Multiplex microbead measurements for the characterization of cat and ragweed allergen extracts

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Background: Current assays for allergen extracts can measure either overall potency or the levels of individual allergens.

Objective: To develop a multiplex allergen extract potency assay (MAEPA) for allergen extracts that can concurrently measure individual allergens and characterize the overall allergen levels in the mixture.

Methods: Six anti–Fel d 1 and 6 anti–Amb a 1 recombinant antibodies were generated and were covalently bound to carboxy-labeled beads. Antibody-bound beads were then used to measure Fel d 1 and Amb a 1 levels in commercial cat hair and short ragweed pollen (SRP) extracts, respectively, using bead-based flow cytometry. These major allergen levels were compared with those obtained using a conventional antibody-based method. Allergen levels were calculated by comparing the half-maximal effective concentrations of dose-response curves analyzed using 4-parameter fits. Bead-antibody pairs were tested to determine whether the presence of additional bead-antibody pairs affected the apparent potency of the extract.

Results: Allergen contents of cat hair and SRP extracts determined using the MAEPA and anti–Fel d 1 and anti–Amb a 1 antibodies were comparable with potencies determined using conventional methods. Cross-interference from the concurrent use of multiple beads was minimal. Six lots of cat hair extract and 6 lots of SRP extract were tested.

Conclusions: The MAEPA, a bead-based assay using recombinant antibodies, accurately determined Fel d 1 levels in cat hair allergenic extracts and Amb a 1 levels in SRP extracts. The results of this assay are reproducible and are consistent with data obtained using conventional methods.

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INTRODUCTION

Of more than 1,200 allergenic extracts on the market in the United States for the diagnosis and treatment of allergy,[1] only 19 are standardized, and the remaining are marketed without a scientifically valid potency measure. The biological potencies of most current reference-standardized allergen extracts were established using the $ID_{50}EAL$ (intradermal dilution for 50-mm sum of erythema diameters determines the allergy unit) technique.[2,3] The replacement of these references, and comparison with them of manufactured lots, is achieved by 1 of several surrogate assays. For standardized short ragweed pollen (SRP) and cat hair extracts, for which there are single predominant allergens, a radial immunodiffusion (RID) assay with a monovalent antiserum is used to determine the potency of the extracts relative to their respective standards. When there are multiple relevant allergens, as in dust mite and grass pollen allergen extracts, a competition enzyme-linked immunosorbent assay (ELISA) with human antisera is used to assess the overall allergen content of these US products.

The competition ELISA is sensitive and specific in determining overall allergen content, but its performance characteristics for each component allergen are uncertain: when an individual allergen is removed from the extract, the polyvalent sera cannot reliably discern its absence.[4,5] Until each relevant allergen in a mixture is identified, regulators have no choice but to ask manufacturers to measure overall potency. Once important allergens are determined, their measurement requires changes in technology that can be time-consuming and difficult.

The purpose of this study was to develop assays that will have the strengths of RID and competition ELISA and that will allow manufacturers and regulators to determine the amounts of specific allergens in a mixture and its overall allergenicity. Multiplex assays have been used with success for a variety of assays, such as detecting allergen specific IgE[6,7] and the amount of allergen in the environment.[8] Following this approach, we designed a multiplex allergen extract potency assay (MAEPA). Multiple monoclonal antibodies to the predominant allergens in cat hair and SRP allergenic extracts were used to measure the potencies of these extracts. We chose to use SRP and cat hair extracts for this purpose because both extracts have been standardized and the major allergen of each is well-known.[9,10]

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METHODS

Allergens, Allergen Extracts, and Antisera

Recombinant Fel d 1 (rFel d 1) was purchased from Indoor Biotechnologies (Charlottesville, Virginia). Native Amb a 1 (nAmb a 1) was obtained from a repository maintained by Bratton Biotech (Rockville, Maryland). Six cat hair and 6 SRP allergenic extracts were obtained from 6 different manufacturers licensed to distribute extracts in the United States and Europe. The cat hair extracts were received in February 2008; the SRP extracts were received between February 1, 2008 to February 28, 2009. All the extracts contained 50% glycerin and 0.4% phenol and were stored at 4°C to 8°C. Potencies were initially determined on receipt by the Food and Drug Administration using RID,[11] comparing them with the current Center for Biologics Evaluation and Research (CBER) reference standards for cat (E4-cat pelt) and ragweed (E15-ragweed). Hyperimmune serum was obtained from a single New Zealand white rabbit immunized with rFel d 1, nAmb a 1, and yellow jacket venom allergen extract in Freund adjuvants.[12]

