

Quantitative measurement of IgE antibodies to purified allergens using streptavidin linked to a high-capacity solid phase

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Background: Commercially available assays for IgE antibody provide results in international units per milliliter for many allergen extracts, but this is not easily achieved with purified or novel allergens.

Objective: To develop assays for IgE antibody suitable for purified or novel allergens by using a commercially available immunosorbent.

Methods: Streptavidin coupled to a high-capacity immunosorbent (CAP) was used to bind biotinylated purified allergens from mite (Der p 1 and Der p 2), cat (Fel d 1), and dog (Can f 1). Assays for IgE antibody to these allergens were performed on sera from children (asthma and control) as well as adults with atopic dermatitis.

Results: The results were validated by serial dilution of sera with high and low levels of IgE antibody and were quantitated in international units per milliliter by using a standard curve. Values for IgE antibody to Der p 1, Der p 2, and Fel d 1 correlated with values obtained with the allergen extracts ($r^2 = 0.80, 0.84$, and 0.95 , respectively; $P < .001$ in each case). Furthermore, the values for IgE antibody in sera from children with high exposure to mite and cat allergens demonstrated 10-fold higher levels of IgE antibody to Der p 1 and Der p 2 than to Fel d 1 ($P < .001$).

Conclusion: The streptavidin immunosorbent technique provides a new method for quantifying IgE antibody to purified proteins. The results provide evidence about the high quantities of IgE antibody to purified inhalant allergens in patients with atopic dermatitis. In addition, the results demonstrate major differences in IgE antibodies specific for mite and cat allergens among children with high exposure to both allergens. (J Allergy Clin Immunol 2005;115:1029-35.)

Key words: IgE antibody, streptavidin, CAP, atopic dermatitis, purified allergens

Abbreviations used

AD: Atopic dermatitis
FU: Fluorescence units
MW: Molecular weight

In 1967, Ishizaka et al¹ developed an antibody against IgE and established that this new isotype was responsible for skin sensitizing or PK (Prausnitz-Küstner) activity. In the same year, Johansson and Bennich in Sweden identified a myeloma serum with a very high concentration of IgE, ie, ~ 20 mg/mL.² This made it possible to define the structure of IgE and to develop the RAST, which has been used extensively to measure IgE antibody to both unpurified extracts and purified allergens.³⁻⁵ Other *in vitro* techniques to measure IgE include different solid phase techniques and radioimmunoprecipitation.⁶ From the earliest studies, it was clear that both total IgE and the levels of IgE antibody in serum were very low compared with other isotypes of immunoglobulin. Over the years, the RAST technique has been modified extensively, including changes in the solid phase, quantitation in absolute units, the use of monoclonal anti-IgE, and the introduction of fluorescent rather than radioactive detection techniques.^{7,8} In 1992, a high-capacity absorbent solid phase was introduced that had an automated assay system; this technique is referred to as a *fluorometric enzyme immunoassay* or CAP.⁹ Although it is possible to bind purified allergens to the immunosorbent, this must be performed by the company.^{10,11} On the other hand, it is possible to bind streptavidin to the solid phase, and this provides a matrix that will bind biotinylated antigens.^{12,13} We report here the use of the streptavidin immunosorbent to measure IgE antibody to purified major allergens from cat, dog, and dust mite. The results establish a new technique for measuring IgE antibodies to purified proteins. In addition, the results provide a new approach for directly comparing IgE antibody responses with allergens from different sources.

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Supported by National Institutes of Health grants AI-20565 and AI/EHS-P01-AI-50989.

Received for publication June 5, 2004; revised December 16, 2004; accepted for publication December 27, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.12.1131

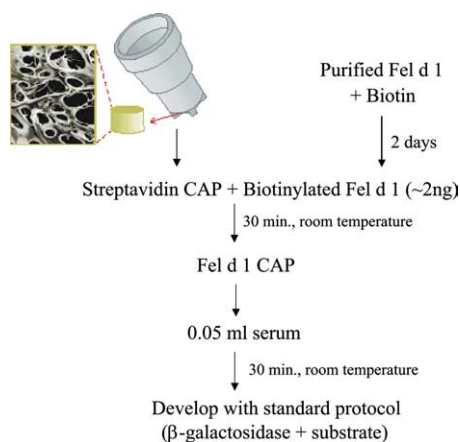


FIG 1. Measurement of IgE antibody to purified allergens using the streptavidin CAP. The standard assay uses galactosidase-labeled rabbit anti-IgE followed by 4-methylumbelliferyl-β-D-galactoside and measurement of the fluorescent signal. This technique used in parallel with the Pharmacia standard curve gives results in international units per milliliter.

