



NEW HORIZONS — ALLERGY —

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Diagnostic tests based on Streptavidin ImmunoCAP™: A versatile tool for detection of IgE antibodies to new allergens

Summary

Streptavidin ImmunoCAP™ is a new tool for coupling of biotinylated allergens, which can be used for detection of IgE antibodies (IgE ab) to new allergens. This article describes how to prepare allergens for binding to Streptavidin ImmunoCAP. IgE ab values, obtained with Streptavidin ImmunoCAP coupled with wheat and rye flour, were on average as high as the results with conventional ImmunoCAP of wheat (f4) and rye (f5) and higher than Enzyme-Allergo-Sorbent-Test (EAST™) results. Using Streptavidin ImmunoCAP, the advantages of the ImmunoCAP technology in comparison to EAST detection of IgE ab can now also be utilized with new allergens, or with allergens for which tests are not commercially available.

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Introduction

IgE antibody recognition of allergens is the central event in the pathogenesis of common allergy, causing immediate symptoms such as conjunctivitis, rhinitis, urticaria, asthma or anaphylaxis. Identification of the offending allergen(s) is therefore an essential part of the clinical diagnosis of IgE-mediated allergy, secondary prevention and immunotherapy. Beside *in vivo* diagnostics with skin tests and specific allergen challenge, the detection of allergen-specific IgE ab in serum plays a key role. However, in the case of allergens for which no commercial validated test system is available, researchers have to produce their own diagnostic tools, usually by coupling the allergen in question to a solid phase. Until recently, only the manufacturer could perform the coupling of allergens to ImmunoCAP™ (Phadia AB, Uppsala, Sweden). Therefore, measurements of IgE ab to new allergens were mostly performed with microtiter plates or cellulose discs as solid phase. Recently, Streptavidin ImmunoCAP was introduced as a tool for the researcher to measure IgE ab to new allergens of interest. It is the aim of this article to show how to use Streptavidin ImmunoCAP and to compare the results of IgE ab measurements with conventional ImmunoCAP and an alternative coupling method (EAST).

How to prepare and couple allergens to Streptavidin ImmunoCAP™

Protein extracts were biotinylated for 2 h on a rotator at room temperature with a 5-fold to 50-fold molar excess of D-biotinoyl-ε-aminocaproic acid N-hydroxysuccinimide ester (NHS-Biotin). Unbound biotin reagent was removed by gel filtration on PD-10 columns. Prewashed Streptavidin ImmunoCAP were loaded with 50 µL labeled protein in varying concentrations (from 80 µg/mL to 2.6 mg/mL), incubated for 30 minutes, washed and used for measurement of IgE ab in patient sera.

While the relation between biotin and protein was critical (best results were obtained with a 5-fold molar excess, e.g. wheat flour allergen in **Figure 1**), the amount of labeled protein added for binding to Streptavidin ImmunoCAP, within the range studied here, had little effect on IgE ab binding. With protein concentrations from 0.17 to 2.6 mg/mL, IgE ab values were stable and reproducible (CV below 10%). As a rule of thumb, we use the biotinylated protein solutions with an OD₂₈₀ value of 1.5 (≈1 mg protein/mL) for coupling.