Preparation of Single-Chain Fragment-Variable Antibodies

Single-chain fragment-variable (scFv) monospecific recombinant antibodies were produced by means of polymerase chain reaction amplification of the variable region of heavy and light chains of a chicken antibody repertoire as described elsewhere.[12] The scFv antibodies expressed from pCOMB3X contain a His tag at the N-terminus and a hemagglutinin recognition sequence at the C-terminus. Sequences encoding allergen-specific scFv antibodies in the pCOMB3X vector were electroporated into TOP10 (Invitrogen, Carlsbad, California) strain of *Escherichia coli*. Transformed clones were selected on LB plates containing 50 μg/mL of carbenicillin. Single colonies were grown in SB media at 37°C in a shaking incubator until the middle of the log phase was reached. Synthesis of scFv antibody was induced for 14 to 16 hours at 30°C in the presence of 1mM isopropyl β-D-1-thiogalactopyranoside. Bacteria were lysed using lysing matrix B (Qbiogene, Cambridge, England), and scFv antibodies were purified from bacterial lysate using nickel chelation chromatography (Ni-NTA Spin Kit; Qiagen Inc, Valencia, California).

Allergen-Specific ELISA

Purified scFv antibodies were tested for allergen binding by means of ELISA. Briefly, 96-well polystyrene plates (Nalge Nunc International, Rochester, New York) were coated with antigen (E4-cat pelt, 1:400; E15-ragweed, 1:500; rFel d 1, 0.5 μg/mL; or nAmb a 1, 1.5 μg/mL) in phosphate-buffered saline (PBS) at 4°C overnight. Wells were washed and then were blocked in 3% ovalbumin in PBS/Tween. Antibodies (in 1% ovalbumin in PBS/Tween) were added to a final concentration of 2 μg/mL (1 hour, 25°C), and bound antibody was detected with antihemagglutinin antibody conjugated to horseradish peroxidase (1:1,000 vol/vol in 3% ovalbumin in PBS/Tween) (Roche Diagnostics Corp, Indianapolis, Indiana) and horseradish peroxidase substrate.

DNA Sequencing

Purified plasmid (100–200 ng) from each clone was combined with 3 pg of forward primer ompseq or 3 pg of reverse primer gback[13] and was sequenced (ABI 3.1 DNA Sequencing Kit, 3130XL DNA Sequencer; Applied Biosystems, Foster City, California) at the Facility for Biotechnology Resources core facility (CBER). The sequences were aligned to verify consistent sequence results from both primers using ClustalW.[14]

Coupling of Antibody to Beads

Recombinant scFv antibodies were coupled to polystyrene carboxy-labeled beads (Bio-Rad Laboratories, Hercules, California) internally dyed with red and infrared fluorophores, which allow the identity of each bead to be determined after laser excitation. Microbeads were primed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysulfosuccinimide to generate an *N*-hydroxysulfosuccinimide ester. Primed beads were then washed with 50mM 2-(N-morpholino) ethanesulfonic acid (pH 5.0); 10 μg of scFv antibody was added to 2.5×10^6 beads and was allowed to couple for 2 hours at room temperature. Beads bound to antibody were washed and stored in PBS with 0.1% bovine serum albumin, 0.02% Tween, and 0.05% sodium azide.

Microbead-Based Mutiplex Assay

All the reactions were performed in 96-well filter plates (Millipore, Billerica, Massachusetts). The plates were rinsed in PBS containing 1% bovine serum albumin. Antibody-coated beads (5,000/well for each bead-antibody) were incubated in the presence of serial 1:2 dilutions of SRP or cat hair allergen extracts for 30 minutes. Bound allergen was detected by sequential washes and incubations with polyclonal rabbit anti–rFel d 1 or anti–nAmb a 1,[12,15] biotinylated anti–rabbit IgG (H+L) (KPL Inc, Gaithersburg, Maryland), and streptavidin–R-phycoerythrin (Pierce, Rockford, Illinois). After the final 30-minute incubation, the amount of label bound to each bead type was detected using the Bio-Plex 200 System (Bio-Rad). Each assay was performed in duplicate (SRP extracts) or triplicate (cat hair extracts).

Epitope mapping experiments were performed in a similar manner. Each antibody (5 μg) was biotinylated using *N*-hydroxysulfosuccinimido biotin (Thermo Scientific, Rockford) according to the manufacturer's protocol. Allergen extract mixtures were serially diluted and were incubated with the appropriate set of 6 bead-bound antibodies. After a 30-minute preincubation period, each biotinylated antibody type was individually added to a preincubated dilution series. Biotinylated antibodies were then detected on washed beads using streptavidin–R-phycoerythrin.

