ImmunoCAP cellulose displays cross-reactive carbohydrate determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels

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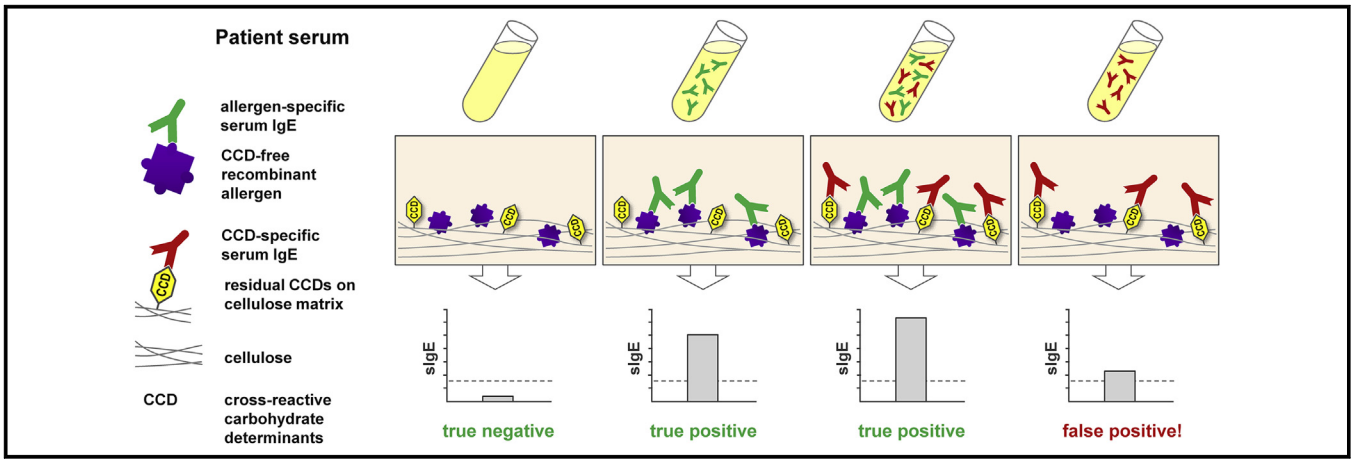
GRAPHICAL ABSTRACT



Background: Cross-reactive carbohydrate determinants (CCDs) in plants and insect venoms are a common cause of irrelevant positive test results during *in vitro* allergy diagnosis. We observed that some CCD-positive sera show nonspecific IgE binding even with CCD-free recombinant allergens when using the Phadia ImmunoCAP platform.

Objective: We investigated whether cellulose used as an allergen carrier in ImmunoCAP harbors residual N-glycans, causing nonspecific background binding in CCD-positive sera.

Methods: IgE binding to 6 samples of blank ImmunoCAPs coupled to either streptavidin (SA-CAP-1 or 2) or nonallergenic maltose-binding protein (MBP; MBP-CAP-1 to 4) and binding to a panel of 4 recombinant allergens were compared in CCD-positive sera before and after inhibition with a CCD inhibitor (MUXF^3–human serum albumin).

Results: Of 52 CCD-positive sera (bromelain, 1.01-59.6 kilounits of antigen per liter [kU$_A$/L]) tested on SA-CAP-1, 35 (67%) showed IgE binding of greater than 0.35 kU$_A$/L (0.41-4.22 kU$_A$/L). Among those with anti-CCD IgE levels of greater than 7.0 kU$_A$/L, 90% (26/29) were positive. IgE binding to SA-CAP-1 correlated with IgE binding to bromelain ($r = 0.68$) and was completely abolished by serum preincubation with the CCD inhibitor (n = 15). Binding scores with SA-CAP-2 and MBP-CAP-1 to MBP-CAP-4 were generally lower but strongly correlated with those of SA-CAP-1 and bromelain. IgE reactivity of 10 CCD-positive sera (14.0-52.5 kU$_A$/L) with the recombinant allergens rPhl p 12, rFel d 1, rAra h 2, and rPru p 3 was positive to at least 1 allergen in 8 of 10 (0.36-1.63 kU$_A$/L) and borderline in 2 of 10 (0.21-0.25 kU$_A$/L). Binding correlated with antibody binding to bromelain ($r = 0.61$) and to all blank ImmunoCAPs ($r > 0.90$) and could be completely blocked by the CCD inhibitor. Overall, mean background binding to cellulose CCDs corresponded to 2% to 3% of the reactivity seen with bromelain.

Conclusions: Cellulose used as a solid-phase allergen carrier can contain varying amounts of CCDs sufficient to cause false-positive test results up to 2 kU$_A$/L with nonglycosylated recombinant allergens in patients with high levels of anti-CCD IgE antibodies. (J Allergy Clin Immunol 2018;141:372-81.)