METHODS

Purified proteins

Fel d 1, Der p 1, and Der p 2 were purified from extracts by using mAbs. Can f 1 was kindly provided by Indoor Biotechnologies (Charlottesville, Va).^{14,15} The concentration of purified allergen in micrograms per milliliter was determined by ELISA.

Biotinylation of antigen

Biotinylation was performed by using Sulfo-succinimidyl-6-(biotinamido) Hexanoate (Enzotag; Enzo Biochemical Inc, New York, NY) and a modification of the standard protocol (Pierce Endogen, Rockford, Ill).^{16,17} Typically, 5 μg Enzotag in 0.12 mL sterile water was added to 40 μg protein in 1 mL PBS. Assuming a molecular weight (MW) for the allergens of 30,000 d, this represents a molar ratio of 10 molecules Enzotag for each protein molecule. The tubes were covered with foil to prevent light exposure and incubated at room temperature for 4 hours. The mixture was then dialyzed to remove free biotin and Enzotag at 4°C for 2 days against PBS, with 1 change after 24 hours.

Sera

Sera were obtained from the Asthma and Allergic Diseases Clinic and from children age 10 to 11 years enrolled in an International Study of Asthma and Allergies in Childhood-based study in New Zealand.^{18,19} The sera from the clinic included sera from 9 patients with severe atopic dermatitis (AD). Severity scores were >20 using the SCORAD index, a composite of objective (extent and intensity of lesions) and subjective (daytime pruritus and sleep loss) findings. These sera had been assayed for IgE antibody to mite, cat, and dog by using CAP FEIA (Pharmacia, Uppsala, Sweden) reagents d1, e1, and e2, respectively. The sera from New Zealand were chosen because the children were known to have had very high exposure to both mite and cat allergens. Previous results showed that the titers of IgE antibody to mite in these sera (geometric mean for positives, 21 IU/mL) was much higher than for cat (1.7 IU/mL).¹⁹ These experiments were approved by the University of Virginia Human Investigation Committee.

Assay for IgE antibody using streptavidin immunosorbent

Biotinylated allergens (1 μg/mL) were diluted in sample diluent at 1:4, 1:8, 1:16, and 1:32. Fifty microliters of each dilution was then

applied to streptavidin CAP (kindly provided by Staffan Ahlstedt and Michael Land, Pharmacia Diagnostics, Kalamazoo, Mich). Five positive and 5 negative sera were tested at each allergen dilution. The results were optimal at 1:16 to 1:32, and a dilution factor of 1:25 (ie, 2 ng antigen/CAP) was used for all subsequent assays (Fig 1). Serum samples are routinely assayed undiluted, and the standard curve gives values as high as 100 IU/mL. Our preliminary data showed that values greater than 80 IU/mL were less consistent, and therefore, all sera with values >80 IU/mL were reassayed at dilutions of 1:5 and 1:20. One international unit is equivalent to 2.4 ng IgE; therefore, 100 IU/mL represents 240 ng IgE antibody/mL. Because the assays were performed with 0.05 mL serum, this represents 12 ng IgE antibody added to the solid phase. Given the known MW of IgE (ie, 180 kd) and assuming a MW of ~30 kd for the allergens, 12 ng IgE antibody is the molar equivalent of 2 ng allergen.