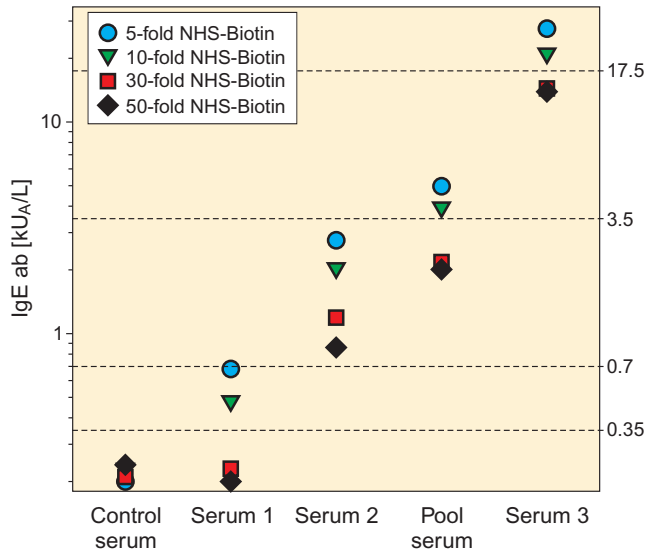


Figure 1. Influence of molar excess of biotin on IgE ab values. Wheat flour protein extracts were coupled with different molar excess of NHS-biotin and the same protein concentration to Streptavidin ImmunoCAP™. The results from four wheat sensitized patients' sera and a control serum show the highest IgE ab values with the lowest biotin excess.

Comparison between Streptavidin ImmunoCAP™ and conventional ImmunoCAP

Twenty-nine sera from patients with IgE ab to wheat flour and 30 sera from patients with IgE ab to rye flour were selected. Eighteen sera from patients without IgE ab to the respective allergens were used as negative controls. Eight control sera had a total IgE level below 100 kU/L, the others between 121 and 496 kU/L.

Wheat and rye flour extracts were biotinylated with a 5-fold molar excess, assuming an average molecular weight of 30 kDa for cereal proteins, bound to Streptavidin ImmunoCAP at a concentration of 1 mg/mL. The IgE ab values obtained with these allergen- Streptavidin ImmunoCAP and the patient sera are shown in comparison to the values of conventional ImmunoCAP (f4 and f5) in **Figure 2**. The values were closely correlated, r^2 wheat flour = 0.948 and r^2 rye flour = 0.932. None of the negative controls showed a reaction with the allergen- Streptavidin ImmunoCAP.

Comparison between Streptavidin ImmunoCAP™ and EAST™

The same wheat and rye flour extracts used for biotinylation and binding to Streptavidin ImmunoCAP were coupled at a concentration of 1 mg/mL to CNBr-activated cellulose discs [1]. The patient sera described above were analyzed for IgE ab with EAST (Allergopharma, Reinbek, Germany). The EAST-values were correlated to the Streptavidin ImmunoCAP values (r^2 wheat flour = 0.883; r^2 rye flour = 0.893), but they were in most cases lower (**Figure 3**) and in 16 cases (30%) the EAST values were below the detection limit.

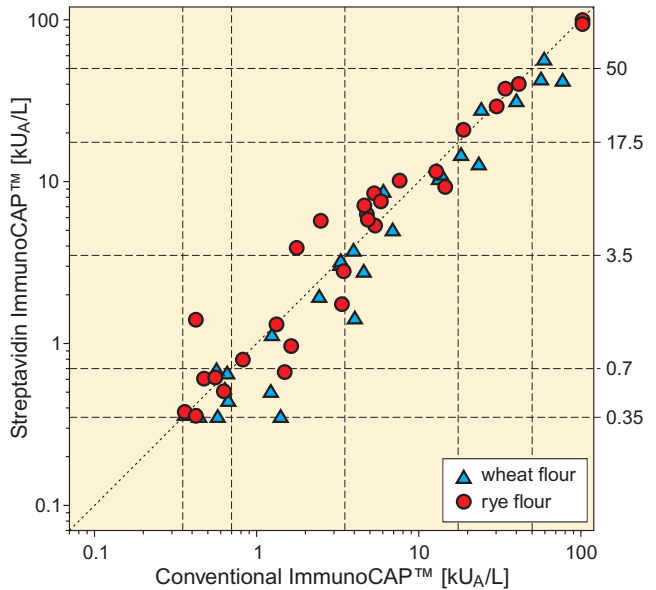


Figure 2. Comparison of IgE ab results obtained with conventional ImmunoCAP™ (f4 wheat and f5 rye) and own extracts of the same allergens bound to Streptavidin ImmunoCAP.

