

Use of Thiocyanate Elution to Estimate Relative Avidity of Allergen Specific IgE Antibodies

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ABSTRACT

Although several methods for estimating avidity of antigen-antibody reactions are available, most are impractical for the study of human IgE antibodies because of a requirement for pure allergen and antibody in relatively large amounts. To determine the relative avidity of specific IgE antibodies for Dermatophagoides pteronyssinus allergens, seven concentrations of the chaotropic thiocyanate ion were used to disrupt epitope-antibody binding in a specific IgE immunoassay system, using sera from 16 allergic patients with marked skin test reactivity to a standardized D. pteronyssinus extract. Relative avidity, the molarity of thiocyanate required to produce a 50% decrement in binding, ranged from 0.29–3.1. Within assay coefficient of variation (CV) was 9.9% and between assay CV was 13%. D. pteronyssinus specific IgE levels ranged from 0.66–141 kUa/L, not correlating with relative avidity ($\rho = -0.12$). Thiocyanate elution appears to be a useful method for estimating relative avidity of specific IgE antibodies for the myriad epitopes of the allergenic proteins in an allergen extract. It could be used to study the immunochemistry of specific IgE assays; avidity maturation in allergen immunotherapy and in asymptomatic but sensitized patients; and preseasonal versus postseasonal changes in avidity within individuals. With a suitable solid phase, it could be modified to examine avidity at the epitope level. (Allergy and Asthma Proc 18:359–362, 1997)

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Antibody avidity is the sum of all attractive (electrostatic, hydrogen bonding, hydrophobic, Van der Waals) and repulsive (hydrophobic, ionic, other forces related to protein structure) forces holding an antigen-antibody complex together.¹ The term “affinity” is usually reserved to describe a specific monovalent interaction between a single pure epitope and a single pure antibody. Avidity, on the other hand, is a broader term that does not imply a monovalent interaction.

Several methods for measuring affinity or avidity have been described.² They include ammonium sulfate precipitation, equilibrium dialysis, hemolytic plaque inhibition, fluorescence quenching, and affinity electrophoresis. These require reasonably pure allergen and antibody, both in relatively large amounts.

The study of the affinity of IgE antibodies for allergen epitopes in humans has been difficult because of the limited amount of IgE in human sera. Ahlstedt and coworkers used ammonium sulfate precipitation to examine avidity of sera from birch pollen allergic individuals for birch allergens.^{2–4} Avidity of specific IgE antibodies was determined by performing ammonium sulfate precipitation before and after immunosorption of IgE and calculating the difference. The authors found no correlation between antibody avidity and patients’ symptoms. More recently, a method using saturation plots to estimate the affinity constant has been reported.⁵

The chaotropic thiocyanate ion disrupts antigen-antibody binding; proposed mechanisms are 1) reduction in the hydrophobicity of apolar groups causing dissociation in the aqueous phase; 2) decreasing the strength of ionic bonds by production of electrostatic shielding of charge groups; and 3) decreasing solubility of proteins by altering their folding.⁶ Low thiocyanate concentrations disrupt low avidity interactions, and disruption of high avidity binding requires a higher thiocyanate concentration.

In ELISA assays for specific IgG and IgA, use of increasing concentrations of thiocyanate to elute antibody from binding to a solid phase antigen has been described and used as a method for estimating relative avidity.⁷⁻⁹ This present study was designed to examine whether thiocyanate elution might be used to examine relative avidity of allergen specific IgE antibodies in humans, using a semi-automated assay system. Additionally, we report the relationship between estimated level of *Dermatophagoides pteronyssinus* specific IgE antibody and relative avidity.

MATERIALS AND METHODS

Patients were selected from the allergy clinic population using the following criteria: 1) either gender, any race; 2) age 18–50; 3) diagnosis of perennial allergic rhinitis by an allergist-immunologist; 4) positive epicutaneous skin test to a standardized *D. pteronyssinus* allergen extract (Hollister-Stier, Spokane, Wash.) with wheal diameter at least 5 mm greater than the negative control; 5) no prior allergen immunotherapy. All gave informed consent for phlebotomy, and the protocol was approved by the Human Assurance Committee.