Key words: *CCDs, cellulose, component-resolved diagnosis, cross-reactive carbohydrate determinants,* in vitro *diagnosis, IgE measurement, ImmunoCAP, N-glycans*

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Abbreviations used
CCD: Cross-reactive carbohydrate determinant
CRD: Component-resolved diagnosis
HSA: Human serum albumin
kU_A/L: Kilounits of antigen per liter
LTP: Lipid transfer protein
MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight
MBP: Maltose-binding protein
MS: Mass spectrometry

N-glycans constitute a highly diverse group of asparagine-linked oligosaccharides on eukaryotic glycoproteins involved in proper protein folding, stability, intracellular traffic, secretion, and function.[1] The universal core structure of N-glycans, consisting of 2 molecules of GlcNAc (N-acetylglucosamine) and 1 distal mannose, is typically modified by the attachment of 1 or more additional sugar residues. With respect to allergy, 2 particular core modifications not present in human tissues and thus highly immunogenic have been identified as a target of specific IgE: α(1,3)-fucosylation of the innermost GlcNAc residue (found in plants, insects, and parasitic worms) and (less importantly) β(1,2)-xylosylation of the terminal mannose residue (found in plants and mollusks).[2] Because of the widespread occurrence of these allergenic N-glycans on otherwise unrelated glycoproteins of animal and plant origin, the term cross-reactive carbohydrate determinants (CCDs) has been coined.[3]

The significance of N-glycans in allergy does not consist so much of representing important triggers of allergic reactions but rather of representing IgE-reactive structures interfering with proper *in vitro* diagnosis. Despite a limited number of studies advocating a functional role of CCDs comparable with that of peptide epitopes, there is broad agreement from experimental studies and clinical practice that CCDs largely lack any clinical relevance but unfavorably obscure *in vitro* diagnosis.[4,5] Different strategies have been recommended in the past to overcome the problem, including screening for serum anti-CCD IgE antibodies, as well as CCD inhibition, by using natural extracts or specifically designed semisynthetic CCD inhibitors.[6] The recent implementation of recombinant molecule-based allergy diagnosis has solved many of the diagnostic problems associated with N-glycans. As a consequence, despite glycosylated allergen extracts still being used widely, the CCD issue seems to gradually disappear from the clinician's view.

The Phadia ImmunoCAP (Thermo Scientific, Uppsala, Sweden) is a well-established *in vitro* test system for measurement of allergen-specific serum IgE launched in 1989, which since then has been used frequently as a reference assay when exploring the analytic performance of other diagnostic platforms.[7,8] The test setup is based on a solid-phase allergen carrier consisting of polymerized cellulose to which the allergens are covalently bound. The major innovation of ImmunoCAP compared with predecessor tests also using cellulose as an allergen carrier was use of a 3-dimensional encapsulated cellulose sponge instead of 2-dimensional paper discs, allowing coupling of much higher allergen amounts as a prerequisite for improved sensitivity.[9]

Apart from hundreds of allergen extracts available for conventional IgE determination, ImmunoCAP is the leading singleplex IgE assay with respect to component-resolved diagnosis (CRD), with more than 100 recombinant or purified allergens on hand. Routinely using these novel molecular tools in daily practice, over time, we encountered some peculiar cases of CCD-positive patients seemingly reacting in a nonspecific manner with an unexpectedly wide range of recombinant allergens but not with the same allergens in the ImmunoCAP ISAC microarray. Exemplary CCD inhibition in some of these sera revealed that IgE reactivity against the recombinant allergens was completely abolished by the CCD blocker. This prompted us to investigate in a systematic manner the potential role of the ImmunoCAP cellulose matrix as the origin of the observed carbohydrate-directed reactivity.

METHODS

Reactivity of CCD-positive sera with allergen-free streptavidin CAPs

To test the hypothesis that serum anti-CCD IgE antibodies can bind to N-glycans present on the ImmunoCAP cellulose allergen carrier, 52 CCD-positive sera with varying levels of anti-CCD antibodies (bromelain, 1.01-59.6 kilounits of antigen per liter [kU_A/L]) were tested on a blank ImmunoCAP in which the cellulose sponge was coupled with streptavidin only (SA-CAP-1). Streptavidin-conjugated ImmunoCAPs are offered by Phadia as a research tool allowing the coupling of self-prepared biotinylated allergens.[10] The investigated batch (lot 7204) was purchased from Phadia in 2005 and continuously stored since then at 4°C.

Fifteen of the CCD-positive sera were also tested on SA-CAP-1 after serum preincubation with a CCD blocker (see below). Inhibition with recombinant streptavidin (Roche Diagnostics, Mannheim, Germany) at a concentration of 100 μg/mL was carried out in 3 selected serum samples to exclude antibody binding to streptavidin.

CCD inhibition

Blocking of IgE antibodies against CCDs was done by preincubating sera with a commercially available semisynthetic CCD inhibitor made up of purified $MUXF^3$ glycopeptides obtained from pineapple stem bromelain and coupled to human serum albumin ($MUXF^3$-HSA; www.proglycan.com). On average, the neoglycoprotein carries 7 $MUXF^3$ glycans per HSA molecule[6] and was used in a concentration of 20 μg/mL of serum, as recommended by the manufacturer. In addition, dose-dependent inhibition (0.2, 2.0, and 20.0 μg/mL) was carried out in selected sera.