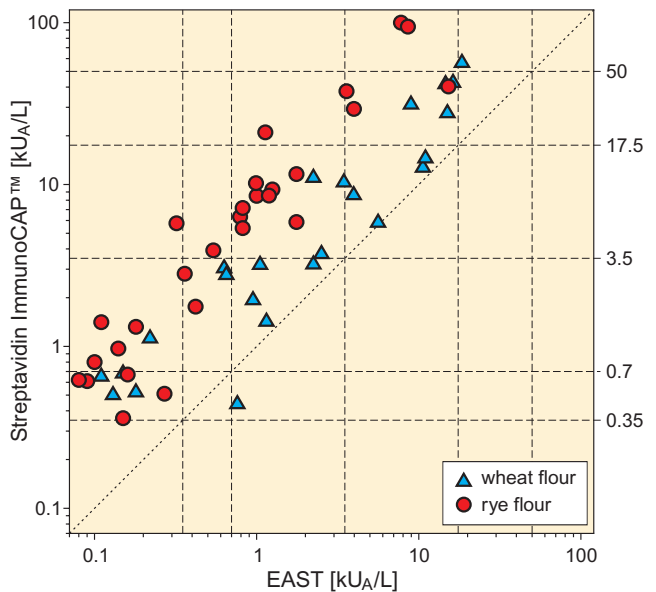


Figure 3. Comparison of EAST™ IgE ab results obtained with own extracts of wheat and rye flour coupled to cellulose discs and results of the same allergens bound to Streptavidin ImmunoCAP™.

Conclusion

Before binding of allergens to Streptavidin ImmunoCAP the allergens have to be labeled with biotin. For labeling, a ~5-fold molar excess of biotin can be used; higher amounts resulted in lower IgE ab values, possibly as a consequence of masking or disruption of epitopes.

The IgE ab values obtained with wheat and rye flour extracts bound to Streptavidin ImmunoCAP were nearly identical to the commercial f4 and f5 tests, although the allergen extracts used were not identical and the principle of coupling was different: biotin-streptavidin binding in one case and covalent coupling to the flexible hydrophilic CNBr-activated cellulose derivative in the other.

However, the main advantage of the ImmunoCAP™ systems in comparison to cellulose discs (EAST™), is the increased surface of the ImmunoCAP, which can bind three times as much protein as the discs, seems to take effect also in the Streptavidin ImmunoCAP. This optimal solid phase ensures binding of all relevant antibodies and is thus a prerequisite for quantitative measurements of IgE ab.

The IgE ab values of the Streptavidin ImmunoCAP were higher than the EAST results in nearly all cases. In fact, 16 cases had a negative IgE ab result in EAST while being positive with Streptavidin ImmunoCAP, indicating a great difference in the sensitivity of the two tests. Similar differences were found after Phadia introduction of ImmunoCAP when compared to their former cellulose disc based technology [2-4]. All studies found an increased sensitivity, higher test results with no or only minor loss of specificity.

As our results with the Streptavidin ImmunoCAP, using cereal proteins as allergens, show the same improvement compared to EAST, we expect the Streptavidin ImmunoCAP to become a valuable tool for measurement of IgE binding to new or commercially unavailable allergens.

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Use of Streptavidin ImmunoCAP™ for the evaluation of Der p 1 and Der p 2 specific IgE antibodies

Summary

The ever-increasing number of allergens identified baffles allergists trying to establish their clinical relevance. Moreover, many of these allergens exist in different isoforms, which may vary in their geographic distribution and differ in their interactions with the immune system [1]. Both scientists and clinicians urgently require simple and validated tests to determine the presence of sensitization to such newly identified allergens in individual patients or the prevalence of sensitization in relevant populations.

Evidence is accumulating that allergenic components present in an allergen source extract exhibit different immunological properties. An example of this is provided by extract of *Dermatophagoides pteronyssinus*, the common house dust mite. Sensitization to Der p 1 in a mouse model of asthma results in very different patterns of immune responses compared to Der p 2 (manuscript in preparation). This is due, at least in part, to multiple extra-immune properties of Der p 1, related to its cysteine protease activity [2]. Thus, in order to understand particular disease mechanisms of allergy, there is a need for complex mixtures of allergens to be deciphered at the level of their individual components.