Sera were collected and frozen at -70°C until analysis. Specific IgE level estimates were performed by direct fluorescence enzyme immunoassay (Pharmacia CAP System, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) using the *D. pteronyssinus* solid phase. In this assay, a $50\ \mu\text{L}$ aliquot of patient serum is incubated for 30 minutes with allergens covalently bound to a cellulose sponge solid phase. Following washing, the solid phase is incubated for 150 minutes with a β -galactosidase enzyme-labeled mixture of polyclonal and monoclonal anti-human IgE antibodies. After washing, the enzyme substrate (0.01% 4-methylumbelliferyl- β -D-galactoside) is added for a 10-minute incubation. The reaction is terminated with 4% sodium carbonate, and fluorescence is measured in a fluorometer. Results are expressed quantitatively by comparison to a total IgE reference curve calibrated to the World Health Organization IgE reference standard using cubic spline interpolation. All equipment, computer software, and reagents are commercially available and were purchased from the manufacturer. All incubations are performed at room temperature. A previous dilution experiment had demonstrated that a log-log plot of concentration versus response the *D. pteronyssinus* solid phase was linear from 0.35 kUa/L up to 50 kUa/L. Accordingly sera with levels greater than 50 kUa/L were diluted and reassayed in order to give a better estimate of specific IgE level. Measurements of allergen specific IgG were not performed. The thiocyanate elution procedure was an adaptation of the basic Pharmacia CAP System specific IgE immunoassay method. For each patient, the assay was set up in 24 replicates. Following the initial incubation of serum with the solid phase, the solid phases were washed 6 times, and $100\ \mu\text{L}$ of potassium thiocyanate (Sigma Chemical Co., St. Louis, Mo.) in concentrations 0M, 0.1M, 0.5M,

1M, 2M, 4M, 6M, and 8M in PBS, pH 7.4 were added to triplicate wells containing solid phase. After another 30 minute incubation and washing, the assay was completed by the usual method as described above. The molar concentration of thiocyanate required to produce a 50% decrement in fluorescence response (versus PBS alone) was calculated by back extrapolation from an elution curve plotted in a log-log coordinate system (Fig. 1).

Within assay variability was measured by performing thiocyanate elution on a single patient's serum five times in the same assay run. Between assay variability was measured by performing thiocyanate elution on a single patient's serum in five different runs on five separate days. The same lots of reagents and solid phases were used in all experiments. The relationship of estimated specific IgE level and relative avidity was examined by Spearman rank correlation.

To examine the effect of thiocyanate on the solid phase antigen conjugate, six replicates were pretreated with 8M thiocyanate and six replicates were treated with PBS, both for a 30-minute period. After the solid phases were washed, an arbitrarily selected single patient serum was added and the assay was finished by the standard protocol. Mean response of the thiocyanate treated solid phases was compared to that of the PBS treated solid phases by the Student *t* test.

RESULTS

Sixteen patients were studied. Estimated serum IgE antibodies to *D. pteronyssinus* ranged from 0.66 to 141 kUa/L (mean 22.7, median 12.3). Relative avidity ranged from 0.29 to 3.1 (mean 2.27, median 2.35). Within assay coefficient of variation (CV) was 9.9%, and the between assay CV was 13%.

Use of a buffer control in the thiocyanate elution procedure permitted evaluation of the effect of the extra washing

