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Detection and Inhibition of IgE for cross-reactive carbohydrate determinants evident in an enzyme-linked immunosorbent assay for detection of allergen-specific IgE in the sera of dogs and cats

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Background – It has been demonstrated recently that immunoglobulin (Ig)E specific for cross-reactive carbohydrate determinants (CCD) is present in the serum of allergen-sensitized dogs and cats, and that these CCD-specific antibodies might confound serological testing.

Hypothesis/Objective – The objective was to document the prevalence of CCD detectable in a monoclonal cocktail-based enzyme-linked immunosorbent assay designed for the detection of allergen-specific IgE in the sera of dogs and cats, and to define a means for successful inhibition of these CCD.

Methods and materials – The incidence of reactivity to bromelain and a commercially available inhibitor of carbohydrate-specific antibodies (RIDA-CCD) was evaluated in 100 dog sera samples before and after inhibition with RIDA-CCD and a proprietary inhibitor containing carbohydrates derived from bromelain (BROM-CCD). Subsequently, sera from 600 dogs and 600 cats were evaluated using a serum diluent with and without BROM-CCD.

Results – Both the RIDA-CCD and BROM-CCD inhibitors demonstrated successful reduction of CCD reactivity, although a more efficient profile of inhibition was evident with BROM-CCD. Mite reactivity in dog and cat sera was largely unaffected; however, substantial inhibition for pollen allergens (trees, grasses and weeds) was shown. After BROM-CCD inhibition, 1% of canine samples and 13% of feline samples were rendered completely negative for allergen reactivity.

Conclusions and Clinical Importance – The results demonstrate that BROM-CCD is effective in reducing reactions with irrelevant carbohydrates, and that inhibition of CCD reactivity might substantially alter the outcome of the *in vitro* reactivity profile used for selection of allergens to be included in an immunotherapeutic regime.

Introduction

Most allergen extracts that are currently used for skin testing as well as for detection of serum immunoglobulin (Ig)E are complex mixtures of allergenic and nonallergenic substances, including proteins, glycoproteins, polysaccharides, lipids, nucleic acids, low molecular weight (LMW) metabolites, salts and pigments.[1] The majority of allergens are proteins or glycoproteins, yet in certain rare circumstances, pure carbohydrates or LMW chemicals can act as allergens.[1,2] In some cases, although a subject's IgE specifically reacts with the carbohydrate moiety of the glycoprotein, the reaction is monovalent in nature and subsequent cross-linking of these epitopes with mast cell-bound IgE and mast cell degranulation does not occur. Consequently *in vivo* reaction with these carbohydrate-specific antibodies does not result in noticeable clinical signs.[3,4] However, reaction with these molecules results in a false positive interpretation for many allergen extracts when evaluated using *in vitro* assays intended for detection of allergen-specific IgE.[5–9] The relevant structure of the epitopes responsible for these false positive reactions has been characterized as a 1,3-fucose linked to the amide nitrogen of an asparagine residue of the protein.[10–12] These specific N-glycans are widely distributed among pollens and invertebrate animals, yet are lacking in mammals[11–13] in which they can be strongly antigenic.

Over the past several years these cross-reactive carbohydrate determinants (CCD) have been defined and characterized in humans,[6–13] where the prevalence of anti-CCD IgE has been estimated in the range 20–70%. This prevalence increases proportionally to an increasing number of pollen sensitivities.[8] Only recently has it been demonstrated that IgE specific for CCDs are present not only in the serum of allergen-sensitized dogs (approximately 24%), but also in the serum of approximately 13% of healthy dogs.[14,15] In addition, although good agreement has been demonstrated between intradermal

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testing (IDT) and serum testing in dogs with no detectable anti-CCD IgE, sera containing anti-CCD IgE showed no agreement with IDT.[16]

Allergen-specific IgE serology is now used widely in human and veterinary clinical practice, where its utility lies in identifying allergens for avoidance or inclusion in allergen-specific immunotherapy (ASIT). However, false positive reactions to CCD limit the use of serology in selecting allergens for these purposes. Reducing the reaction of serum IgE with CCDs during testing has been shown to effectively reduce the incidence of false positive reactions in humans.[9,12] A well-known and characterized enzyme-linked immunosorbent assay (ELISA) for the detection of allergen-specific IgE in dogs and cats is the *mac*ELISA manufactured by Stallergenes Greer (Lenoir, NC, USA).[17] In light of the recent description of CCD in dogs[14–16] Stallergenes Greer has developed a new inhibitor of CCD-reactive antibodies and compared its utility to a commercially available CCD inhibitor. In the present study we evaluate the utility of the two CCD inhibitors on the responses evident in serum samples derived from dogs with clinical allergy. In addition, we define the prevalence of CCD in serum samples of dogs and cats suspected of clinical allergy. We hypothesized that inhibition of CCD would reveal evidence of false positive reactions in sera that demonstrate extensive pollen reactivities.

Methods and materials

Cross-reactive carbohydrate inhibitors

RIDA CCD Inhibitor (product ZA0601) was manufactured by R-Biopharm AG (Darmstadt, Germany) and purchased from its affiliated distributor in the United States (Gold Standard Diagnostic; Davis, CA, USA). This product consists of glycopeptides containing a maximum of four amino acid residues which are purified from the plant glycoprotein bromelain and coupled to human serum albumin. A second preparation of CCD Inhibitor (BROM-CCD) containing the carbohydrate components present in bromelain was prepared in-house and remains a proprietary product of Stallergenes Greer.