CCD-dependent reactivity with ImmunoCAP recombinant allergens

Ten sera with high levels of anti-CCD IgE antibodies (14.0-52.5 kU_A/L) were tested on a panel of 4 structurally unrelated recombinant allergens, rBet v 2 (birch pollen profilin), rPru p 3 (peach nonspecific lipid transfer protein [LTP]), rFel d 1 (cat uteroglobulin), and rAra h 2 (peanut 2S albumin), with and without prior CCD inhibition to demonstrate that CCD-positive sera have false-positive test results with nonglycosylated recombinant molecules in the ImmunoCAP system because of interference with cellulose glycans.

False-positive results with recombinant allergens in patients with Hymenoptera venom allergy

Double positivity to honeybee and wasp venom is a common obstacle in the diagnosis of insect sting allergy and often caused by CCDs.[11,12] Seven CCD-positive patients with a history of anaphylaxis after a Hymenoptera sting (*Vespula* species, n = 5; honeybee, n = 2) and 1 CCD-positive control subject

TABLE I. IgE reactivity with recombinant allergens tested by using ImmunoCAP singleplex and the ISAC microarray before and after inhibition with MUXF^3-HSA in 2 patients with high anti-CCD IgE levels

	ImmunoCAP (kU/L)		ISAC microarray (ISU-E)	
	Before CCD inhibition	After CCD inhibition	Before CCD inhibition	After CCD inhibition
Case 1				
Bromelain/MUXF^3-CCD	44.60	0.38	8.53	<0.3
Almond	49.00	0.24	—	—
rAra h 1 (peanut 7S)	0.42	0.04	<0.3	<0.3
rAra h 2 (peanut 2S)	1.63	0.00	<0.3	<0.3
rAra h 3 (peanut 11S)	1.16	0.01	<0.3	<0.3
rAra h 9 (peanut LTP)	0.53	0.00	<0.3	<0.3
rCor a 8 (hazelnut LTP)	0.58	0.00	<0.3	<0.3
rBet v 1	0.94	0.02	<0.3	<0.3
rBet v 2	1.12	0.04	<0.3	<0.3
rFel d 1	—	—	2.13	2.14
nDer f 1	—	—	0.81	1.00
rDer f 2	—	—	3.95	4.03
Case 2				
Bromelain/MUXF^3-CCD	24.60	1.17	20.0	<0.3
Vespula species venom	34.00	17.40	—	—
rVes v 1	11.80	9.72	—	—
rVes v 5	48.70	47.6	6.2	8.9
Honeybee venom	27.60	1.25	—	—
rApi m 1	1.63	0.11	<0.3	<0.3
Birch pollen	19.60	0.11	—	—
rBet v 1	0.75	0.03	<0.3	<0.3
rBet v 2	0.74	0.04	<0.3	<0.3
rBet v 4	1.00	0.00	<0.3	<0.3
Grass pollen	25.00	1.11	—	—
rPhl p 1	0.42	0.04	<0.3	<0.3
rPhl p 7	0.47	0.02	<0.3	<0.3
rPhl p 12	0.88	0.05	<0.3	<0.3
Mugwort pollen	21.60	0.21	—	—
nArt v 1	1.28	0.02	<0.3	<0.3
Ragweed pollen	25.7	0.49	—	—
nAmb a 1	1.94	0.04	<0.3	<0.3

Apart from strong inhibition of antibody binding to bromelain and allergen extracts, the CCD inhibitor also inhibits antibody binding to recombinant allergens positive in ImmunoCAP (but negative in ISAC), indicating that this reactivity is due to background binding with cellulose glycans.

without venom allergy were tested by using ImmunoCAP with whole venoms and the recombinant major venom allergens rApi m 1, rVes v 1, and rVes v 5 before and after serum inhibition with the CCD blocker.

Batch-to-batch variation in cellulose CCDs

Because CCD-dependent antibody binding to recombinant ImmunoCAP allergens varied to some extent within single patients, we explored whether this might be due to batch-to-batch variation between different cellulose samples. Therefore 15 sera were additionally tested on a second lot of streptavidin-conjugated CAPs (SA-CAP-2, lot BZV0F) as well as on 4 lots of blank ImmunoCAPs coupled with nonallergenic maltose-binding protein (MBP; MBP-CAP 1-4; production years, 2012-2013). All material was kindly provided by Thermo Fisher Scientific. In addition, CCD inhibition was carried out with SA-CAP-1 and MBP-CAP-1 to MBP-CAP-4 in selected sera.

Distribution of anti-CCD IgE levels in CCD-positive serum samples

Around 20% to 25% of atopic patients have anti-CCD IgE antibodies, although mostly at low concentrations.[6,13] To estimate the proportion of sera with low- versus high-level anti-CCD IgE among CCD-positive patients, we analyzed all ImmunoCAP bromelain tests (k202) performed in our laboratory between 2008 and 2014.

Identification on N-glycans in cotton cellulose fibers using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Cotton linters processed to different degrees, coniferous kraft cellulose, hardwood kraft cellulose, and hardwood sulfite cellulose were analyzed by using mass spectrometry (MS) to investigate the presence of CCDs in cellulose sources. The material was digested with pepsin in formic acid and N-glycans subsequently released by PNGase A. After purification by means of cation exchange, gel filtration, and reverse-phase solid-phase extraction, the samples were analyzed with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. In parallel, aliquots were mixed with an isotope-labeled N-glycan (AAF = $Gal_2Man_3GlcNAc_5$; Gruber et al, unpublished work). The glycans were mixed with 5 pmol standard per gram of original cellulose and analyzed by using electrospray MS.[14]

RESULTS

Two case histories from daily practice

As an example of the effect of cellulose-dependent false-positive test results with recombinant allergens in daily practice, 2 case histories are presented.