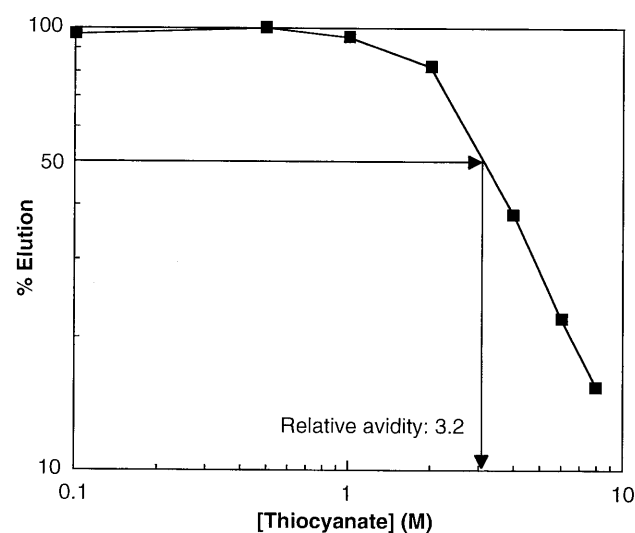


Figure 1. Example of thiocyanate elution plot.

step introduced in the procedure. Previously estimated specific IgE level correlated well with the specific IgE level from the buffer control in thiocyanate elution (Pearson $r = 0.98$, $p < 0.001$, $n = 15$). One patient was excluded from this comparison because his level of 141 kUa/L had previously been determined by dilution; the sample was not diluted for the thiocyanate elution procedure.

There was no correlation (Spearman $\rho = -0.12$) between estimated specific IgE level and relative avidity (Fig. 2). A single patient with an estimated *D. pteronyssinus* specific IgE level of 141 kUa/L produced a biphasic elution curve (Fig. 3).

Treatment of the solid phase with 8M thiocyanate produced a small decrement in binding relative to treatment

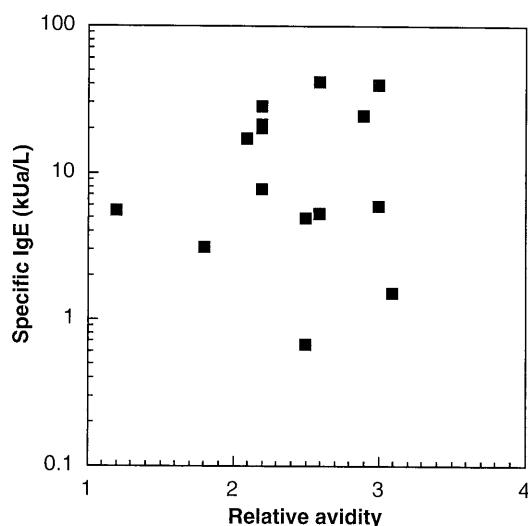


Figure 2. Lack of correlation of relative avidity with estimated specific IgE level ($\rho = -0.12$, $n = 16$).

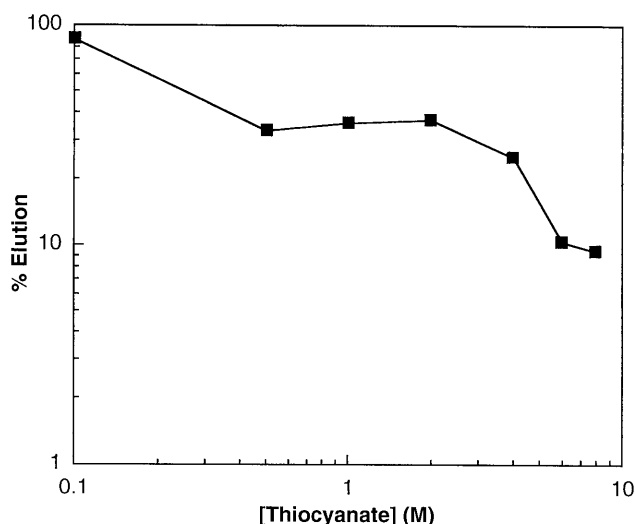


Figure 3. An atypical biphasic elution curve seen in one patient, suggesting the presence of at least two populations of specific IgE antibody.

with PBS (5801 versus 6757 FU). This difference was not statistically significant ($t = 1.627$, $df = 5$, $p = 0.17$).