Sera samples

The sera samples used throughout were derived from dogs and cats suspected of clinical allergy and had previously been submitted by veterinarians for evaluation using Stallergenes Greer *mac*ELISA for detection of allergen-specific IgE. Samples with a volume >2.0 mL were stored frozen (–20°C) for up to one year before being used in this study. A total of 600 sera derived from dogs which had been evaluated previously for allergen-specific IgE reactivity to mites or pollen allergens on the Stallergenes Greer *mac*ELISA were utilized. Five hundred and ninety-six individual samples known to be reactive to pollens and mites were included; four samples shown to be nonreactive to mites and pollens also were included in the evaluation to ensure the integrity of the assay. Likewise, 600 samples derived from individual cats also were utilized, yet these feline samples were selected randomly from those that had been submitted for diagnostic evaluation using *mac*ELISA (e.g. no prior test results were known). Sufficient volume (>2 mL) for evaluation was the sole criterion for inclusion in the present study.

Sample evaluations – *mac*ELISA

The operational characteristics and procedures for the *mac*ELISAs have been described previously.[17–19] Following incubation of allergen-coated wells with an appropriately diluted serum sample, allergen-specific IgE is detected using a secondary antibody mixture of biotinylated monoclonal anti-IgE antibodies, streptavidin alkaline phosphatase as the enzyme conjugate, and *p*-nitrophenylphosphate (*p*NPP) as substrate reagent. Specific IgE reactivity to the allergens is then estimated by determining the absorbance of each well measured at 405 nM using an automated plate reader. All results are expressed as ELISA absorbance units (EAU) which are background-corrected observed responses expressed as milli absorbance.[17] The buffers used throughout included: (i) well-coating buffer: 0.05 m sodium carbonate bicarbonate buffer, pH 9.6; (ii) wash buffer: phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20, and 0.05% sodium azide; and (iii) serum and reagent diluent buffer: PBS, pH 7.4, containing 1% fish gelatin, 0.05% Tween 20 and 0.05% Proclin. For inhibition evaluations, RIDA-CCD inhibitor was added directly to the sera samples at the defined concentrations recommended by the manufacturer, whereas the BROM-CCD was added to the diluent buffer at the defined concentration of 2.5 mg/mL before addition of serum sample.

Calibrators

Calibrator solutions of predetermined reactivity in ELISA were prepared as three-fold serial dilutions (calibrators 1–5) of a sera pool highly reactive to most pollen allergens; calibrators specific for dogs were prepared from a canine sera pool and calibrators specific for the cat ELISA were prepared from a reactive feline sera pool. The reactivity of these calibrators to a mixture of grass pollen allergens was determined in each respective assay run. Replicates of each were evaluated in each respective assay run and served as a standard response curve for normalizing results observed with the various samples. All results were expressed as EAU.

Allergen panel

The allergen panel for this study included wells previously coated with either RIDA-CCD inhibitor or bromelain at 2.5 μg/mL; this optimal well-coating concentration for each was determined by dilution titration. A four-well North American Screen Panel (NA Screen) containing separate mixtures of mite, grass, weed and tree allergens was used to evaluate serum for the presence of specific IgE reactivity. The respective wells were coated with a mixture of four mites, six grasses, eight weeds or 12 trees at optimum coating concentrations for each allergen in the admixture. The protocol for coating and storage of wells has been described previously.[17–19]

Assays for CCD activity

The first evaluations completed were designed to compare the utility of RIDA-CCD and BROM-CCD inhibitor preparations. Samples were evaluated, in triplicate, for reactivity to the individual inhibitors using wells coated with the individual inhibitors as well as the NA Screen. Simultaneously, the ability of BROM-CCD to inhibit the reactivity of the 100 sera samples to bromelain and RIDA-CCD was assessed; each sample was diluted in assay buffer containing 2.5 mg/mL BROM-CCD and evaluated, in triplicate, on wells coated with either Bromelain or RIDA-CCD. Finally, to compare the utility of BROM-CCD and RIDA-CCD in removing presumed CCD reactivity, these same 100 samples were evaluated on the NA panel of allergens. Following the manufacturer's protocol, the RIDA-CCD inhibitor was added directly to the serum sample and incubated for ≥30 min before the serum samples subsequently were diluted and evaluated by ELISA. BROM-CCD was included in the sample diluent at a concentration of 2.5 mg/mL and samples were diluted directly into this diluent before addition to the allergen-coated wells.

In order to evaluate the incidence of reactivity to CCDs in populations of dogs and cats, individual samples from 600 dogs and 600 cats were evaluated using duplicate wells in ELISA with and without BROM-CCD containing sample diluent.

Statistics

A coefficient of variation was calculated as the ratio of standard deviation and means of the responses observed for the calibrator solutions within different runs. Pearson's correlation statistic was used for comparison among individual allergens. Statistical analyses were conducted using Excel (2016; Microsoft; Redmond, WA, USA).

Results

Overall, the assay variance observed throughout remained indistinguishable from the acceptable range documented previously for this assay.[17–19] The average intraassay variance observed with the calibrators throughout the multiple assay runs was calculated to be 4.8% (range 1.3–14.4%). The interassay variance calculated for the multiple runs was 5.9% (range 4.6–7.4%). As expected, the greatest variance was evident with the individual calibrator solutions at the lower end of the dose–response curve.