Case 1. A 33-year-old female patient with allergic rhinoconjunctivitis from cat and house dust mite presented with an episode

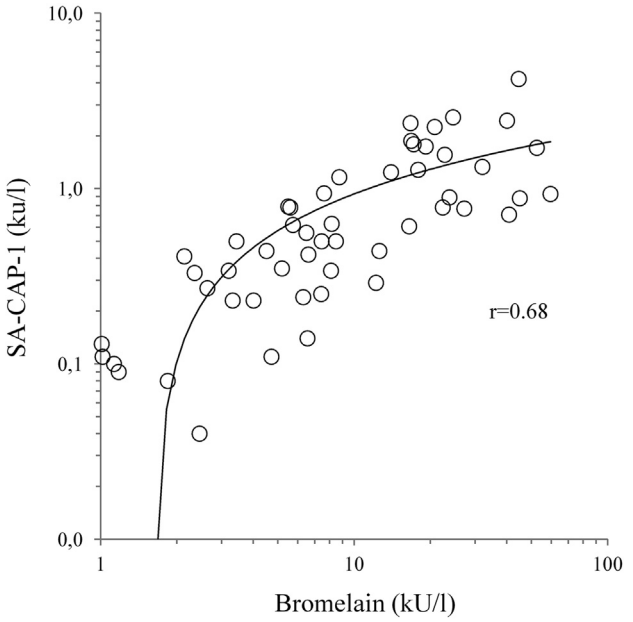

FIG 1. Correlation between IgE binding to CCDs (ImmunoCAP bromelain) and allergen-free ImmunoCAPs coupled with streptavidin (SA-CAP-1) in 52 CCD-positive sera with varying amounts of serum anti-CCD IgE antibodies (1.01-59.6 kU_A/L).

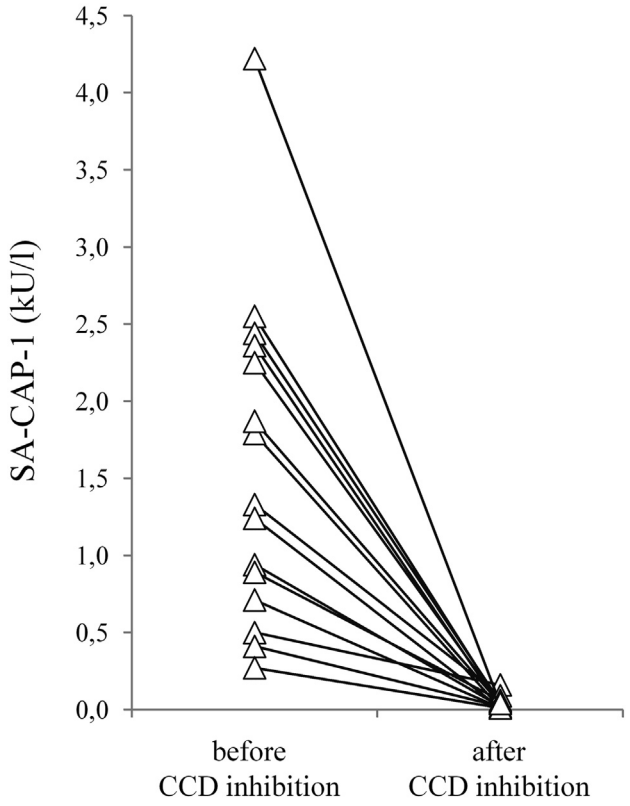

FIG 2. IgE binding to allergen-free ImmunoCAPs coupled with streptavidin (SA-CAP-1) in 15 CCD-positive sera before and after serum inhibition with a CCD inhibitor ($MUXF^3$-HSA).

of urticaria and angioedema possibly associated with ingestion of nut-containing chocolate or peanut-containing ice cream. All types of nuts had been consumed without previous problems.

Routine assessment revealed positive skin test reactions to cat and dust mites but only questionable reactions to peanut and hazelnut. In any case, positive ImmunoCAP results for the recombinant peanut 2S albumin Ara h 2 (1.63 kU_A/L) and hazelnut LTP Cor a 8 (0.58 kU_A/L) suggested true nut allergy (Table I). Subsequent prick-prick testing with nuts and seeds (almond, macadamia, cashew, hazelnut, peanut, pistachio, brazil nut, walnut, pecan, sesame, cucumber seeds, and poppy seeds) again revealed only questionable reactions, whereas CAP results turned out to be positive also to other LTPs, storage proteins, and profilin. Screening for anti-CCD IgE antibodies revealed a result of 44.6 kU_A/L to bromelain. Results for all recombinant allergens became completely negative after serum inhibition with a CCD blocker. Results for testing native serum on the Phadia ISAC microarray platform were positive for Fel d 1, Der p 1, Der f 1, Der f 2, and $MUXF^3$-CCD but negative for storage proteins, LTPs, and profilins. We concluded that all positive ImmunoCAP results to nut allergens were caused by clinically irrelevant anti-CCD antibodies and that the patient does not have true nut allergy. Dietary restrictions and prescription of self-injectable epinephrine are not required.