Biotinylated allergens were used within 1 month, and the specific immunosorbents were made on the day of the assay. The assay uses rabbit anti-IgE labeled with β-galactosidase and 0.01% 4-methylumbelliferyl-β-D-galactoside. Fluorescence was detected with a Fluorescent-96 fluorometer (Pharmacia) with results expressed as fluorescence units (FU). The assay is routinely run in parallel with a standard curve that allows conversion from FU to IgE antibody in international units per milliliter. The background for these assays using sera from nonallergic individuals was ~400 FU, which is equivalent to <0.35 IU/mL. With each of the purified allergens, the background was equal to or below the background for the standard, ie, ~400 FU (<0.35 IU/mL). (For detailed protocol information, contact lmn2q@virginia.edu or cae9m@virginia.edu.)

Statistics

The prevalence of different titers of IgE antibody to different allergens was compared by using the Mann-Whitney *U* test. Correlation between IgE antibodies to purified versus unpurified allergens was assessed by using the Pearson rank correlation.

RESULTS

To measure IgE antibody to purified allergens, streptavidin-linked immunosorbents were prepared with biotinylated Der p 1, Der p 2, Fel d 1, or Can f 1 by using ~2 ng of allergen for each assay. Serial dilutions of sera with high titer IgE antibody to cat or *Dermatophagoides pteronyssinus* were assayed by using Der p 1, Der p 2, and Fel d 1 immunosorbents (Fig 2, A). The results show that the dilution curves were parallel to the control curve. Because many sera have low levels of IgE antibody, we also performed serial dilutions on sera with lower levels of IgE antibody (Fig 2, B). The results show that these curves titrate out comparably with the control curve. The parallel dilution curves seen in Fig 2 support our use of the streptavidin CAP technique to derive units for IgE antibody from the standard curve at both high and low levels of IgE antibody. This standard curve uses a serum pool with IgE antibody calibrated relative to the World Health Organization International #75/502 standard. To evaluate further the streptavidin-based assay, we compared the levels for IgE antibody to Fel d 1 in 17 sera with those obtained by using a radioimmunoprecipitation assay with ¹²⁵I-labeled Fel d 1.⁶ The results showed a close

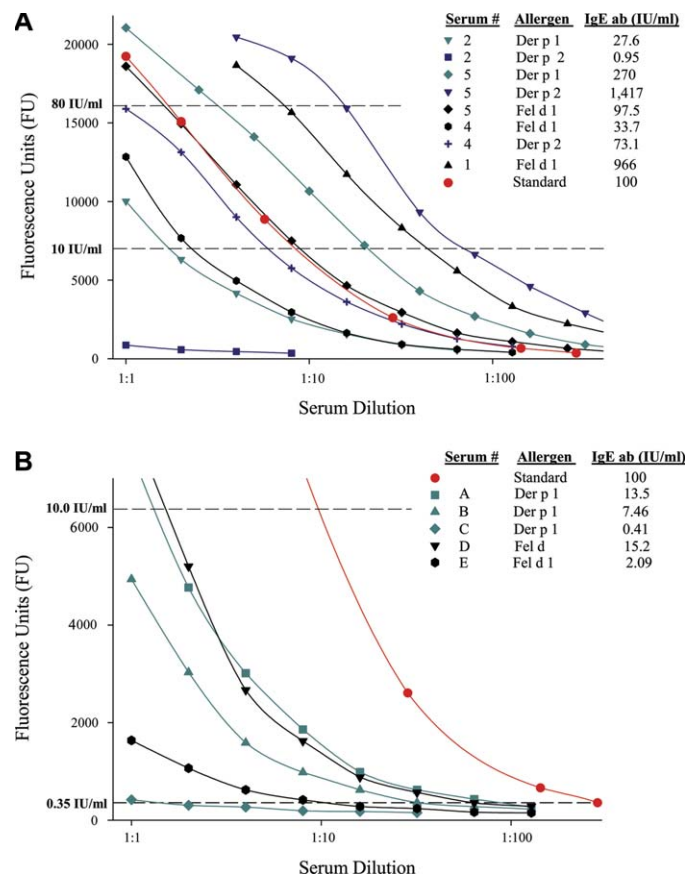


FIG 2. A, FU observed with serial dilutions of sera from 4 patients with high titer IgE antibody (Table II). The standard curve (red circles) is also shown; values for IgE antibody in international units per milliliter were calculated from the standard curve. B, Serial dilutions of 5 sera from patients with levels of specific IgE antibody less than 20 IU/mL. The standard curve is also shown (red circles). Values for IgE antibody in international units per milliliter were calculated from the standard curve by using the values in FU from the undiluted sera.