Heat-Treated Extracts

Undiluted allergenic extracts were incubated at 80°C for 1 minute or at 97°C for 1 hour, after which the extracts were

Table 1. Apparent Relative Potencies of a Cat Hair Extract as Determined Using Different Combinations of Bead-Bound Anti–Fel d 1 Recombinant Antibodies

Antibody name	Relative potency					
	Individual		3 combined			All 6 combined
F7	1.62	1.62		2.77		1.68
F8	1.82		2.35		1.67	1.89
F9	1.28		1.39	0.71		1.44
F10	0.81		0.81	0.88		0.88
F11	1.2	1.20			1.05	0.91
F12	1.88	1.88			1.25	1.61

immediately cooled to 4°C on ice. Extracts were then diluted and assayed as described previously herein.

Determinations of Relative Potencies

Relative potency (RP) was calculated only for assays in which slopes of test extracts were sufficiently similar to slopes of standards when graphed on a log-linear scale. Raw data were exported from Bio-Plex Manager software to Excel 2003 (Microsoft Corp, Redmond, Washington) and then into GraphPad Prism 5 (GraphPad Software, La Jolla, California) for analyses using 4-parameter curve fitting. To determine appropriate cutoff values for slope differences, the slopes from reference extracts were analyzed independently 17 times for cat hair extract or 19 times for SRP extract. The 99% confidence intervals (CIs) were calculated for the slopes of each extract. For each antibody tested, the difference between the upper and lower limits of the slopes was calculated. The greatest percentage difference was then used as the maximum allowable difference for the slopes when comparing an extract with its reference standard for all antibodies used for that extract. In this way, the maximum allowable difference of the slope of the test extract compared with the slope of the reference was independently calculated to be 29% for cat hair and SRP extracts (at the 99% CI). Slopes within this range were considered equivalent, and half-maximal effective concentrations were compared with calculated RPs.[15]

RESULTS

Initial Antibody Characterization

For this assay, recombinant antibodies to SRP allergen Amb a 1 and cat allergen Fel d 1 were covalently coupled to chemically derivatized polystyrene beads. Antibody-coated beads are then exposed to test allergen extracts, and bound allergen is detected on the washed beads with rabbit antiserum. In preliminary work (not shown), we determined that 4 pg of recombinant antibody per bead consistently resulted in reproducible detection of Amb a 1 and Fel d 1. The amount of antibody was optimized using a checkerboard titration of antibody and bead ratios. Anti–Fel d 1 antibodies were tested in 17 separate experiments with the reference extract. The mean (SD) of the slopes from these tests was 1.73 (0.13). Anti–Amb a 1 antibodies were tested in 19 separate experiments, and the mean (SD) slope was 1.60 (0.42).

Concurrent Use of Multiple Antibody-Coated Microbeads

One potential advantage of this assay is that several beads, each coated with a distinct monoclonal antibody, can be used concurrently. With typical allergen extracts, which may contain multiple distinct allergens, these multiplex assessments may be performed with little concern about potential interactions among the different determinants. However, cat hair and SRP allergen extracts, each of which is being tested for the presence of a single immunodominant molecule, pose a worst-case scenario for potential cross-interference because one bead may compete with the other. We assessed this interference for each scFv antibody by analyzing them individually, in groups of 2 or 3, and all together.

Table 1 illustrates the data obtained from studies performed with 6 anti–Fel d 1 antibodies. The Fel d 1 content of cat hair extract as measured by antibody F10 was unaffected by the presence of microbeads coated with other anti–Fel d 1 antibodies (coefficient of variation [CV] = 5%). For antibodies F7 and F9, the presence of other antibodies seemed to have a much greater effect (CV = 28%–30%), whereas the other antibodies' binding was affected to an intermediate degree (CV = 12%–17%).

Similar results were obtained using the anti–Amb a 1 antibodies (Table 2). Assays using antibodies A3 and A4 resulted in a much higher apparent RP when tested individually or paired together as opposed to when they were grouped with 2 or all other scFv antibodies (CV = 51% and 44%, respectively). The remaining scFv antibodies uniformly yielded RPs of 0.29 to 0.59 whether assayed individually or in groups. Least affected by the concurrent use of other antibodies were antibodies A5 (CV = 11%) and A2 (CV = 15%).