Case 2. A 46-year-old male patient presented with anaphylaxis after a sting by an unidentified insect. ImmunoCAP results were double positive for *Vespula* species (34.0 kU_A/L) and honeybee (27.6 kU_A/L) venom, strongly positive for rVes v 1 and rVes v 5, and moderately positive to rApi m 1 (1.63 kU_A/L), suggesting true double sensitization (Table I). Because ImmunoCAP results were also highly positive to pollen extracts, the serum was tested for major and minor pollen allergens, all of which produced positive scores of between 0.42 and 1.94 kU_A/L, suggesting multiple genuine pollen sensitization. Subsequent inhibition of the patient's serum with a CCD blocker completely abrogated IgE binding to pollen allergens and to rApi m 1 but not rVes v 1 and Ves v 5. ISAC results were positive for rVes v 5 but negative for rApi m 1 and all pollen marker allergens. We concluded that the patient is single positive to wasp venom and does not require double immunotherapy with both venoms. He does not have true pollen allergy.

Reactivity of CCD-positive sera with allergen-free streptavidin CAPs (SA-CAP-1)

Of 52 CCD-positive sera tested, 35 (67%) bound with a score of greater than 0.35 kU_A/L to SA-CAP-1 (0.41-4.22 kU_A/L), and all but 4 (92%) showed a score of greater than 0.10 kU_A/L. Among samples with anti-CCD IgE levels of greater than 7 kU_A/L, 90% (26/29) were positive at greater than 0.35 kU_A/L.

IgE binding to SA-CAP-1 correlated significantly with IgE binding to bromelain ($r = 0.68$, Fig 1). The mean IgE-binding score obtained with SA-CAP-1 corresponded to 7.6% ± 4.3% of the score obtained with bromelain, but values varied individually between less than 1% and greater than 15%. Sera with high anti-CCD IgE levels tended to show less relative binding to SA-CAP-1 compared with samples with low-level anti-CCD, possibly because of a restricted number of CCD epitopes offered by the cellulose.

CCD inhibition by $MUXF^3$-HSA completely abolished IgE binding to SA-CAP-1 in all 15 samples (range, 0.27-4.22 kU_A/L before inhibition and 0.01-0.09 kU_A/L after inhibition). Serum preincubation with streptavidin had no effect (Fig 2). Dose-response curves for SA-CAP-1 and bromelain performed in 3

TABLE II. IgE binding of 10 CCD-positive sera with bromelain, allergen-free streptavidin-CAP (SA-CAP-1), and 4 recombinant allergens before (−CCD) and after (+CCD) CCD inhibition

		SA-CAP-1 (kU/L)		rPhl p 12 (kU/L)		rFel d 1 (kU/L)		rAra h 2 (kU/L)		rPru p 3 (kU/L)		Mean binding with recombinant allergens	
Patient no.	Bromelain (kU/L)	−CCD	+CCD	−CCD	+CCD	−CCD	+CCD	−CCD	+CCD	−CCD	+CCD	kU/L	Bromelain
1	44.6	4.22	0.04	1.12	0.04	2.97*	1.86	1.63	0.00	1.21	0.03	1.32	2.96%
2	52.5	ND	ND	1.51	0.05	1.49	0.03	1.02	0.01	1.47	0.03	1.37	2.61%
3	40.1	2.44	0.05	1.00	0.05	0.91	0.02	0.89	0.08	1.09	0.06	0.97	2.43%
4	32.1	1.33	0.09	0.36	0.03	0.29	0.02	0.36	0.01	0.38	0.06	0.35	1.08%
5	24.6	2.55	0.04	0.88	0.05	1.00	0.12	0.76	0.09	1.03	0.07	0.92	3.73%
6	17.2	1.79	0.01	0.51	0.02	0.61	0.00	0.37	0.00	0.41	0.02	0.48	2.76%
7	16.7	2.36	0.02	0.71	0.01	0.81	0.00	0.80	0.00	0.74	0.02	0.77	4.58%
8	14.0	1.24	0.01	0.40	0.03	0.48	0.00	0.31	0.00	0.40	0.02	0.40	2.84%
9	40.9	0.71	0.01	0.25	0.02	0.14	0.01	0.09	0.01	0.25	0.01	0.21	0.51%
10	15.4	0.89	0.06	0.21	0.02	0.09	0.01	0.09	0.00	0.16	0.01	0.14	0.89%

ND, Not determined.
*Patient 1 had true cat allergy (data for rFel d 1 were excluded from statistical analysis).

selected patients are shown in Fig E1 in this article's Online Repository at www.jacionline.org.

CCD-dependent reactivity with ImmunoCAP recombinant allergens

Of 10 selected CCD-positive sera tested on a panel of 4 recombinant allergens, 8 bound with a score of greater than 0.35 kU_A/L to at least 1 of the components (0.36-1.63 kU_A/L, Table II). The remaining 2 sera (patients 9 and 10) showed borderline results of up to 0.25 kU_A/L. Mean IgE binding to the 4 recombinant allergens correlated strongly with IgE binding to SA-CAP-1 ($r = 0.98$) and bromelain ($r = 0.61$, Fig 3). Serum inhibition with $MUXF^3$-HSA completely abrogated reactivity with the recombinant allergens in all sera apart from reactivity with Fel d 1 in patient 1, who was truly allergic to cat (Table II).

