Major Allergen Analyses: Comparability and Clinical Relevance of Polyclonal vs. Monoclonal Antibodies to Alternaria Allergen Alt a 1

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Abstract

Detection and quantitation of prominent allergenic proteins in commercial extracts is an important step to improving the quality, consistency and clinical utility of these products. Current assays employing allergen-specific animal (mouse monoclonal and/or rabbit polyclonal) antibodies and recombinant or purified native allergen preparations in two-site (sandwich) configurations are capable of detecting nanogram quantities of most target allergens. However, for many allergens, the relationships between the specific antigenic sequences or structural regions recognized by these antibodies and those responsible for allergenic (human IgE-binding) interactions in allergic patients have not been examined in sufficient detail.

The binding specificities of mouse and rabbit antibodies to the major Alternaria allergen Alt a 1 were examined in this study using two-site ELISA methods optimized to each antiserum and competition (inhibition) formats of human IgE immunoblotting and ImmunoCAP analyses. Alt a 1 ELISA using mouse or rabbit antibodies produced different results (outside 1SD ranges) for 73% (8/11) of Alternaria extracts tested. Coincubation of the mouse or rabbit antibodies with an allergic human serum pool prior to immunoblotting induced no noticeable changes in human IgE binding to Alt a 1 or other Alternaria proteins and only minimal reductions in rabbit or mouse IgG interactions with these extract components. Maximum inhibition of human IgE binding to Alternaria ImmunoCAP allergens exceeded 80% with rabbit anti-Alt a 1 but was less than half that value (below 40%) with mouse anti-Alt a 1. Rabbit antibodies directed at multiple Alternaria antigens displayed a maximum inhibition level above that observed with mouse anti-Alt a 1.

These data indicate that polyclonal Alt a 1-specific rabbit antibodies recognize multiple distinct and non-overlapping IgE epitopes and provide a more complete assessment of the presence of important allergenic structures on this protein compared to a monoclonal, site-specific mouse anti-Alt a 1 reagent. Demonstration of the competitive binding characteristics of allergen-specific antibodies supports selection and validation of the best available reagents for major allergen ELISA analyses and facilitates improved correlations with current extract standardization methods and dose units.

Materials and Methods

Glycerinated Alternaria extract concentrates (1:10-1:20 w/v) were obtained from all 7 FDA-regulated U.S. allergen manufacturers (Greer Labs, Lenoir, NC; ALK Abello, Port Washington, NY; Allergy Labs, Oklahoma City, OK; Allermed Labs, San Diego, CA; Antigen Labs, Liberty, MO; Hollister-Stier Labs, Spokane, WA; and Nelco Labs, Deer Park, NY). These products are designated as A-G in no specific order. Cellular extracts from four different *A. alternata* strains cultured at Greer (shown as 1-4) were also examined.

Two-site (sandwich) ELISA methods for Alt a 1 were performed using either mouse monoclonal antibodies (McAb) or rabbit polyclonal antibody (PcAb) fractions as antigen capture and biotinylated probe reagents. Monoclonal antibody reagent pairs were purchased from Indoor Biotechnologies (Charlottesville, VA). Rabbit antiserum to Alt a 1 hypoallergen was kindly provided by Hari Vijay, Ph.D. (Health Canada, Ottawa) and modified for use at Greer by ammonium sulfate precipitation and biotinylation. Both assays employed the same recombinant Alt a 1 standard (Indoor, 1000 ng/mL) and detection reagents (avidin-AP conjugate, pNPP substrate). Serial 2-fold or 3-fold dilutions of standard and test extracts produced consistent dose-response curves, with all dilutions falling within the optimal (highest correlation) standard curve included in calculations of mean interpolated concentrations and interdilutional standard deviations.

SDS-PAGE was performed using 12% acrylamide gels in a Bio-Rad Mini-Protean II slab cell (0.5 µg of test extract protein per lane). Immunoblots with mouse or rabbit antibodies and a human allergic serum pool were conducted after transfers to PVDF membranes (Millipore Immobilon-P^{SQ}). Immunoblot inhibitions involved co-incubation of mouse or rabbit antibodies with the human serum pool and detection of human IgE, mouse IgG or rabbit IgG binding patterns after development with antibody-specific AP conjugates and BCIP/NBT substrate.