The reactivity profiles of the samples to the various mixed allergens used for comparison of RIDA-CCD and BROM-CCD are presented in Table S1. Considering the defined cut-off level of 150 EAU, 45 of the samples were reactive to all allergens tested, 14 were reactive to mites only and 11 samples were not reactive to any of the tested allergens. Seventy samples were documented with positive responses to mites, 68 to grasses, 73 to weeds and 61 to trees. Increasing the cut-off level to 2,400 EAU yielded 29 samples reactive to mites, 32 to grasses, 17 to weeds and 20 to trees. The data presented in Table S1 also provide information regarding the relative reactivity of the samples for the various allergens tested.

Comparison of results for the RIDA-CCD and BROM-CCD inhibitors on the NA screen demonstrate that the magnitude of responses evident with these samples were similar when evaluated on wells individually coated with either RIDA-CCD or BROM-CCD; the Pearson correlation was calculated as 0.878. Almost identical responses ($r = 0.985$) were noted for 72 samples when evaluated on RIDA-CCD- and BROM-CCD-coated wells. However, somewhat divergent responses were noted with the remaining 28 samples ($r = 0.361$). The results (Table S2) for the majority of these 28 sera (≈90%) indicate that the magnitude of responses yielded with the RIDA-CCD-coated wells were approximately three-fold greater than those of BROM-CCD-coated wells. Only three of the samples yielded responses to BROM-CCD that were substantially greater than those evident with RIDA-CCD.

The results presented in Table 1 demonstrate that the majority of reactivity to bromelain in all samples is inhibited by BROM-CCD. Over 80% of the bromelain reactivity was lost in 83% of the samples tested and > 60% of the bromelain reactivity was inhibited in 13% of the samples. Bromelain reactivity was reduced by ≥20% in all samples tested. However, no apparent inhibition (<20%) of RIDA-CCD reactivity was evident in 16% of the samples that were incubated with BROM-CCD. Inhibition of ≥80% of the RIDA-CCD reactivity was demonstrable in only 35% of the samples that were evaluated, and inhibition of RIDA-CDD reactivity in the remaining 49 samples ranged from 21% to 80%.

The results presented in Table 2 demonstrate that the sera reactivity is decreased in some sera samples following inhibition of CCD using either BROM-CCD or RIDA-CCD. Only slight inhibition was evident with mites following inhibition with either BROM-CCD or RIDA-CCD. However, the magnitude of responses evident with pollens indicated that BROM-CCD appears to offer a greater inhibition of reactivity than that evident with RIDA-CCD inhibitor. For example, nearly complete inhibition of grass results occurred in 72% of the samples following inhibition with BROM-CCD, whereas only 29% of the samples were similarly inhibited with RIDA-CCD. Superior inhibition with BROM-CCD also was evident with weed and tree pollens.

Table 1. Reactivity to bromelain- or RIDA-cross-reactive carbohydrate determinant inhibitors (BROM-CCD or RIDA-CCD)-coated wells following inhibition of sera samples with BROM-CCD

	Number of samples/ coated wells	
% Inhibition	BROM-CCD	RIDA-CCD
>80	83	35
61–80	13	22
41–60	1	14
21–40	3	13
0–20	0	16
Total	100	100

The results presented in Table S3 demonstrate that mite reactivity was reduced in some dog serum samples and indicate that cross-reactive carbohydrate components are evident for some mite allergens. However, the majority of samples (approximately 93%) that contain large quantities of anti-mite reactivity (reactivity range >2,400) were not affected by either inhibiting substance. Complete inhibition was lacking in this reactivity category, yet both inhibitors yielded results that indicate the reactivity of one sample was reduced by ≥20% and one sample was reduced by at least 60%.

The results presented in Table S4 demonstrate that substantial reactivity to grass allergens is directed to carbohydrate moieties. Reactivity was dramatically reduced (>50%) with both CCD inhibitors, with BROM-CCD being slightly more efficient in reducing this reactivity. Sample reactivity was substantially removed (>80%) from approximately 72% (49 of 68) of the reactive samples that were treated with BROM-CCD and nearly 30% (20 of 68) of the samples treated with RIDA-CCD.

The results presented in Table S5 document that reactivity to weed pollen allergens also are readily inhibited by specific carbohydrate moieties. BROM-CCD and RIDA-CCD effectively eliminated (>80%) the reactivity of the samples that were shown to be reactive to weed pollens; however, inhibition with BROM-CCD was substantially greater. The reactivity of approximately one half (36 of 73) of the BROM-CCD-treated samples was inhibited substantially (>80%); similar inhibition was noted in <30% (21 of 73) of the RIDA-CCD-treated samples.

The results presented in Table S6 demonstrate that both BROM-CCD and RIDA-CCD effectively reduce a substantial portion of the reactivity to tree pollen allergens present in various sera samples. The reactivity to tree pollen allergens of approximately 45% (28 of 61) of the individual samples was reduced substantially (>80%) following treatment with BROM-CCD, whereas similar inhibition following RIDA-CCD treatment was evident in <25% (15 of 61) of the samples tested.

The reactivity profiles for the 600 canine samples that were evaluated in a sample diluent that contained or was deficient in BROM-CCD are presented in Table S7.