False-positive results to recombinant allergens in patients with Hymenoptera venom allergy

Seven CCD-positive patients with venom allergy were tested. As expected, all sera showed strong reactivity to both venoms. Antibody binding to the nonculprit venom was inhibited by $MUXF^3$-HSA by 75% to 95% in all sera. Among the 5 patients with wasp venom allergy, 4 of 5 had positive results to rApi m 1 (0.45-1.63 kU_A/L), and 1 of 5 had a borderline result. Reactivity with rApi m 1 was strongly inhibited by $MUXF^3$-HSA, whereas binding to rVes v 1 and 5 was not. Likewise, both patients with bee venom allergy showed antibody binding to rVes v 1 and rVes v 5 (0.43-1.00 kU_A/L), which could be blocked by the CCD inhibitor (Table III and see Fig E2 in this article's Online Repository at www.jacionline.org).

In summary, true double sensitization would have been erroneously diagnosed in all 7 patients on the basis of ordinary CRD without CCD inhibition. The CCD-positive control serum reacted with all recombinant venom allergens (0.88-1.14 kU_A/L) and became completely negative after CCD inhibition (0.05-0.08 kU_A/L).

Batch-to-batch variation in cellulose CCDs

Fifteen sera reacting with the streptavidin CAP (SA-CAP-1) were selected and retested on a second lot of streptavidin-coupled CAP (SA-CAP-2) and on 4 blank ImmunoCAP cellulose batches conjugated with MBP (MBP-CAP-1 to MBP-CAP-4; Table IV and see Table E1 in this article's Online Repository at www.jacionline.org). Antibody binding to the new cellulose batches correlated strongly with that to SA-CAP-1 ($r = 0.94\sim0.99$), although binding scores were generally lower and accounted, on average, for only 22% to 32% of those obtained with SA-CAP-1. Overall, the magnitude of antibody binding to SA-CAP-2 and to the 4 MBP-CAPs was similar to that seen with recombinant allergens, whereas the very strong binding with SA-CAP-1 appears to be a solitary case. Excluding SA-CAP-1, the mean reactivity with cellulose CCDs corresponded to 2.4% to 3.1% of the bromelain reactivity, although individual variation was between less than 1% and 10%. Regression curves again showed that mean relative binding correlated with anti-CCD IgE concentrations, being lowest in sera with high anti-CCD IgE levels and highest in those with low levels (>20 kU_A/L, ~2%; 10-20 kU_A/L, ~2.5%; 5-10 kU_A/L, ~4%; and <5 kU_A/L, ~5-6%).

CCD inhibition strongly diminished antibody binding to SA-CAP-2 and all MBP-CAPs to values between 0.00 and 0.06 kU_A/L, confirming that antibody binding was caused by CCDs (data not shown).

Distribution of anti-CCD IgE levels in CCD-positive serum samples

Because interference with CCDs of the cellulose matrix appeared to become a significant problem only with high serum anti-CCD IgE levels, we explored the distribution of anti-CCD IgE antibody levels among CCD-positive patients to estimate how often false-positive ImmunoCAP results might be expected in daily practice. In total, 517 patients with a positive bromelain test result were recorded. Three hundred seventy-three (72.1%) of 517 of the sera had low anti-CCD IgE levels of between 0.35 and 3.5 kU_A/L (corresponding to former class 1 and 2), 117 (22.6%) of 517 had medium levels of between 3.5 and 17.5 kU_A/L (former class 3), and 27 (5.2%) of 517 had high levels of greater than 17.5 kU_A/L (class 4-5). Seventy-nine (15.3%) of 517 had anti-CCD IgE levels of 7 kU_A/L or greater, and 52 (10.1%) of 517 had levels 10 kU_A/L or greater.

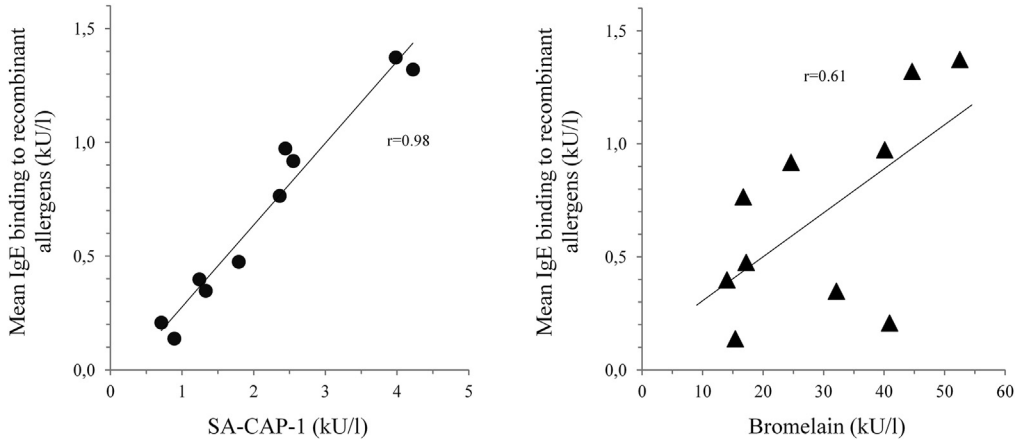

FIG 3. Correlation of IgE binding to recombinant allergens with IgE binding to allergen-free streptavidin-CAP (SA-CAP-1; *left panel*) and bromelain *(right panel)* in 10 sera with high anti-CCD IgE levels.