The ImmunoCAP™ System (Phadia AB, Uppsala, Sweden) represents a suitable assay platform for such purposes, owing to its robustness and quantitative performance. The inherent advantages of the system can now be utilized for rapid screening of numbers of allergens, even if available only in limited quantities, by making use of Streptavidin ImmunoCAP. As biotin labeling of proteins can be carried out by standard methods and at low protein concentration, the approach offers an attractive addition of versatility of the assay system.

In the course of studies of immune responses to *D. pteronyssinus*-derived allergens, we have made use of Streptavidin ImmunoCAP. We present here results obtained by assessing serum samples of house dust mite sensitive patients with recombinant Der p 1 and Der p 2 molecules, using conventional ImmunoCAP and Streptavidin ImmunoCAP side-by-side. We find that the Streptavidin ImmunoCAP test is easy to use and has the capacity to generate results fully comparable to ImmunoCAP tests prepared using conventional coupling chemistry.

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Material and methods

Recombinant allergens

Coding sequences for full-length pro-Der p 1 and for Der p 2 were generated by RT-PCR and inserted into the pPICZαC expression vector (Invitrogen, Carlsbad, CA). Positive clones were identified by PCR using Der p 1 and Der p 2 specific primers and verified by DNA sequencing. Plasmid cDNA was purified and transformed into the yeast *Pichia pastoris* using the Easy Select *Pichia* Expression kit (Invitrogen). Cells were first cultured in buffered minimal glycerol medium and then transferred to minimal medium containing methanol for induction of protein expression. Mature glycosylated rDer p 1 was purified on a HiTrap SP HP column (Amersham Biosciences, Uppsala, Sweden). Recombinant Der p 2 was purified by gel filtration using a Superdex 75 column (Amersham Biosciences). Protein peaks corresponding to rDer p 2 were concentrated and assessed by SDS-PAGE and reactivity to mono- and polyclonal antibodies of murine and human origin. Correct folding of rDer p 1 and rDer p 2 was evaluated using conformation-dependent specific mAbs produced in our laboratory.

Protein biotinylation

Recombinant allergens were dialyzed against 50 mM sodium carbonate buffer, pH 9.2. Primary amines were engaged by N-hydroxysulfosuccinimide ester-modified biotin using the EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). A molar ratio of Sulfo-NHS-LC-Biotin to allergen of 25:1 was used for an incubation of 5 hrs at room temperature. Unreacted biotin was removed by extensive dialysis against 150 mM phosphate buffer containing 0.5 M NaCl. This protocol resulted in the addition of an average of 4 and 2 biotin molecules per molecule of rDer p 1 and rDer p 2, respectively, as determined using the EZ Biotin Quantitation Kit (Pierce).

Protein immobilization on Streptavidin ImmunoCAP™

Streptavidin ImmunoCAP washing and loading with biotinylated allergen were performed following a specific menu provided within the ImmunoCAP 100 instrument software. For routine assays, 10 µg/mL of biotinylated allergen in PBS

was added to the Streptavidin ImmunoCAP™ and incubated for 30 minutes at room temperature, prior to washing and running the assay.

Protein immobilization using conventional chemistry

Experimental IgE antibody assays specific for rDer p 1 and rDer p 2 were prepared by covalent immobilization of purified recombinant allergen onto activated cellulose (Axén *et al.*, 1988), at a concentration chosen to achieve an adequate linear measuring range and a low level of background binding for negative sera.

Analysis of serum samples of mite-sensitized donors

To compare assays prepared by the streptavidin-biotin and conventional chemistry methods, analyses were performed using a collection of sera from mite-sensitized subjects. Thirty sera reactive to rDer p 1 and 30 sera reactive to rDer p 2 were analyzed for IgE antibodies on either type of test in parallel, using an ImmunoCAP 100 instrument. All measurements presented are mean values of duplicate determinations. All assay procedures were performed according to the manufacturer's instructions and calculation of assay parameters were carried out by the ImmunoCAP instrument software.