Table 2. Evaluation of sera reactivity to mite, grass, weed and tree allergen extract mixtures following inhibition with either bromain- or RIDA-cross-reactive carbohydrate determinant inhibitors (BROM-CCD or RIDA-CCD, respectively)

				BROM-CCD		RIDA-CCD	
Allergen	Reactivity range	N	% Inhibition	N	% Inhibited	N	% Inhibited
Mite mix							
Dermatophagoides farinae	<150	30		30	NA	30	NA
Dermatophagoides pteronyssinus	>150	70	>80	4	5.7	1	1.4
Tyrophagus putrescentiae			60–80	4	5.7	1	1.4
Acarus siro			40–60	6	8.6	2	2.9
			20–40	7	10.0	9	12.9
			0–20	49	70.0	57	81.4
Grass mix							
Bermuda (*Cynodon dactylon*),	<150	32		32	NA	32	NA
Johnson (*Sorghum halepense*),	>150	68	>80	49	72.1	20	29.4
Kentucky Blue (*Poa pratensis*),			60–80	7	10.3	18	26.5
Meadow Fescue (*Festuca pratensis*)			40–60	2	2.9	5	7.4
Perennial Rye (*Lolium perenne*)			20–40	1	1.5	6	8.8
Quack Grass (*Elytrigia repens*)			0–20	9	13.2	19	27.9
Weed mix							
Cocklebur (*Xanthium strumarium*)	<150	27		27	NA	27	NA
Dock/Sorrel (*Rumex crispus*/*R. acetosella*)	>150	73	>80	36	49.3	21	28.8
English Plantain (*Pantago lanceolata*)			60–80	14	19.2	10	13.7
Lamb's Quarters (*Chenopodium album*)			40–60	7	9.6	11	15.1
Ragweed (*Ambrosia trifida*/*A. artemisiifolia*)			20–40	8	11.0	8	11.0
Goldenrod (*Solidago* sp.)			0–20	8	11.0	23	31.5
Tree mix							
Maple (*Acer negundo*/*A. saccharum*)	<150	39		39	NA	39	NA
Oak (*Quercus velutina*/*Q. rubra*/*Q alba*)	>150	61	>80	28	45.9	15	24.6
Ash (*Fraxinus pennsylvanica*/*F. Americana*)			60–80	19	31.1	9	14.8
Pine (*Pinus strobus*/*P. echinata*/*P. taeda*)			40–60	6	9.8	8	13.1
Cedar (*Juniperus virginiana*)			20–40	1	1.6	10	16.4
Mulberry (*Morus rubra*).			0–20	7	11.5	19	31.1

Considering the positive/negative cut-off (150 EAU) that has been established for this ELISA, only four of the 600 samples were defined as being negative for all of the allergens tested, while 356 of 600 samples (59.3% of all test samples) were defined as being reactive to all tested allergens. Rates of reactivity to individual classes of allergen were as follows: mite reactivity in 552 samples, grass pollen reactivity in 465 samples, weed pollen reactivity in 499 samples and tree pollen reactivity in 409 samples. As expected, the reactivity profile evident in the BROM-CCD-inhibited canine serum samples that contained anti-CCD antibodies was changed. In spite of the inhibition reactivity noted above, all (100%) of the reactive canine samples remained reactive to at least some of the allergens included within the test panel. In fact, 224 of the canine samples remained reactive (EAU> 150) to all of the allergens following inhibition with BROM-CCD and eight of the samples maintained reactivity in excess of 2,400 EAU for all allergens tested. Considering reactivity to pollens only, 507 of the 596 (85.1%) pollen-reactive samples remained reactive to at least one pollen while the number of mites-only reactive samples increased from 76 to 89 (117.1%).

The results presented in Table S7 also provide an estimate of the sample reactivity character in relation to increasing concentration of allergen-specific IgE. In this regard, 206 (24.6%) samples yielded reactivity >300 EAU for all allergens tested, which indicates an approximate three-fold increase in the concentration of specific IgE when compared to the 150 EAU cut-off category. Likewise, a nine-fold increase in specific antibody was evident in 107 (17.8%) of all test samples that yielded a signal of >600 EAU for all allergens tested. Forty-one (6.8%) samples yielded responses in excess of 1,200 EAU and 11 samples (1.8%) yielded responses greater than 2,400 EAU for all allergens tested, which indicates that these sample contain at least 27-fold and 81-fold, respectively, more allergen-specific IgE than the quantity of allergen-specific IgE in those samples that yielded responses in the 150–300 EAU category. The reactivity of the remaining samples spanned the array of potential reactivity profiles within each of the cut-off categories. Considering that the selection criterion dictated that the samples be derived from dogs suspected of clinical allergy and which previously had been shown to be reactive to mites or pollen allergens, this reactivity profile was not unexpected. These results also document that substantial reactivity of a given sample can be reduced dramatically without changing the positive/negative interpretation for a given sample (Table 3).

The results observed following BROM-CCD inhibition of feline sera samples are presented in Table 4. Similar to the canine samples, mite reactivity in approximately 92% (273 of 298) of the feline samples remained unaffected and 6.4% of the samples (19 of 298) exhibited an inhibition response of <40%; only six samples yielded responses indicative of >40% inhibition. However, complete inhibition of grass reactivity was noted in approximately 85% (198 of 232) of the grass-reactive samples, 62% (131 of 210) of the weed-reactive samples and 59%

Table 3. Inhibition of allergen-reactive sera in a population of dog sera samples evaluated with diluent containing 2.5 mg/mL bromain cross-reactive carbohydrate determinant

	Allergen-coated wells							
	Mites		Grasses		Weeds		Trees	
% Inhibition	N	%	N	%	N	%	N	%
>80	0	0.0	142	30.5	50	12.2	77	15.4
60–80	2	0.4	106	22.8	86	20.9	109	21.8
40–60	8	1.4	84	18.1	96	23.4	84	16.8
20–40	28	5.1	49	10.5	65	15.8	86	17.2
<20	514	93.1	84	18.1	114	27.7	144	28.8
Total positive	552	100	465	100	411	100	500	100