TABLE III. ImmunoCAP IgE binding to Hymenoptera venoms and recombinant venom allergens before and after CCD inhibition in 7 CCD-positive patients with venom allergy and a CCD-positive subject without a history of venom hypersensitivity

			Honeybee venom		rApi m 1		*Vespula* species venom		rVes v 1		rVes v 5	
Patient no.	Culprit insect	CCD	−CCD	+CCD	−CCD	+CCD	−CCD	+CCD	−CCD	+CCD	−CCD	+CCD
1	w	24.6	27.60	1.25	1.63	0.11	34.00	17.40	11.80	9.72	48.70	44.60
2	w	38.6	19.10	0.38	0.67	0.02	36.20	14.10	7.01	6.48	13.50	12.70
3	w	2.14	7.39	1.89	0.19	0.02	8.44	3.44	5.34	5.24	5.70	5.43
4	w	17.9	55.8	3.06	0.45	0.06	46.70	14.90	2.31	1.96	>100	>100
5	w	6.70	7.50	1.50	0.55	0.11	37.40	34.60	30.20	29.90	14.00	13.70
6	b	40.1	45.3	47.5	94.9	93.5	3.65	0.22	1.00	0.10	0.94	0.13
7	b	7.62	19.1	7.19	1.02	0.56	6.03	0.67	0.48	0.06	0.43	0.11
8	None	31.0	33.2	2.02	0.98	0.06	7.24	0.56	1.14	0.05	0.88	0.08

Figures are in kilounits of antigen per liter.
b, Honeybee; *+CCD*, after inhibition; *−CCD*, without inhibition; *w*, wasp (*Vespula* species).

TABLE IV. Mean reactivity of 15 CCD-positive sera with 6 samples of allergen-free ImmunoCAPs coupled with either streptavidin (SA-CAP-1 and SA-CAP-2) or MBP (MBP-CAP 1-4) and mean CCD-dependent antibody binding to recombinant allergens

	IgE binding (kU/L)		Bromelain reactivity (%)	
Solid phase	Mean ± SD	Range	Mean ± SD	Range
Bromelain	20.17 ± 14.25	1.02-44.6		
SA-CAP-1	1.51 ± 1.09	0.11-4.22	9.29 ± 3.86	1.74-14.53
SA-CAP-2*	0.48 ± 0.39	0.04-1.25	2.42 ± 1.00	0.84-3.92
MBP-CAP-1	0.40 ± 0.25	0.06-0.96	3.05 ± 2.43	0.49-10.21
MBP-CAP-2	0.44 ± 0.31	0.06-1.22	3.05 ± 1.67	0.56-5.96
MBP-CAP-3	0.41 ± 0.28	0.04-1.09	2.73 ± 1.50	0.54-6.38
MBP-CAP-4	0.33 ± 0.22	0.05-0.79	2.36 ± 1.49	0.51-5.96
Mean binding with recombinant allergens	0.53 ± 0.35	0.09-1.94	2.38 ± 1.26	0.34-7.89

*Only 10 of the sera were tested on SA-CAP-2.

Identification on N-glycans in cotton cellulose fibers with MALDI-TOF MS

By using MALDI-TOF MS, typical plant N-glycans were detected in the unprocessed and medium-processed cotton samples investigated. In unprocessed cotton several xylosylated N-glycans with and without α1,3-linked fucose were identified ($MMXF^3$, $GnGnXF^3$, $MGnXF^3$, MMX, and MGnX). In processed cotton linters the predominant N-glycan detected was of the $MMXF^3$ type, along with several high-mannose glycans (Fig 4). Highly processed cotton linters and all wood cellulose samples investigated contained smaller amounts of $MMXF^3$, which amounted to 0.2 ± 0.05 pmol of glycan/g of cellulose compared with 200 pmol/g in unprocessed cotton linters. With a hypothetical glycoprotein of 50 kDa, 0.2 pmol would constitute 10 ng of glycoprotein per gram of cellulose.