ImmunoCAP analyses (Phadia, Portage, MI) were conducted at Greer on an ImmunoCAP100 instrument using Phadia reagents and incubation conditions. Human IgE ImmunoCAP inhibitions were performed by co-incubation of a fixed dilution of human allergic serum with varying levels of mouse or rabbit antibodies. Rabbit antisera directed against multiple Alternaria, Aspergillus niger, Aureobasidium or short ragweed antigens were prepared at Greer and evaluated as control sera.

Alt a 1-specific ELISA

The Alt a 1 concentrations of Alternaria extracts A-G and 1-4 analyzed by McAb and PcAb assays are summarized below. Comparisons of mean Alt a 1 levels (µg/mL) and standard deviations (SD) for up to 5 valid dilutions of each extract demonstrated that 5/7 (71%) commercial products and 3/4 (75%) non-commercial, alternate strain samples produced results outside of 1SD ranges between the two methods (shown in red). Most extracts produced higher values in the McAb assays. Two products displayed specific reactions with either the McAb reagents (F) or the PcAb reagents (4).

Extract	[Alt a 1] µg/mL ± 1SD		PcAb % of McAb
	McAb	PcAb	
A	0.025 ± 0.006	0.013 ± 0.004	52
B	9.66 ± 0.54	6.21 ± 0.63	64
C	0.30 ± 0.03	0.22 ± 0.04	73
D	1.43 ± 0.35	0.60 ± 0.27	42
E	0.05 ± 0.00	0.08 ± 0.03	160
F	10.23 ± 1.25	0.80	8
G	3.20 ± 0.46	3.69 ± 0.53	115
1	3.38 ± 0.07	2.09 ± 0.31	62
2	0.99 ± 0.14	0.85 ± 0.15	86
3	4.99 ± 0.37	1.87 ± 1.01	37
4	< 0.001	0.47 ± 0.35	> 100

Immunoblot Analyses

To assess the comparative Alternaria protein specificities of the anti-Alt a 1 McAb and PcAb ELISA reagents, and their relationships with the human IgE specificities of a human allergic serum pool, immunoblots were performed with the same Alternaria extracts subjected to Alt a 1 ELISA analyses. Direct (uninhibited) and competition (cross-inhibited) blots were developed with antibody-specific detection reagents, yielding profiles for human IgE, mouse IgG and rabbit IgG binding interactions with Alt a 1 and other Alternaria proteins. Antibody and serum dilutions producing optimal band pattern resolution (1:100,000 mouse/rabbit antibodies, 1:5 human serum) were employed. Extracts were loaded at moderate levels (0.5 µg protein per lane) to optimize detection and band pattern development using the immunoblot assay conditions currently established and validated in the Greer R&D laboratory.

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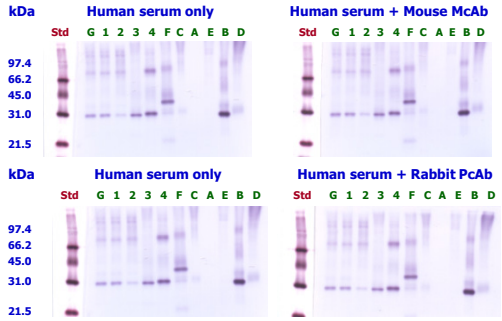
Immunoblot Inhibitions

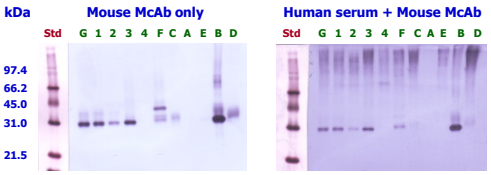
Coincubation of Alternaria IgE-positive human allergic serum with either McAb or PcAb anti-Alt a 1 reagents on immunoblots with Alternaria extracts produced no apparent changes in human IgE binding specificities or intensities compared to an uninhibited control (human serum only).