Table 4. Inhibition of allergen-reactive sera in a population of cat sera samples evaluated with diluent containing 2.5 mg/mL bromain cross-reactive carbohydrate determinant

	Allergen-coated wells							
	Mites		Grasses		Weeds		Trees	
% Inhibition	N	%	N	%	N	%	N	%
>80	2	0.7	198	85.3	131	62.4	101	59.1
60–80	1	0.3	20	8.6	48	22.9	31	18.1
40–60	3	1.0	5	2.2	17	8.1	20	11.7
20–40	19	6.4	4	1.7	8	3.8	13	7.6
<20	273	91.6	5	2.2	6	2.9	6	3.5
Total Positive	298	100	232	100	210	100	171	100

(101 of 171) of the tree-reactive samples. Substantial inhibition (60–80%) also was noted in 8.6% of the grass-reactive samples, 22.9% of the weed-reactive samples and 18.1% of the tree-reactive samples. Inhibition of reactivity following BROM-CCD was lacking in only five of 232 (2.2%) of grass-reactive samples, six of 210 (2.9%) of weed-reactive samples, and six of 171 (3.5%) of tree-reactive samples.

Only 350 of the feline samples were identified as being reactive to any of the allergens tested (EAU > 150), while 250 were defined to be nonreactive to all of the allergens tested (Table S8). Reactivity to all allergens tested was noted in 142 samples (23.7%) and mite-only reactions were yielded for 108 samples (18.0%); the remainder of the samples spanned the array of potential reaction profiles. Mite reactivity was noted in 301 individual samples, while 232 samples reacted to grasses, 201 samples reacted with weeds and 169 samples reacted with trees. Because the test samples for the cat population were selected randomly from samples received and presumed to be allergic, the overall reaction profile might represent the expected reaction profile for a population of cat samples submitted for *in vitro* testing. The reactivity profile evident for the BROM-CCD-inhibited cat samples (Table S8) demonstrates that 305 of the 350 (87.1%) reactive cat samples remained reactive to at least one of the allergens in the test panel. However, only 69 of 242 samples remained reactive to at least one pollen and the number of mites-only reactive samples more than doubled from 108 to 236 (218.5%).

Discussion

It has been documented previously that a three-fold increase in specific IgE results in a two-fold increase in the EAU magnitude.[17–19] Thus, the relative quantity of specific IgE needed to yield a response of 300 EAU will be approximately three times the amount required to yield a positive response at the cut-off level of 150 EAU. The quantity of specific IgE required to yield a response of 2,400 EAU will be ≥81 times that which is required to generate a signal of 150 EAU and a 200-fold increase in specific IgE will be required to reach the upper limit of detection for the assay. Overall, the selected sample populations (Tables S1, S7 and S8) contain reactivity to each of the allergens that spans the range of reactivity defined for the assay.

The observation that a substantial number of samples yield a signal of greater magnitude when evaluated on RIDA-CCD-coated wells than on BROM-CDD-coated wells (Table S2) indicates that the number of carbohydrate-reactive sites are greater on the RIDA-CCD inhibitor. However, the reactivity of these samples to grasses, weeds and trees are more readily inhibited with BROM-CCD (Tables 1 and 2). Considering the difference in quantity of inhibitory substances used, the apparent discrepancies in results are not surprising. On the one hand, the amount of BROM-CCD (2.5 mg/mL) included in the inhibitory diluent is likely in great excess of what is required. On the other, the recommended amount of RIDA-CCD inhibitor for use results in a serum concentration of 20 μg/mL before dilution (1:6) for evaluation in the assay; hence, the concentration in the allergen reaction mixture is reduced to 3.33 μg/mL. Although each of the RIDA-CCD inhibitory molecules is purported to contain approximately four carbohydrate epitopes, it is unlikely that this amounts to the same number of accessible reactive epitopes that are presented in the BROM-CCD used at 2.5 mg/mL. Thus, samples with carbohydrate cross-reactive antibodies will more likely be completely inhibited

with BROM-CCD than with RIDA-CCD, especially those samples that contain relatively large quantities of anti-carbohydrate-specific IgE. This likelihood is readily apparent in the results presented in Tables S3-S6 where the reactivity response is divided into categories that are indicative of the relative quantity of specific IgE present. These categories are defined by the dose–response profile evident in this ELISA which indicates that a two-fold increase in signal requires a three-fold increase in specific IgE.[17–19] Collectively, these results demonstrate that BROM-CCD effectively results in a more efficient inhibition of the CCD reactivity to pollen allergens and warrants adoption of this inhibitor for routine evaluation of sera samples.

Considering the 150 EAU cut-off defined for the assay and comparing the BROM-CCD inhibited and noninhibited dog sera sample results (Table S7), reactivity to all allergens in the panel was noted in 224 of 356 (62.9%) of the canine samples that were inhibited with BROM-CCD. A similar reduction in the incidence of reactive samples for all (higher) cut-off categories also was noted. The number of samples that were nonreactive to all test allergens at the 150 EAU cut-off remained unchanged following evaluation in the presence of BROM-CCD. In both cases <1% (four of 600) of the samples were classified as negative to all allergens. However, the number of samples nonreactive to all allergens increased as the EAU value was increased. For example, 12 of the population samples were defined as nonreactive at the 300 EAU cut-off level before BROM-CCD inhibition and 15 samples were similarly characterized following inhibition with BROM-CCD. This trend continued at each higher EAU cut-off category, such that the number of samples classified as entirely negative in each category increased approximately 1.7-fold following inhibition with BROM-CCD. These results and those observed with the remainder of the samples are consistent with the hypothesis that individual allergen reactivity in some samples is inhibited while reactivity of the other allergens is not inhibited, or inhibited to a lesser degree. However, it is equally likely that these results also might imply that when the level of allergen-specific IgE is extremely high, it is more likely that a substantial portion of that reactivity is due to CCD rather than "true" sensitization to the pollen allergens. Yet, it is important to note the possibility that carbohydrate moieties might, in some cases, actually represent allergenic epitopes. In such cases, use of CCD inhibitors for *in vitro* testing for allergen-specific IgE can potentially result in false negative interpretation.