Antibody-Specific Inhibition

The antibodies were then analyzed to determine whether they detect different or overlapping epitopes. In these experiments, biotinylated monoclonal antibodies competed for allergen with bead-bound antibodies. When anti–Amb a 1 antibody A3 is conjugated to beads and preincubated with SRP extract, subsequent binding of biotinylated antibodies A3

Table 2. Apparent Relative Potencies of a Ragweed Extract as Determined Using Different Combinations of Bead-Bound Anti–Amb a 1 Recombinant Antibodies

Antibody name	Relative potency							
	Individual		Pairs			3 combined		All 6 combined
A1	0.39	0.38				0.36		0.55
A2	0.39	0.41				0.29		0.40
A3	1.34		1.08			0.53		0.44
A4	1.05		1.02				0.46	0.47
A5	0.43			0.51		0.40		0.43
A6	0.43			0.59		0.42		0.43

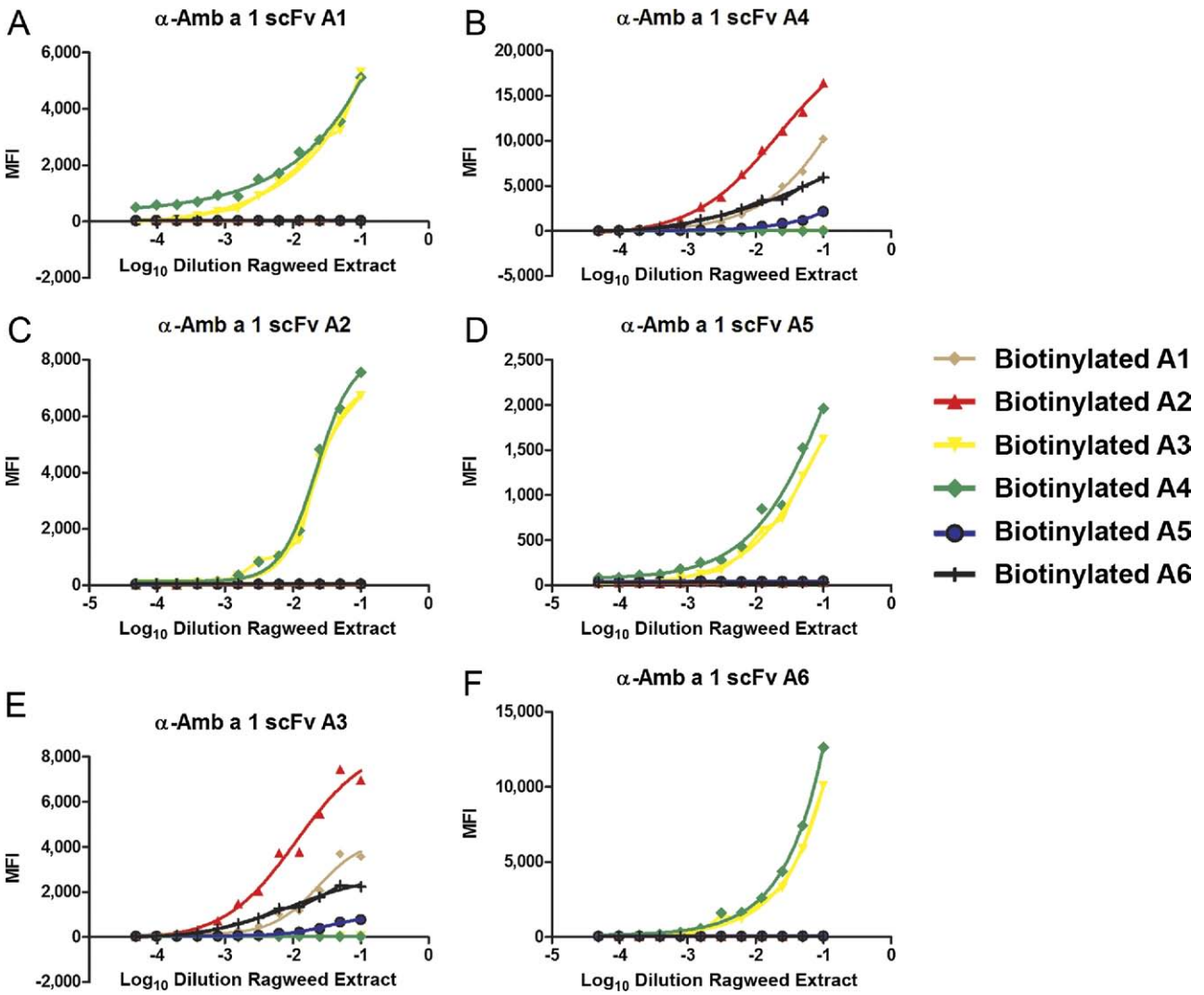


Figure 1. Dose-response curves showing the presence or absence of binding by biotinylated anti–Amb a 1 single-chain fragment-variable (scFv) antibodies after preincubation of short ragweed pollen extract with bead-bound anti–Amb a 1 scFv antibodies. Biotinylated anti–Amb a 1 scFv antibodies A3 and A4 bind to Amb a 1 in short ragweed pollen extract only when this extract has been preincubated in the presence of bead-bound anti–Amb a 1 scFv antibodies A1, A2, A5, and A6. MFI indicates median fluorescence intensity.