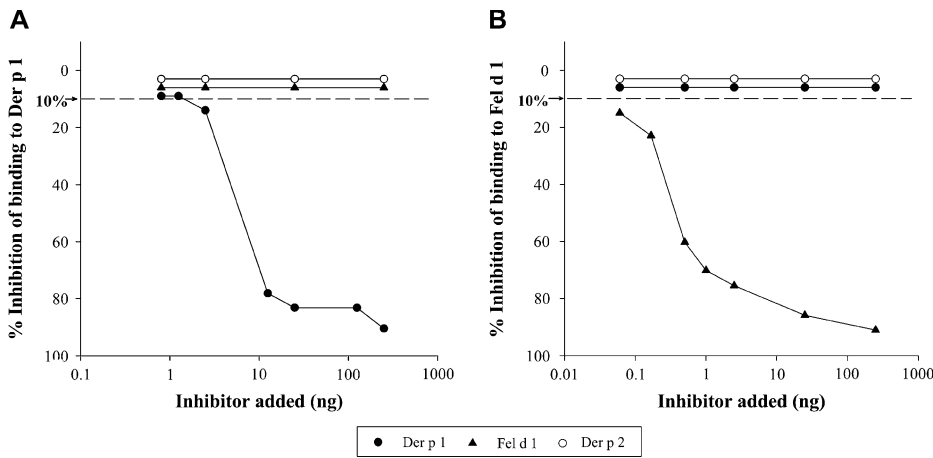


FIG 3. Sera were incubated with several serial dilutions of purified allergen overnight and then added to purified allergen CAP. Results are shown for Der p 1 CAP (A) and Fel d 1 CAP (B). Results for Der p 2 also showed specific inhibition (data not shown).

quantitative correlation between the 2 assays ($r=0.96$; $P<.01$).

Inhibition experiments were performed by incubating purified allergens with sera before adding them to Fel d 1, Der p 1, or Der p 2 immunosorbents. The results for Der p 1 (Fig 3, A) and Fel d 1 (Fig 3, B) show greater than 85% inhibition of specific binding using 10 ng specific inhibitor, ie, Der p 1 inhibiting