Varying degrees of inhibition to the various classes of allergens can range from total inhibition to no inhibition. Thus, the reactivity profile that is evident in a sample following inhibition could logically fall within a different reactivity profile. This is readily evident in the responses noted for the mite and grass-only reactivity profile, where the number of samples falling into this category increased three- to 10-fold at all EAU cut-off levels after BROM-CCD inhibition. As expected, total inhibition of all pollen reactivity results in a measurable increase (approximately 20%) in the number of samples that are reactive to mite allergens only. In light of these results, it is obvious that definition of an absolute effect of inhibition of CCD must be at the individual sample level and the defined allergen reactivity. Although the proportion of samples with reactivity to at least one allergen group (at the defined assay cut-off of 150 EAU) remains essentially unaltered at the population level, the reactivity profile of each individual sample following inhibition with BROM-CCD will be a better reflection of the hypersensitivity profile for that animal. Consequently, selection of the allergens for inclusion in an immunotherapeutic regimen will be more precise and ensure that nonessential allergens are not included in the treatment prescription. Although the results of this study demonstrate CCD reactivity using mite, grass, weed and mixtures, we have demonstrated subsequently that responses to >100 individual grass, weed and tree allergens, in some samples, are inhibited dramatically with BROM-CCD (data not shown).

Comparing the results for the cat population before and after (Table S8) inhibition with BROM-CCD demonstrates that substantial reactivity to CCDs occurs within cat serum and that BROM-CCD effectively removes that reactivity. After CCD inhibition, 45 of 350 (13%) formerly positive serum samples lost all reactivity at the assay cut-off of 150 EAU. The dramatic decrease in the number of samples that are reactive to all allergens following inhibition, combined with the observed substantial increase in the number of mites only reactive samples following BROM-CCD inhibition indicates that a substantial proportion of reactivity to pollen allergens in the cat population is due to CCD epitopes.

The observation that IgE antibodies to the cross-reactive carbohydrate determinants are not involved with skin reactions[4,6,9,12] indicates that better agreement between IDT and *in vitro* assessments of allergen reactivity might result from a comparison with the results following inhibition of carbohydrate-specific reactivity. In a preliminary study of 31 dogs, it was demonstrated that agreement with IDST and IgE serology was markedly enhanced following blocking of the anti-CCD IgE antibodies.[16] A multi-institute study to address this specific concept is currently underway using a substantially larger number of dogs.

Collectively, the results presented herein demonstrate that reactivity to CCD is present in a large proportion of dog and cat sera submitted for evaluation of allergen sensitization. The results also confirm the hypothesis that CCD may result in false positive reactivity when serum samples are evaluated for allergen-specific IgE and demonstrate that BROM-CCD is effective in reducing this nonspecific reactivity. Nevertheless, inhibition of CCD reactivity might substantially alter the outcome of the *in vitro* reactivity profile used for selection of allergens to be included in an ASIT regime.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Reactivity profile and incidence of reaction for a population of 100 dog sera samples* used for comparison of individual wells coated with two CCD inhibitors.
Table S2. Difference in magnitude of response for individual dog sera samples used for comparison of wells coated independently with two differing CCD inhibitors.
Table S3. Reactivity of dog sera samples to a mite mixture containing *D. farinae*, *D. pteronyssinus*, *T. putrescentiae* and *A. siro* following inhibition with either BROM-CCD or RIDA-CCD.
Table S4. Reactivity of dog sera to a grass extract mixture following inhibition with either BROM-CCD or RIDA-CCD.
Table S5. Reactivity of dog sera to a weed extract mixture following inhibition with either BROM-CCD or RIDA-CCD.
Table S6. Reactivity of dog sera to a tree extract mixture following inhibition with either BROM-CCD or RIDA-CCD.
Table S7. Reactivity profile and incidence of reaction at varying cutoff levels for a population of dog sera samples evaluated with or without BROM-CCD in the diluent.
Table S8. Reactivity profile and incidence of reaction at varying cut-off levels for a population of cat sera samples evaluated with or without BROM-CCD in the diluent.

Résumé

Contexte – Il a été démontré récemment que les immunoglobulines (Ig)E spécifiques des CCD (cross-reactive carbohydrate determinants) sont présents dans le serum des chiens et chats sensibilisés aux allergènes et que ces anticorps spécifiques-CDD peuvent perturber les tests sérologiques.

Hypothèses/Objectifs – L'objectif était de documenter la prévalence des CDD détectables dans un test ELISA pour mélange monoclonal pour la détection des IgE spécifiques d'allergènes dans les sera de chiens et chats et de définir des moyens d'inhibition efficace de ces CDD.