and A4 are completely blocked (Fig 1E). Likewise, bead-bound A4 blocks the binding of biotinylated A3 and A4 (Fig 1B), suggesting (as do the data in Table 2) that A3 and A4 recognize overlapping epitopes. Conversely, bead-bound A1 inhibits the binding of biotinylated A1, A2, A5, and A6 (Fig 1A) but not A3 or A4. The same pattern is repeated for bead-bound A2, A5, and A6 (Figs 1C–E). These data indicate that A1, A2, A5, and A6 bind overlapping epitopes that are distinct from those recognized by A3 and A4.

A similar study was performed with the anti–Fel d 1 antibodies. Bead-bound anti–Fel d 1 antibody F8 inhibits only biotinylated F8 but does not inhibit binding of any of the remaining 5 anti–Fel d 1 antibodies (Fig 2D). Conversely, these 5 antibodies inhibit the binding of each biotinylated anti–Fel d 1 except F8 (Fig 2). This suggests that F7, F9, F10, F11, and F12 recognize overlapping epitopes but that F8 binds to a distinct epitope.

Relative Potencies

We then used these scFv antibodies to determine the allergen content of 6 different cat hair (Table 3) and SRP (Table 4) extracts. These assays were performed at least 3 times each, and results were compared with the potencies determined using RID. The mean potencies determined using the MEAPA fell within the 95% CI of the RID for 5 of 6 cat hair extracts tested. The RPs were also determined for 4 separate SRP extracts (extracts J, L, M, and N) and 2 giant and short