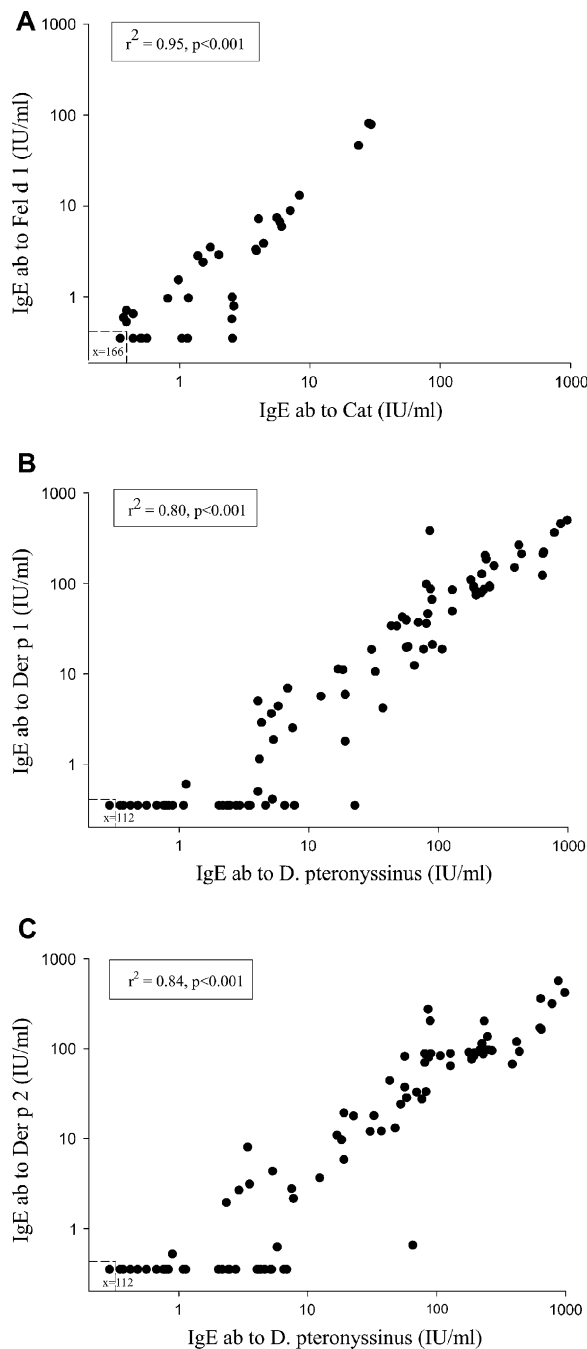


FIG 4. Correlation of IgE antibody (IU/mL) between biotinylated/purified allergen linked to streptavidin CAP and the corresponding assay for the allergen extract in sera from 199 children in New Zealand. **A**, Correlation between Fel d 1 and cat (e1; $r^2 = 0.95$; $P < .001$). **B**, Correlation between Der p 1 and *D pteronyssinus* (d1; $r^2 = 0.80$; $P < .001$). **C**, Correlation between Der p 2 and *D pteronyssinus* (d1; $r^2 = 0.84$; $P < .001$).

binding to Der p 1. By contrast, the other allergens produced $<10\%$ inhibition with 200 ng of inhibitor (Fig 3). Similar specific data were obtained with Der p 2.

IgE antibody to purified allergens in sera from wheezing and nonwheezing children (n = 199)

Sera from children in New Zealand were assayed for IgE antibody to mite and cat allergens. The results for Fel d 1 on 199 sera, including 33 sera positive for cat, showed a strong correlation between IgE antibody to Fel d 1 and IgE antibody to cat ($r^2 = 0.95$; $P < .001$; Fig 4, A). Only a small proportion of the sera assayed had ≥ 10 IU/mL IgE antibody to either cat allergen or Fel d 1 (Table I). For IgE antibody to dust mite, the correlation with IgE antibody results for unpurified allergen was highly significant for both Der p 1 ($r^2 = 0.80$; $P < .001$) and Der p 2 ($r^2 = 0.84$; $P < .001$; Fig 4, B and C). The results demonstrate high titer IgE antibody to Der p 1 and Der p 2, with many sera having IgE antibody ≥ 10 IU/mL to the purified allergens (Table I). The prevalence of IgE antibody ≥ 10 IU/mL to each of the mite allergens was greater than the prevalence of equivalent IgE antibody to Fel d 1 ($P < .001$ in each case). Only 29 of the sera had IgE antibody to both mite and cat allergens. In these sera, the geometric mean of IgE antibody to the purified allergens was 42.3 IU/mL for Der p 1, 37.3 IU/mL for Der p 2, and 2.01 IU/mL for Fel d 1. The quantities of IgE antibody to Fel d 1 in these sera were significantly lower than IgE antibody to Der p 1 or Der p 2 ($P < .001$ for each).

IgE antibody to purified allergens in sera from patients with AD

With the use of RAST, it has been clear for many years that most patients with severe AD have high titers of both total and specific IgE. We have previously reported very high titers of IgE antibody to dust mite and to Der p 1.^{20,21} With the use of the streptavidin technique, it was clear that IgE antibody to the cat allergen Fel d 1 was also very high in some patients with AD (Table II). The results for Fel d 1—for example, 966 IU/mL (patient 1) or 490 IU/mL (patient 3)—are ~ 10 -fold to 100-fold higher than values of IgE antibody to Fel d 1 seen in children from New Zealand (Table I). For dust mite, although the values for IgE antibody to Der p 1 and Der p 2 in some AD sera were also very high, ie, 300 to 1400 IU IgE antibody/mL (Table II; Fig 2, A), there were children with asthma in New Zealand with values that were only 2-fold to 3-fold lower, ie, 200 to 400 IU IgE antibody/mL (Table I; Fig 4, B and C).