Statistical evaluation

The statistical evaluation of the two allergen immobilization methods included analysis of quota between measurements obtained with the two methods across the IgE antibody concentration range and comparative assessment of the coefficient of variation (CV) in duplicate determinations, also across the IgE antibody concentration range.

Results

rDer p 1 and rDer p 2 were coupled to ImmunoCAP matrix, either directly to cyanogen bromide activated cellulose (conventional chemistry) or through biotin labeling and binding to Streptavidin ImmunoCAP. Serum samples from 30 Der p 1 and 30 Der p 2 sensitized individuals were used to perform a direct comparison between tests prepared by conventional coupling chemistry and biotin-streptavidin mediated immobilization.

To determine the solid phase saturation profile of the Streptavidin ImmunoCAP for the two allergens used in this study, serial dilutions of biotinylated allergen were used for immobilization. Two different reactive sera were used to analyze each of the two series of tests and the results are shown in **Figure 1**. It was found that 0.25 - 2 µg of rDer p 1 and rDer p 2, respectively, was sufficient for functional saturation of the solid phase of one Streptavidin ImmunoCAP.

The results of the comparative analysis are shown in **Figure 2A** (rDer p 1) and **Figure 2B** (rDer p 2). An almost perfect correlation between the two methods was found: $r=0.983$ and 0.995 for rDer p 1 and rDer p 2, respectively. On average, tests prepared using conventional coupling chemistry gave 7% lower results for rDer p 1 and 12% higher results for rDer p 2, as compared to biotin-streptavidin coupling. Only one serum gave rise to a positive/negative discrepancy between the two immobilization methods, showing $0.45 \text{ kU}_A/\text{L}$ for rDer p 2 coupled with

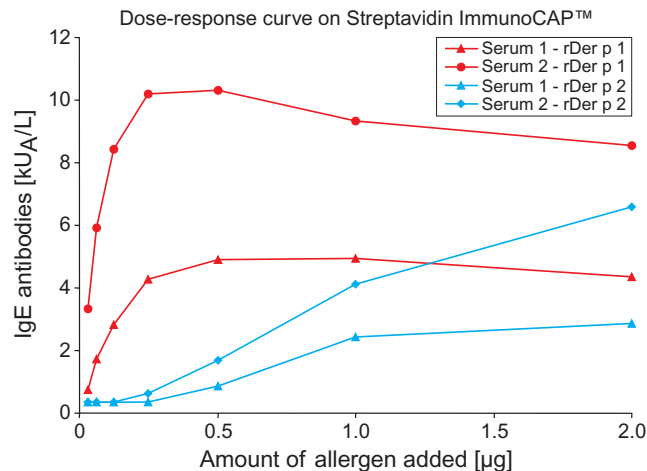


Figure 1. Dose-response relationship between load of biotinylated allergen on Streptavidin ImmunoCAP™ and IgE antibody concentration readout.

conventional chemistry and $<0.35 \text{ kU}_A/\text{L}$ for rDer p 2 coupled with the biotin-streptavidin method.

To reveal in a more sensitive way any difference that may exist between the two methods of allergen coupling to the solid phase, with respect to IgE binding capacity and assay uniformity, assay results were compared across the IgE antibody concentration range. In **Figure 3**, the quota of IgE determinations with the two types of tests were calculated for each serum and plotted against the mean of the same two determinations. **Figure 3A** shows the rDer p 1 results and **Figure 3B** the rDer p 2 results analyzed in this way. Although tests generated by the two methods give overall comparable results, somewhat higher IgE binding to rDer p 1 is observed with streptavidin-biotin coupling, while a slight advantage for conventional chemistry is observed in the case of rDer p 2.

Figure 4 shows intra-assay CV values between duplicates for the two coupling methods, plotted against the mean IgE antibody concentration of each duplicate pair. For both rDer p 1 (**Figure 4A**) and rDer p 2 (**Figure 4B**), the two immobilization chemistries resulted in comparable overall intra-assay CV, with a small advantage for conventional chemistry in both cases. No correlation between CV and IgE antibody concentration can be observed for either of the two allergens or coupling methods.