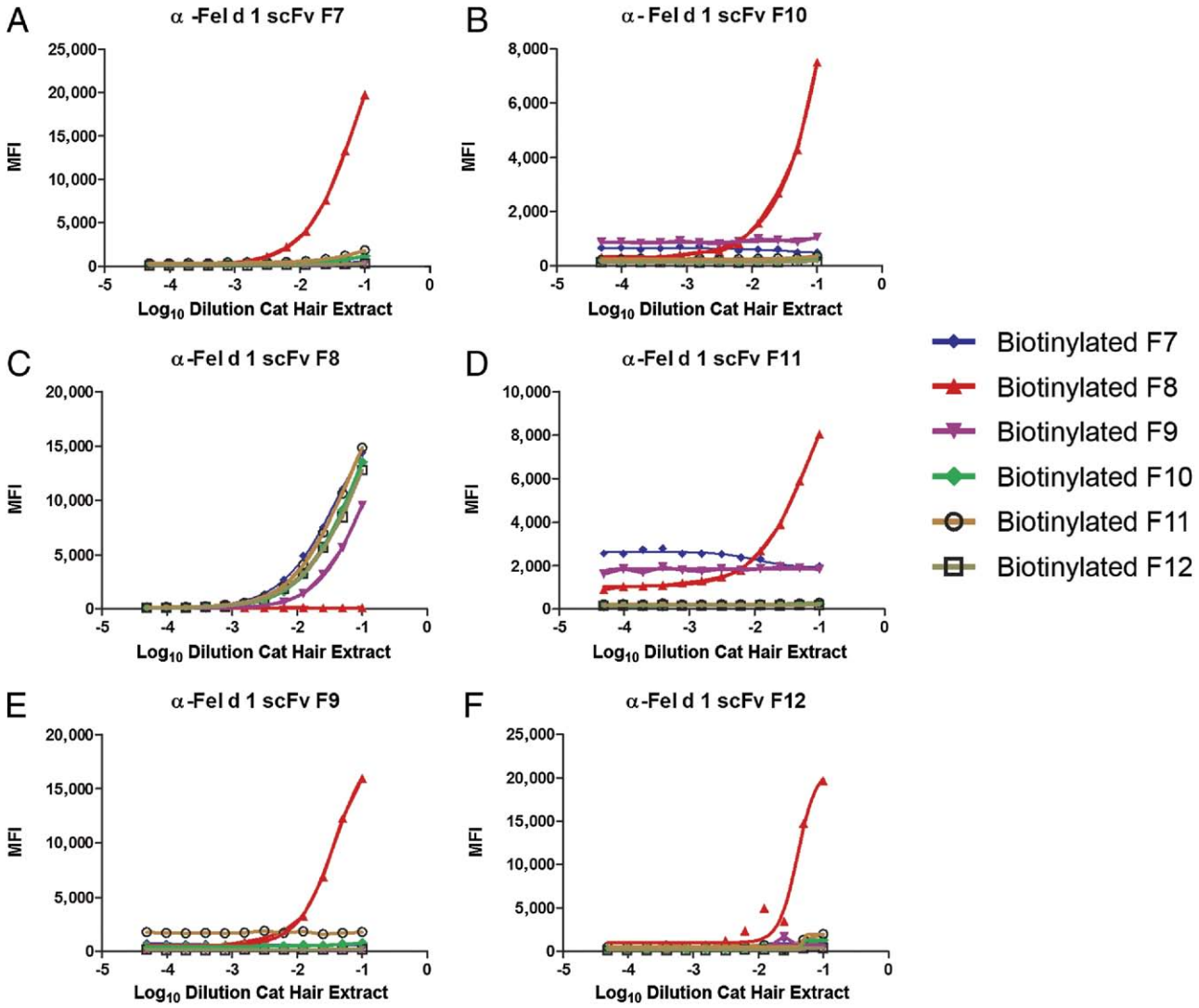


Figure 2. Dose-response curves showing the presence or absence of binding by biotinylated anti–Fel d 1 single-chain fragment-variable (scFv) antibodies after preincubation of bead-bound anti–Fel d 1 scFv antibodies with cat hair extract. Biotinylated anti–Fel d 1 scFv antibody F8 binds to Fel d 1 in cat hair extract that has been preincubated with each of the remaining bead-bound scFv antibodies. MFI indicates median fluorescence intensity.

Table 3. Potencies of 6 Cat Hair Allergen Extracts as Determined by Means of MAEPA and RID

Extract	Fel d 1, mean (95% CI), U/mL	
	MAEPA	RID
B	6.91 (6.19–7.62)	8 (6.0–10.0)
C	17.14 (16.25–18.04)	14 (10.0–18.0)
D	4.76 (3.96–5.56)	6 (4.5–7.5)
E	16.33 (16.22–16.43)	21.5 (16.1–26.9)
F	16.44 (12.47–20.41)	21 (15.7–26.3)
H	6.99 (5.16–8.81)	13 (9.75–16.3)

Abbreviations: CI, confidence interval; MAEPA, multiplex allergen extract potency assay; RID, radial immunodiffusion.

Table 4. Potencies of 6 Ragweed Pollen Extracts as Determined by Means of MAEPA and RID

Extract	Amb a 1, mean (95% CI) U/mL	
	MAEPA (n = 2–5)	RID (n = 3)
J	145 (142–147)	165 (124–206)
K	109 (85.9–131)	98 (74–123)
L	393 (364–422)	360 (270–450)
M	212 (169–255)	248 (186–310)
N	344 (341–346)	383 (287–478)
O	100 (98.0–102.0)	102 (77.7–128.0)

Abbreviations: CI, confidence interval; MAEPA, multiplex allergen extract potency assay; RID, radial immunodiffusion.