Human IgE detection

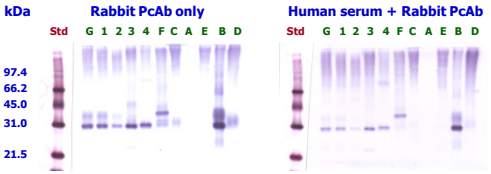


Using the same antibody and serum combinations, mouse McAb and rabbit PcAb anti-Alt a 1 IgG binding to immobilized Alternaria proteins displayed noticeable quantitative but minimal qualitative differences after coincubation with human allergic serum.

Mouse IgG detection



Rabbit IgG detection



ImmunoCAP Inhibitions

The abilities of antibody molecules recognizing the same allergenic protein structures to compete effectively for the identical epitopes are affected not only by the precise specificities and relative concentrations of the competing antibodies but also by the densities or binding capacities of the immobilized antigens.

The minimal inhibition of human IgE binding to Alternaria allergens by mouse or rabbit IgG antibodies on immunoblots may be related to the moderate loads of immobilized extract (0.5 µg protein) and relatively high animal antibody dilutions (1:100,000) required for optimal visualization of immunoreactive bands.

An immunoassay solid phase possessing defined levels of immobilized Alternaria allergens and a capacity for very high human and animal antibody concentrations is available with the Phadia ImmunoCAP system. Alternaria (m6) solid phases typically react with undiluted human serum with specific IgE detection determined using reagents that do not recognize other human or animal antibody subclasses.

To verify the specificity of the Alternaria ImmunoCAP assay, Alternaria-positive human serum was coincubated with rabbit antisera directed against unrelated antigens (short ragweed, Aspergillus, Aureobasidium) at high relative volumes (75 to 95%). Human IgE binding was not inhibited significantly by these sera (≥ 84% recoveries). These data provide an important control and baseline level for competitive inhibition experiments with mouse McAb anti-Alt a 1, rabbit PcAb anti-Alt a 1 and a rabbit antiserum recognizing Alt a 1 and other Alternaria antigens.

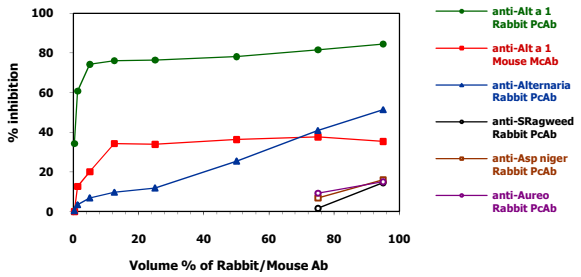
Inhibition of IgE binding to Alternaria ImmunoCAP allergens was considerably higher (> 2X) with rabbit PcAb anti-Alt a 1 (> 80% max) compared to mouse McAb anti-Alt a 1 (< 40% max) at equivalent relative volumes.

Dose-response curves with both anti-Alt a 1 reagents begin to plateau at relatively low volumes (5 to 12.5%).

Rabbit anti-Alternaria (Alt a 1 + other antigens) produced linear increases in IgE inhibition, surpassing the levels found for mouse McAb anti-Alt a 1 at 75-95% relative volumes.

MPN 0907H010

ImmunoCAP Inhibitions



Conclusions

Rabbit PcAb anti-Alt a 1 interact with structural regions that appear to correspond closely to the multiple human IgE-binding epitopes recognized by allergic patients. By comparison, mouse McAb anti-Alt a 1 inhibits a limited number of epitopes (or only one), and is not nearly as effective at covering the multiple and distinct allergenic structures present on the Alt a 1 molecule.

Validation of the IgE inhibition properties of allergen-specific PcAbs or combinations of distinct McAbs is thus critical to the selection of clinically-relevant capture/probe reagents and optimal conditions for major allergen assessments of commercial and investigational extracts.

Recognition and detection of multiple IgE epitopes reduces the likelihood that structural changes (natural or environmental) to any single epitope would impact the accuracy and consistency of major allergen measurements. Minor changes on Alt a 1 regions near or within the McAb binding site can reduce or eliminate McAb binding. Changes (positive or negative) to IgE epitopes remote from the McAb binding site may be detected and accounted for in a PcAb-based assay.

Further comparability studies with additional allergen-specific antibodies and human allergic sera are needed to establish clear performance specifications and analytical requirements for high-quality major allergen reagents and assays.