Discussion

The results presented here demonstrate the utility of the ImmunoCAP matrix for the assessment of single allergens with a coupling method mediated by biotin-streptavidin interaction.

The method is simple and can be performed in any laboratory, without the use of potentially harmful reagents, such as cyanogen-bromide, or protective measures. In addition, moderate quantities of the protein of interest are required. For the rDer p 1 and the rDer p 2 assays, 2 µg of allergen or less was sufficient to prepare one allergen-specific ImmunoCAP test. This represents a definite advantage when limiting amounts of material are available, such as for newly identified allergens being analyzed for clinical significance.

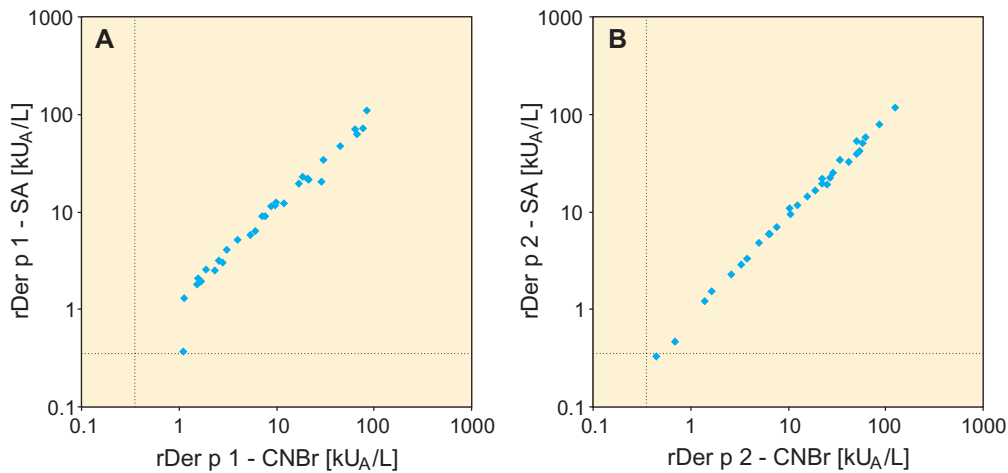


Figure 2. Comparison of IgE antibody results obtained with ImmunoCAP™ tests carrying conventionally (X-axis) and biotin-streptavidin (SA) coupled allergen (Y-axis). **A:** rDer p 1; **B:** rDer p 2.

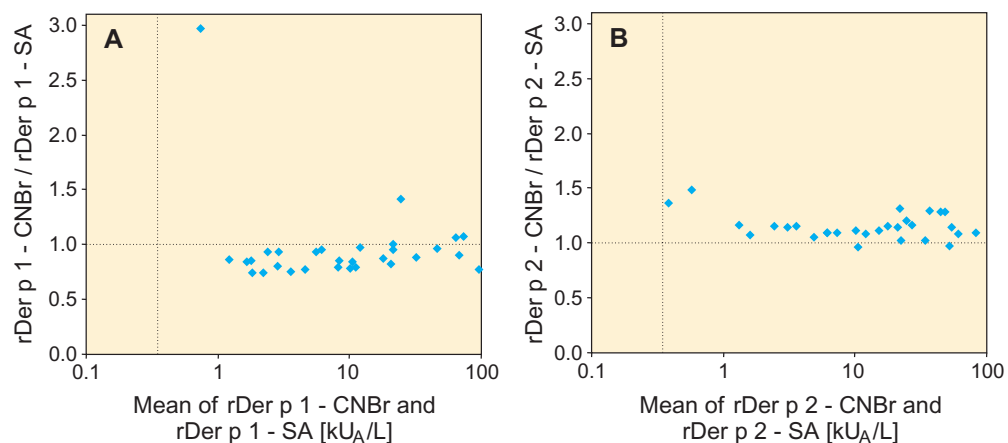


Figure 3. Quota of IgE antibody measurements obtained with ImmunoCAP™ tests carrying conventionally (CNBr) and biotin-streptavidin (SA) coupled allergen, across the range of concentrations present in the serum specimens analyzed. **A:** rDer p 1; **B:** rDer p 2.