Matériels et méthodes – L'incidence de la réactivité à la bromelaïne et un inhibiteur des anticorps spécifiques des carbohydrates (RIDA-CCD) disponible commercialement a été évalué pour 100 sera de chien avant et après inhibition avec RIDA_CCD et un inhibiteur exclusif contenant des carbohydrates dérivés de la bromélaïne (BROM-CDD). Ensuite, les sera de 600 chiens et 600 chats ont été évalués à l'aide de diluant sérique avec ou sans BROM-CCD.

Résultats – Les deux inhibiteurs RIDA-CCD et BROM-CCD ont montré une efficacité dans la réduction de la réactivité des CCD, bien que un profil d'inhibition plus efficace soit évident pour BROM-CCD. La réactivité des acariens dans les sera de chien et chat était largement non affectée ; cependant, une inhibition conséquente des allergènes de pollens (arbres, herbacées, graminées) a été montrée. Après inhibition BROM-CCD, 1% des échantillons canins et 13% des échantillons félins ont été complètement négativés pour la réactivité allergénique.

Conclusions et importance clinique – Les résultats démontrent que BROM-CCD est efficace pour réduire les réactions avec des carbohydrates non-pertinents, et que l'inhibition de la réactivité des CCD pourrait altérer de façon importante les résultats des profils de réactivité *in vitro* utilisés pour la sélection des allergènes à inclure dans une immunothérapie.

Resumen

Introducción – se ha demostrado recientemente que la inmunoglobulina (Ig) E específica para determinantes de carbohidratos de reacción cruzada (CCD) está presente en el suero de perros y gatos sensibilizados a alérgenos, y que estos anticuerpos específicos de CCD pueden confundir las pruebas serológicas.
Hipótesis/Objetivo – el objetivo era documentar la prevalencia de CCD detectable en un ensayo inmunoabsorbente ligado a enzimas basado en cócteles monoclonales diseñado para la detección de IgE específica de alérgenos en el suero de perros y gatos, y definir un medio para una inhibición eficiente de estos CCD.
Métodos y materiales – se evaluó la incidencia de reactividad a la bromelaína y a un inhibidor de anticuerpos específicos de carbohidratos (RIDA-CCD) disponible en el mercado en 100 muestras de suero de perro antes y después de la inhibición con RIDA-CCD y un inhibidor patentado que contiene carbohidratos derivados de la bromelaína (BROM-CCD). Posteriormente, se evaluaron los sueros de 600 perros y 600 gatos utilizando un diluyente de suero con y sin BROM-CCD.
Resultados – tanto los inhibidores de RIDA-CCD como de BROM-CCD demostraron una reducción exitosa de la reactividad de CCD, aunque fue evidente un perfil de inhibición más eficiente con BROM-CCD. La reactividad de los ácaros en los sueros de perros y gatos no se vio afectada en gran medida; sin embargo, se demostró una inhibición sustancial de los alérgenos del polen (árboles, pastos y malas hierbas). Después de la inhibición de BROM-CCD, el 1% de las muestras caninas y el 13% de las muestras felinas resultaron completamente negativas para la reactividad de alérgenos.
Conclusiones e importancia clínica – los resultados demuestran que BROM-CCD es eficaz para reducir las reacciones con carbohidratos irrelevantes y que la inhibición de la reactividad del CCD podría alterar sustancialmente el resultado del perfil de reactividad *in vitro* utilizado para la selección de alérgenos que se incluirían en un régimen inmunoterapéutico.

Zusammenfassung

Hintergrund – Es wurde kürzlich gezeigt, dass Immunglobulin (Ig)E spezifisch gegen kreuzreaktive Kohlenhydratdeterminanten (CCD) im Serum Allergen-sensibilisierter Hunde und Katzen vorkommt und dass diese CCD-spezifischen Antikörper die serologische Testung stören könnten.
Hypothese/Ziel – Das Ziel dieser Studie war es, die Prävalenz von CCD zu dokumentieren, die in einem monoklonalen Cocktail-basierten Enzym-linked Immunosorbent Assay designed für die Detektion von Allergen-spezifischen IgE in Sera von Hunden und Katzen erkannt wurden und einen Weg zur erfolgreichen Inhibition dieser CCD zu definieren.
Methoden und Materialien – Die Inzidenz der Reaktivität auf Bromelain und einen kommerziell erhältlichen Inhibitor von Kohlenhydrat-spezifischen Antikörpern (RIDA-CCD) wurde in 100 Hunde Serumproben vor und nach der Inhibition mit RIDA-CCD und einem eigenen Inhibitor, welcher Kohlenhydrate aus Bromelein (BROM-CCD) enthielt, evaluiert. In der Folge wurden Sera von 600 Hunden und 600 Katzen mittels Serumverdünner mit und ohne BROM-CCD evaluiert.
Ergebnisse – Sowohl die RIDA-CCD als auch die BROM-CCD Inhibitoren zeigten eine erfolgreiche Reduzierung der CCD Reaktivität, obwohl ein effizienteres Inhibitionsprofil mit BROM-CCD offensichtlich war. Die Milbenreaktivität in Hunde- und Katzen Sera war weitgehend unbeeinflusst; es konnte jedoch eine deutliche Inhibition für Pollenallergene (Bäume, Gräser und Unkräuter) gezeigt werden. Nach BROM-CCD Inhibition zeigten sich 1% der Hundeproben und 13% der Katzenproben keine Allergenreaktivität mehr.
Schlussfolgerungen und klinische Bedeutung – Die Ergebnisse zeigen, dass BROM-CCD bei der Reduzierung von Reaktionen mit irrelevanten Kohlenhydraten wirksam sind, und dass die Inhibition der CCD Reaktivität das Ergebnis des *in vitro* Reaktivitätsprofils, welches zur Auswahl der Allergene, die zur Immuntherapie eingesetzt werden, drastisch verändern könnte.