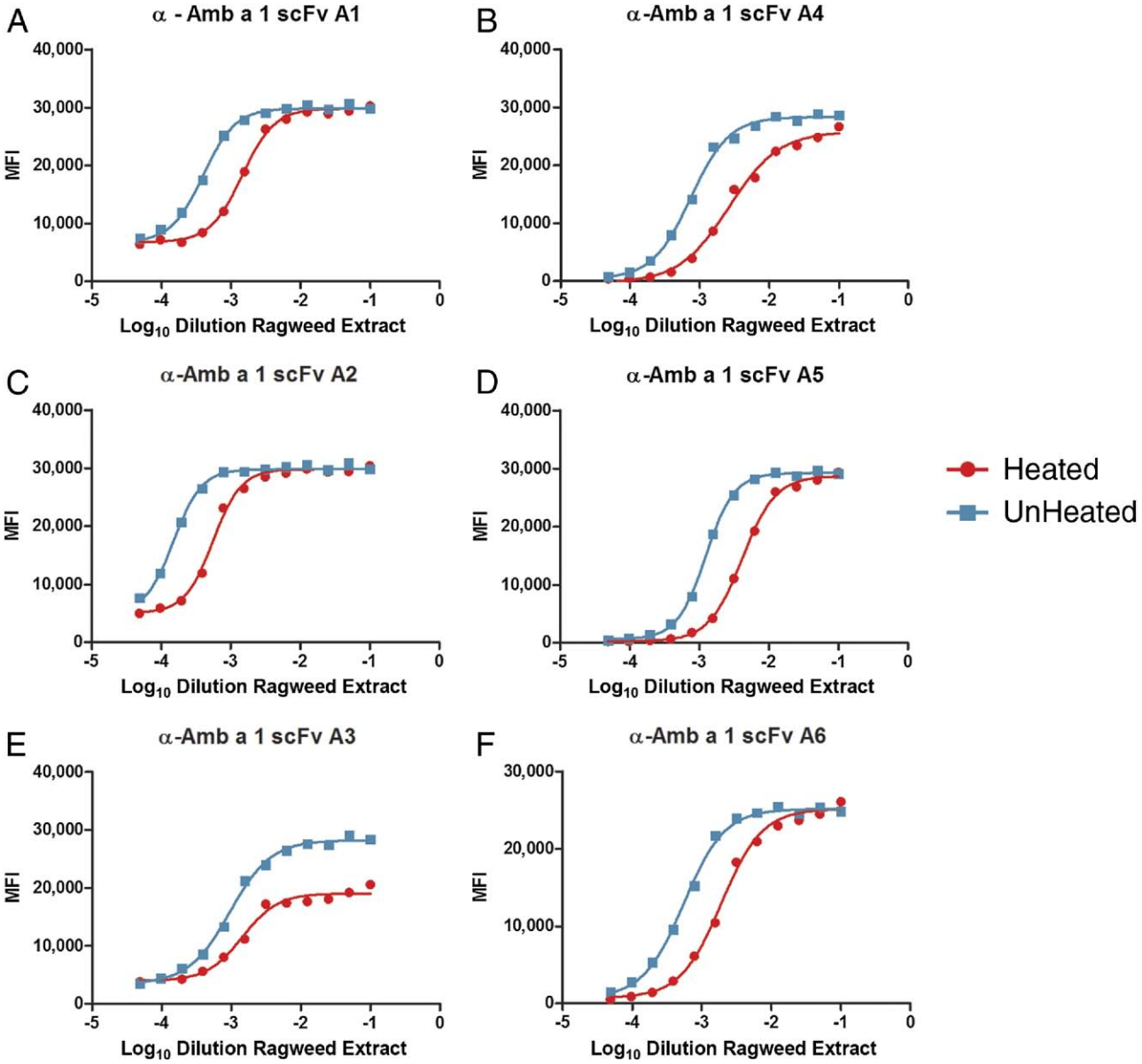


Figure 3. Comparison of dose-response curves of anti–Amb a 1 single-chain fragment-variable (scFv) antibodies binding to short ragweed pollen extracts with and without heat treatment. Although binding of all 6 scFv antibodies was decreased, the binding of anti–Amb a 1 scFv antibodies A3 and A4 were most highly affected. MFI indicates median fluorescence intensity.

ragweed mixed extracts (K and O). For all 6 extracts, the mean Fel d 1 contents calculated by MAEPA were within the 95% CI of the RID assay results.

Heat Treatment of Cat and SRP Allergen Extracts

To further characterize the binding properties of these antibodies, allergen extracts were heat-treated and tested using MAEPA. Fel d 1 is a thermostable allergen, and only a partial loss of antibody binding was observed even after incubation of cat allergen extracts at 97°C for 1 hour (data not shown). The same treatment completely ablated binding of all 6 anti–Amb a 1 antibodies to SRP allergen extracts (data not shown). When SRP extracts were heated for 1 minute at 80°C, all 6 antibodies had reduced binding, with a shift in the dose-response curve of the MAEPA and an apparent loss in allergen potency (Fig 3). The heat treatment lead to an apparent 74% loss in RP of the heat-treated extract compared with the unheated extract. In addition, antibodies A3 and A4 had a decreased maximum level of binding (Figs 3B and E), suggesting a reduced number of available epitopes, usually through irreversible

epitope loss. These data indicate that the epitope recognized by A3 and A4 is more thermolabile than are those bound by A1, A2, A5, and A6.

