study was supported by a research grant for the development of Health and Medical Technology (HMP-99-M-09-0006), Ministry of Health and Welfare, Korea.

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