DISCUSSION

The technique described here provides a new method for quantitative measurements of IgE antibody against purified allergens, partially purified allergens, or novel allergens. The technique has sensitivity comparable with that of the CAP assay using unpurified allergens, and the specificity is supported by the correlation with IgE antibody to the unpurified allergens and the inhibition experiments. Examples of the possible uses of the technique include the following:

- (1) Investigation of IgE antibody to a newly identified allergen source or a therapeutic mAb

TABLE I. Quantities of IgE antibodies to purified mite and cat allergens in sera from 199 children†

IgE Ab IU/mL	Mite (d1)	Der p 1	Der p 2	Cat (e1)	Fel d 1
>50	40** (35)‡	28** (25)	33** (30)	0 (—)	2 (2)
≥10-50	11* (4)	18* (12)	14* (8)	3 (3)	2 (2)
≥2-10	23* (15)	9, NS (8)	9, NS (6)	13 (11)	12 (9)
≥0.35-2	13, NS (7)	6, NS (2)	4, NS (3)	17 (14)	10 (9)
<0.35	112 (37)	138 (51)	139 (51)	166 (70)	173 (76)

Significant increase in prevalence of sera with IgE antibody to mite, Der p 1, or Der p 2 relative to cat or Fel d 1 is indicated: ** $P < .001$; * $P < .05$; NS, not significant. The mean level of Der p 1 was 23.1 μ g allergen/g dust. Fifty percent of the children had a cat, and the mean concentration of cat allergen in these houses was 40.8 μ g Fel d 1/g dust. The mean concentration was 3.3 μ g Fel d 1/g dust in houses without a cat. The quantity of IgE antibody to Fel d 1 was not influenced by the presence of an animal.

†Allergen levels in dust samples from the homes of the children have been reported elsewhere.¹⁹

‡The parenthetical values represent the number of wheezing children in each group.

TABLE II. IgE antibodies (IU/mL) to purified inhalant allergens in sera from 9 patients with severe AD

Serum # (age, sex)	Total serum IgE	Mite, d1 CAP*	Der p 1, biotin	Der p 2, biotin	Cat, e1 CAP*	Fel d 1, biotin	Dog, e2 CAP*	Can f 1, biotin
1 (M, 41)†	21,835	445	345	549	810	966	35.8	20.7
2 (F, 48)†	6411	15.4	27.6	0.95	211	90.3	222	233
3 (F, 40)	17,413	1.67	0.57	0.44	1120	490	99.6	193
4 (F, 41)†§	2793	87.1	33.7	73.1	32.1	33.7	9.71	26.2
5 (F, 30)†	11,880	2673	270	1417	65.8	97.5	579	1353
6 (F, 56)‡	3733	0.5	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35
7 (F, 50)§	187	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35
8 (F, 51)	8931	6.48	0.64	<0.35	29.7	70.5	26.9	39.7
9 (M, 43)	6361	859	128	165	2.17	2.78	7.84	71.9

*Sera assayed against Pharmacia CAP d1 (catalog #14-4107-01), e1 (14-4109-01), and e2 (14-4121-01).

†Sera used for serial dilution studies (Fig 2, A).

‡Patient #6 has 60 IU/mL IgE antibody to egg (f1).

§Patient #7 had persistent *Staphylococcus aureus* infection of the skin but no defined inhalant or food allergies.

- (2) Analysis of IgE antibody to fractions from an allergen extract during purification
- (3) Investigation of the IgE antibody response to multiple purified or recombinant allergens to define their relative importance, such as multiple cockroach allergens
- (4) Investigation of the quantitative relationship among allergen specific IgE antibody, IgE antibody to the unpurified extract, and total IgE

The experiments reported here used purified allergens, but we have also had successful results with biotinylated cat extract (Greer Laboratories, Lenoir, NC) and with biotinylated extract made from defatted *Harmonia axyridis* (ladybugs) prepared in our laboratory.²² Biotinylation is not difficult, and the current technique is ideal for testing an allergen that is not established or is only available in small quantities.