DISCUSSION

In the United States, existing assays to measure allergen content in complex allergen mixtures (radioallergosorbent test inhibition, competition ELISA, and quantitative skin testing) produce a signal that is an integral of multiple antigen-antibody interactions, but the individual components that contribute to the overall potency signal cannot be resolved. In contrast, existing assays for individual allergens (RID in the United States and 2-site ELISA elsewhere) provide reliable allergen-specific information, but their use as potency assays depends on data that support the immunodominance of the measured allergen molecule in the target patient population. These data are convincing for cat hair and SRP allergen extracts and others, but for many important allergen extracts it seems likely that 3 or more specific allergen molecules are pathogenic in susceptible individuals. Thus, for standardized house dust mite and grass pollen allergen extracts, CBER and US manufacturers have continued to use an assay (competition ELISA) to assess overall allergen content, although its ability to detect specific allergen is limited.[4,5]

The purpose of this project was to develop a microbead-based multiplex assay to accurately measure the allergen content of complex allergen mixtures. Using the MAEPA, we hope to measure the overall allergen content of complex allergen mixtures as an integral of discrete component signals that can be resolved now but could be characterized as specific immunodominant allergens are identified. As a first step, we have now shown that recombinant antibodies against allergens of interest can be used to detect relevant proteins in 2 relatively simple allergen extracts and can accurately and consistently determine the potencies of these mixtures.

We chose to study 12 different scFv antibodies, 6 that recognize cat allergen Fel d 1 and 6 that recognize SRP allergen Amb a 1, based on their binding properties to the allergens of interest. The antibodies were then studied individually and concurrently to assess possible interference. The binding of anti–Fel d 1 antibodies F7 and F9 was significantly affected by the presence of other antibody-bead conjugates, but the binding of the other anti–Fel d 1 antibodies seemed to be relatively unaffected. Likewise, the binding of 2 anti–Amb a 1 antibodies (A3 and A4) decreased significantly in the presence of the other antibodies, but the binding of the other 4 anti–Amb a 1 antibodies was unaffected by the presence or absence of other coated beads (Tables 1 and 2).

Specific interference, presumably a consequence of epitope overlap and differential affinities, was further assessed by mixing bead-bound antibody with soluble, biotin-labeled antibody. These experiments (Figs 1 and 2) allowed us to delineate 2 epitope clusters on Amb a 1 (A3/A4 and A1/A2/A5/A6) and 2 on Fel d 1 (F8 and F7/F9/F10/F11/F12). Multiple antibody-binding epitopes in Fel d 1[16,17] and Amb a 1[18] have been described.

The MAEPA evaluation of RP after heat treatment was as expected from previous work: Fel d 1 is thermostable[19] and Amb a 1 is thermolabile.[20–22] Notably, mild heat treatment of SRP extract resulted in 2 distinct binding patterns of the anti–Amb a 1 antibodies: the binding of A3 and A4 to heat-treated extract was more markedly affected than was the binding of A1, A2, A5, and A6. These results support our observation that A3 and A4 bind to an epitope on Amb a 1 that is distinct from those bound by the other antibodies (Fig 1).

The Food and Drug Administration has adopted the use of a single-allergen assay, RID, for only 2 allergen extracts: SRP and cat hair/pelt. In contrast, the European Medicines Agency[23] has encouraged manufacturers to measure specific relevant allergens "whenever possible," in addition to IgE inhibition assays, to determine extract potencies. In the absence of single unified reference standards, European manufacturers have developed proprietary potency units based on in-house reference standards, and specific potency can vary widely.[24] In the United States, products for which allergen content is controlled only by overall potency assays can have highly variable levels of specific allergens[25,26] as well. In both cases, the clinical significance of this variability is uncertain. The purpose of MAEPA is to provide overall allergen content data concurrently with specific allergen data, even before the immunologic relevance of those allergens is known. In concept, assays such as MAEPA may provide an approach to harmonizing disparate allergen potency paradigms within Europe and between Europe and the United States.

The MAEPA results correlated well with the potencies determined using the RID method for the SRP and cat hair extracts tested. Furthermore, even using these simple extracts and relatively few antibodies, we could discern specific epitopes that exhibited, in the case of Amb a 1, distinct thermal properties. We will now apply this approach to complex allergen mixtures, such as German cockroach allergen extracts[2,25] and mold allergen extracts.

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1. b
2. d
3. c
4. c
5. d