要約

背景 – 近年、交差反応性炭水化物決定基（CCD）に特異的な免疫グロブリン（Ig）Eがアレルゲン感作犬および猫の血清中に存在し、これらのCCD特異的抗体が血清学的検査を混乱させる可能性があることが示された。
仮説/目的 – 本研究の目的は、犬および猫血清中のアレルゲン特異的IgE検出用に設計された、モノクローナルカクテルベースの酵素結合免疫吸着アッセイ法で検出可能なCCDの保有率を記録し、これらのCCD阻害を成功させる手段を定義することであった。
材料と方法 – ブロメラインおよび市販の炭水化物特異的抗体阻害剤（RIDA-CCD）への反応の発生率を、RIDA-CCDおよび独自のブロメライン由来炭水化物含有阻害剤（ BROM-CCD）による阻害前後に採取し

た犬100頭の血清サンプルで評価した。続いて、BROM-CCDを使用する場合と使用しない場合の血清希釈液を使用して、犬600頭および猫600頭の血清を評価した。
結果 – RIDA-CCD阻害剤およびBROM-CCD阻害剤の両方でCCD反応性の低下が成功したことが示されたが、阻害のより効率的なプロファイルはBROM-CCDで明らかであった。犬および猫血清におけるダニの反応性はほとんど影響を受けなかった。しかし、花粉アレルゲン（木、草、雑草）に対する実質的な抑制が示された。 BROM-CCD阻害後、犬サンプルの1%および猫サンプルの13%が、アレルゲン反応性について完全に陰性になった。
結論と臨床的重要性 – 結果は、BROM-CCDが無関係な炭水化物との反応を減らすのに効果的であり、CCD反応性の阻害が、免疫療法レジメに含まれるアレルゲンの選択に使用されるin vitro反応性プロファイルの結果を大幅に変える可能性があることを示している。

摘要
背景 — 最近已经证明，交叉反应性碳水化合物决定簇(CCD)特异性免疫球蛋白(Ig)E存在于过敏原致敏犬和猫的血清中，并且这些CCD特异性抗体可能混淆血清学检测。
假设/目的 — 目的是记录在基于单克隆鸡尾酒的酶联免疫吸附试验中可检测到CCD的概率，该试验设计用于检测犬和猫血清中的过敏原特异性IgE，并定义成功抑制这些CCD的方法。
方法和材料 — 100份犬血清样本，在用RIDA-CCD和含源自菠萝蛋白酶的碳水化合物的专有抑制剂(BROM-CCD)抑制前后，评价了对菠萝蛋白酶和市售碳水化合物特异性抗体抑制剂(RIDA-CCD)的反应性发生率。随后，使用含和不含BROM-CCD的血清稀释剂评价600只犬和600只猫的血清。
结果 — RIDA-CCD和BROM-CCD抑制剂均证明可成功降低CCD反应性，尽管BROM-CCD的抑制特征更有效。犬和猫血清中的螨虫反应性基本不受影响；然而，显示对花粉过敏原（树木、草和杂草）具有显著抑制作用。BROM-CCD抑制后，1%的犬样本和13%的猫样本过敏原反应性完全为阴性。
结论和临床重要性 — 结果表明，BROM-CCD可有效减少与无关碳水化合物的反应，并且，在过敏原体外试验中抑制CCD反应，可能会显著优化免疫治疗方案中的的选项。

Resumo
Contexto – Demonstrou-se recentemente que a imunoglobulina (Ig) E específica para determinantes de carboidratos com reatividade cruzada (CCD) está presente no soro de cães e gatos sensibilizados com alérgenos, e que esses anticorpos específicos para CCD podem confundir os testes sorológicos.
Hipótese/Objetivo – O objetivo foi documentar a prevalência de CCD detectável em um ensaio imunoabsorvente enzimático baseado em coquetel monoclonal projetado para a detecção de IgE alérgeno-específica em soros de cães e gatos, e definir uma forma de inibição bem-sucedida desses CCD.
Métodos e materiais – A incidência de reatividade à bromelaína e um inibidor comercialmente disponível de anticorpos específicos de carboidratos (RIDA-CCD) foi avaliada em 100 amostras de soros de cães antes e após a inibição com RIDA-CCD e um inibidor de carboidratos derivados da bromelaína (BROM-CCD). Posteriormente, soros de 600 cães e 600 gatos foram avaliados usando um diluente de soro com e sem BROM-CCD.
Resultados – Ambos os inibidores RIDA-CCD e BROM-CCD demonstraram redução bem-sucedida da reatividade do CCD, embora um perfil de inibição mais eficiente tenha sido mais evidente com o BROM-CCD. A reatividade dos ácaros em soros de cães e gatos não foi afetada; entretanto, foi demonstrada inibição substancial para alérgenos de pólen (árvores, gramíneas e ervas daninhas). Após a inibição do BROM-CCD, 1% das amostras caninas e 13% das amostras felinas tornaram-se completamente negativas para reatividade ao alérgeno.
Conclusões e importância clínica – Os resultados demonstram que o BROM-CCD é eficaz na redução de reações a carboidratos irrelevantes e que a inibição da reatividade do CCD pode alterar substancialmente o resultado do perfil de reatividade *in vitro* usado para a seleção de alérgenos a serem incluídos em um regime imunoterapêutico.