The results for purified allergens are given here in the same international units as those used for IgE antibody to unpurified allergens (ie, the commercially available assay) and also for total serum IgE. This is dependent on several assumptions: first, that the dilution curves are parallel; second, that the allergen is not significantly altered by biotinylation; and third, that there is sufficient allergen on the matrix compared with the conventional CAP. We have shown that the dilution curves are parallel between the standard curve and titrated sera using purified allergen

both at high and low levels of IgE antibody. In addition, calculation of the quantity of IgE antibody compared with allergen on the matrix suggests that 2 ng should be sufficient to bind the IgE antibody in 50 μ L serum with as much as 100 IU IgE antibody/mL (see Methods). Quantifying IgE bound depends on the repertoire of IgE antibody present in the serum and the number of IgE epitopes on the molecule. Obviously we cannot specify how many epitopes are present on the molecules, but previous studies with Der p 1 and Fel d 1 using mAbs have demonstrated multiple epitopes.^{14,23} Our results and assay of allergens with mAbs confirm that the allergens used here were not significantly altered by biotinylation. This is in keeping with previous results on immunoglobulins but would need to be confirmed with other allergens.^{13,17}

The original identification of IgE was difficult because of the small quantities of this protein in serum.¹ In addition to being quantitatively lower than all other isotypes, total IgE is more variable and is profoundly influenced by allergic disease. The reasons this isotype of immunoglobulin is present in such small quantities are at least 3-fold: (1) IgE is produced only against a limited number of foreign antigens, (2) the quantity produced against an antigen is limited, and (3) the half-life of IgE in the circulation is less than 2 days.²⁴⁻²⁶ In keeping with this, in most patients, the quantities of specific IgE antibody used to diagnose allergic disease are very low. Even a class III result (ie, 3.5-17.5 IU/mL) is less than 50 ng IgE antibody/

mL. This is particularly obvious when considering the levels of specific IgE antibody to food allergens that have been shown to predict a positive food challenge.^{27,28} For some food allergens, such as peanut, egg, and milk, 2 IU/mL of IgE antibody is predictive, whereas for other allergens, the predictive values are between 4 IU/mL and 10 IU/mL. Although these measurements are highly repeatable as well as clinically useful, they would be on the lower or nonlinear phase of the standard curve (Fig 2, B).

The results reported here using AD sera show that the high titers of IgE antibody to mite, cat, and dog allergens correlate closely with specific IgE to Der p 1, Der p 2, Fel d 1, and Can f 1. In some sera, the IgE antibody to purified allergens was quantitatively greater than IgE antibody to the extracts (Table II). This was true for Can f 1 in several sera, and also for the combination of Der p 1 and Der p 2 in sera 1, 2, and 4. With unpurified extracts containing multiple allergens, it is possible that the quantity of individual allergens on the matrix is inadequate. This is more likely to occur with an allergen such as dust mite in which a large number of different proteins have been recognized as allergens (see www.allergen.org). Preliminary data in our laboratory show that IgE antibody to 5 purified cockroach allergens using streptavidin CAP correlates closely with IgE antibody to German cockroach using CAP (i6, catalog #14-4224-01; Satinover et al²⁹). Although some of the elevation of IgE in AD may be a result of nonspecific factors, it is clear from our current results that specific IgE antibody to these allergens can make a contribution to the total.^{30,31}

In the results on the children from New Zealand, many of the sera contained as much as 400 IU IgE antibody/mL to Der p 1 or Der p 2, which represents 1 µg IgE antibody/mL. Previous results on dust samples from the homes of those children revealed very high concentrations of both mite (mean, 23.1 µg Der p 1/g dust) and cat allergens.^{19,32} It is well recognized that the IgE antibody response to ragweed can represent a significant proportion of the total IgE,³³ but it is less well recognized for perennial allergens. The results show that the quantity of IgE antibody to the cat allergen, Fel d 1, is significantly lower than the titer of IgE antibody to Der p 1 and Der p 2. This is in keeping with previous findings that high exposure to cat allergens does not increase either the prevalence or the quantity of IgE antibody to cat allergens.^{19,34-36} The results here establish that this difference in titer reflects differences in IgE antibody to the major allergens. The technique described here will allow wider investigation of the quantitative relationship between IgE antibody to allergen extracts and the relevant purified allergens. However, the most important feature of the technique described here may be its flexibility, which makes it possible to make quantitative measurements of IgE antibody to a range of different proteins.

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