DISCUSSION

Classic methods for examining antibody avidity are difficult to apply to the study of IgE antibodies in humans. Consequently almost nothing is known about the clinical importance of differences in antibody avidity. In the diagnostic allergy laboratory, it might be used to examine whether different skin test methods (epicutaneous and intracutaneous) favor detection of low avidity IgE antibodies, whether skin testing and immunoassays for specific IgE differ in ability to detect low avidity antibodies, and the effect of various first stage incubation times on performance of specific IgE immunoassays. In the latter situation, one would speculate that a prolonged incubation time would enhance detection of low avidity antibodies, since these take longer to reach equilibrium with the solid phase. Also, choice of solid phase media in specific IgE immunoassays might favor detection of high or low avidity antibodies; in an individual with both high and low avidity antibodies, a solid phase with a low total antibody binding capacity (such as a paper disk) may not capture efficiently the low avidity antibodies. However, clinical relevance of the finding of low avidity antibodies is unknown, and an immunoassay optimized to detect such antibodies might produce positive, but clinically irrelevant, results. Examination of the elution curve of one patient suggests that the high binding capacity of the cellulose sponge solid phase might have permitted capture of at least two populations of *D. pteronyssinus* specific IgE antibodies: a predominant population with very low avidity (0.29), and another with a much higher avidity (about 4.8).

In ELISA, thiocyanate may affect conformation of antibodies coupled to the solid phase.¹⁰ Attempted production of recombinant Der p 1 (the major *D. pteronyssinus* allergen) in bacteria has resulted in a product with limited allergenicity, whereas a product prepared in a yeast vector has greater allergenicity.¹¹ Presumably, protein folding (i.e., conformation) is important for Der p 1 allergenicity; if so, it is conceivable that thiocyanate treatment might have affected Der p 1 or other *D. pteronyssinus* allergens to some degree. In the present study, thiocyanate elution did not appear to have substantial effect on the protein allergens covalently coupled to the solid phase; thiocyanate in a concentration of 8M produced a small but not significant decrement in binding. Unfortunately, the exact specificity of this patient's *D. pteronyssinus* specific IgE antibodies is not known.

The first antibodies produced in a series of antigen injections are of low avidity; over time, antibody avidity increases, a phenomenon termed "avidity maturation."¹² In the course of allergen immunotherapy, it would be interesting to study avidity maturation of the specific IgG response, as well as changes in specific IgE avidity; in rabbits, induc-

tion of tolerance results in production of very low affinity antibodies.¹³

Additionally, the finding of specific IgE in asymptomatic individuals is well documented.¹⁴ Long term followup indicates that these persons are more likely to develop overt allergic disease than are nonsensitized individuals.¹⁵ The importance of avidity maturation of the specific IgE response in this phenomenon is unknown. Also, whether seasonal exposure to aeroallergens affects avidity of IgE antibodies within individuals has not been determined.

The immunoassay system used in this study reports an estimate of specific IgE level based on a calibration curve derived from assay of known amounts of total IgE (calibrated to the World Health Organization standard) captured by an anti-IgE solid phase. Thus, in the nonsaturating conditions used in this study, estimated specific IgE levels reflect actual mass unit levels unless high levels of allergen specific IgE are present. The finding of lack of correlation between specific IgE level and relative avidity was not unexpected, as independence of IgG antibody level and relative avidity has been observed in humans and mice.⁷

Thiocyanate elution, performed in a standard immunoassay for specific IgE, may be a useful means for studying relative avidity in humans. Thiocyanate elution can be performed with less than 2 ml serum. The observed within and between assay CVs are acceptable. The manufacturer reports within and between assay CVs for the basic method ranging from 6–10%; thus, the thiocyanate elution procedure introduces only minimal additional variability. This study did not examine errors that might be introduced by use of different reagent lots, particularly the allergen solid phase materials. Because different production lots of solid phase media might be expected to have compositional differences, the same lot should be used in comparison studies.

As described, thiocyanate elution determines overall avidity of specific IgE antibodies for the myriad epitopes of the allergenic proteins in an allergen extract. While use of this method with purified or recombinant allergen solid phases might provide a more focused evaluation of relative avidity in sensitized individuals, solid phase material pre-

pared with specific antibody epitopes could be used for study of affinity at the epitope level.

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