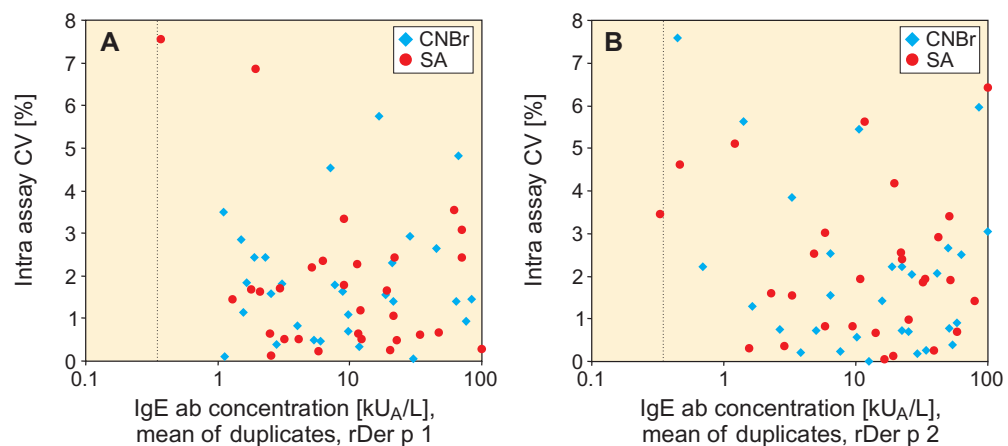


Figure 4. Comparison of intra assay coefficient of variation (CV) of duplicate IgE antibody measurements with conventionally (CNBr) and biotin-streptavidin (SA) coupled allergen, across the range of concentrations present in the serum specimens analyzed. **A:** rDer p 1; **B:** rDer p 2.

The complexity of immune responses and clinical symptoms to allergen exposure prompts an increasing number of clinical investigators to assess the presence of antibodies to individual components of allergen mixtures, so as to establish the relative importance of each of these components at the onset and evolution of clinical reactivity. In this context, Streptavidin ImmunoCAP offers a versatile open system for clinical as well as more basic research purposes.

Assays for virtually any new allergen can be created by the investigator and used to analyze relevant patient samples. For example, the prevalence and clinical significance of antibody responses to cross-reactive allergens in allergies to pollens, fruits and vegetables can be studied. Further, the relevance of allergen isoforms and its corollary, the potential use of some isoforms for therapeutic purposes, should become easy to evaluate, using a combination of direct IgE binding and IgE

inhibition experiments. Along the same lines, mutants of allergens designed to reduce IgE binding could be readily assessed with this assay.

Further, Streptavidin ImmunoCAP™ offers the possibility of simple and accurate quantitation of allergen-specific antibody reagents of animal or human origin. Such antibodies can be made chimeric by substituting their heavy chain for the human Fcε counterpart, or be chemically conjugated with Fcε fragment, which is detected in the assay system. For instance, we use a Der p 2 specific antibody made from a Fab fragment of a BALB/c antibody cloned in frame with human Fcε (unpublished).

We believe that the Streptavidin ImmunoCAP assay, with its high sensitivity for IgE antibodies to individual allergenic proteins, is a significant step forward in the assessment of anti-allergen immune responses. Its validation in comparative studies will allow a meaningful and direct comparison of results obtained in different laboratories, with a medium-term benefit for patients.

One intriguing possibility would be to detect associations between some of the pathophysiological manifestations and humoral reactivity towards different allergenic components. For instance, the pattern of asthma due to house dust mite sensitivity could be linked to the relative importance of the sensitivity to Der p 1 versus Der p 2. It is indeed likely that differences in size, glycosylation and enzymic activity between these two allergens result in distinct *in vivo* behavior and pathological consequences. Experimental models of asthma already provide some insight into this interesting possibility, which could be usefully complemented by the versatile antibody assay system described here.

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