DISCUSSION

CCDs have been recognized as a phenomenon interfering with correct *in vitro* diagnosis since 1981.[3] Although ignored for quite

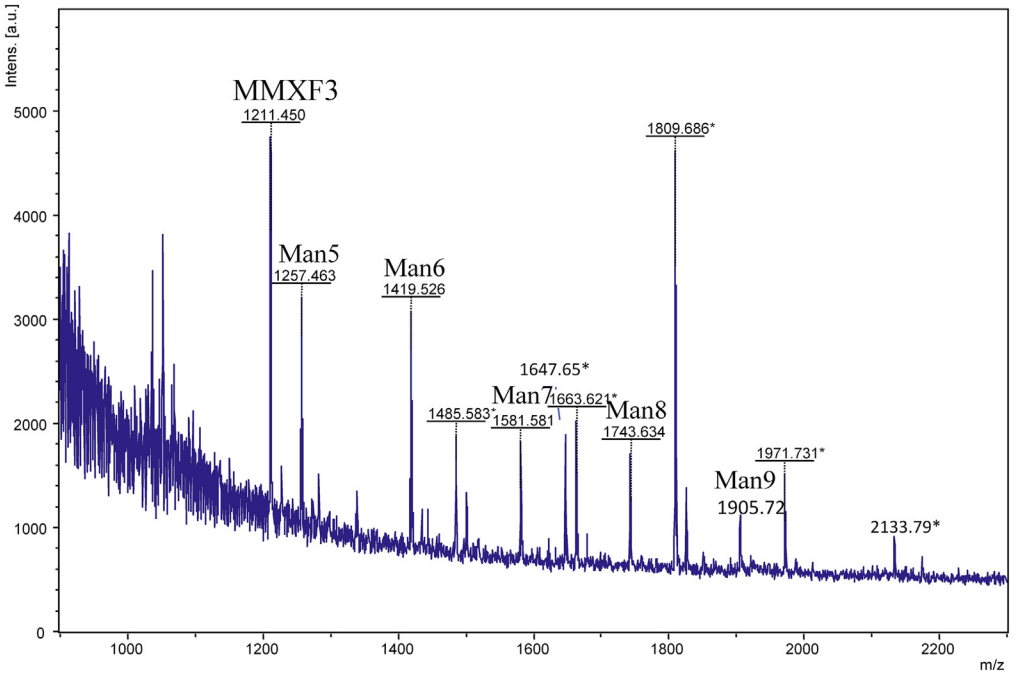

FIG 4. Identification of N-glycans in processed cotton cellulose. Samples were digested with pepsin in formic acid, and N-glycans were released by PNGase A and analyzed by using MALDI-TOF MS after cation exchange purification, gel filtration, and reverse-phase solid-phase extraction. The main peak at 1211.4 corresponds to complex N-glycans of the $MMXF^3$ type. Peaks marked by *asterisks* correspond to background signals derived from pepsin.

a long time, their misleading effect has been demonstrated over the past years within various fields of allergy, including food allergy,[15-17] insect venom allergy,[11,18] pollen allergy,[12] and latex allergy.[19,20]

With the increase in molecular allergology and the introduction of recombinant allergens for CRD, the CCD problem is often believed to have dissolved. However, this is not at all the case. First, (glycosylated) allergen extracts are still widely used in clinical practice and will stay in use further on because many extracts are far from being substituted by components adequately. Furthermore, CRD is not always based on recombinant molecules devoid of CCDs. Quite a lot of components used in commercial test systems, both in singleplex and multiplex systems, such as the ImmunoCAP ISAC, are purified natural proteins, many of which carry CCDs and readily cause irrelevant misleading results.[21] Therefore, despite the CCD problem being mitigated by using modern CRD, there is an ongoing need to call to mind this important subject. The findings of the present study now add a completely novel and surprising aspect to the CCD issue inasmuch as CCD-dependent false-positive test results can be obtained even with CCD-free recombinant allergens in sera with high anti-CCD serum IgE antibodies if cellulose is used as the allergen carrier.

Our study provides good evidence from experiments with allergen-free ImmunoCAP samples, as well as from CCD inhibition tests, that the cellulose matrix used as the solid-phase allergen carrier in the ImmunoCAP system contains residual CCDs in concentrations high enough to cause significant nonspecific background binding of up to 2 kU_A/L in CCD-positive serum samples. We do not know whether similar effects also occur with other test platforms by using cellulose discs as allergen carrier (eg, HYTEC; HYCOR Biomedical, Indianapolis, Ind) or even platforms using nitrocellulose (like most lateral-flow allergy screening tests). In any case, the unique 3-dimensional construction of the ImmunoCAP cellulose sponge designed to enable coupling of high allergen amounts might be specifically prone to such nonspecific background binding.

How big is the problem? Our data show that the observed interference with cellulose CCDs becomes a significant problem only with high serum concentrations of CCD-specific IgE antibodies but hardly with low concentrations. By and large, nonspecific antibody binding of greater than 0.35 kU_A/L starts to become a problem in serum samples with anti-CCD IgE levels of 7 to 10 kU_A/L. IgE antibodies against CCDs are a quite common phenomenon among atopic subjects. In large cohorts of unselected patients, 20% to 25% of samples turned out CCD positive, but the prevalence can be as high as 35% in adolescents and higher than 50% in certain subgroups with pollen, food, and insect venom allergy.[6,11,13] However, the majority of these CCD-positive sera contain low levels of CCD-specific IgE antibodies, which are unlikely to lead to serious background binding. In agreement with this, 72% of all CCD-positive sera identified at our own center had low anti-CCD IgE levels of 0.35 to 3.5 kU_A/L. In any case, 10% to 15% of patients had anti-CCD IgE levels of greater than 7 to 10 kU_A/L, which is similar to figures published by others,[6] and thus will have a substantial risk for false-positive test results.

Assuming a 20% prevalence of CCD positivity among allergic patients, around 2% to 3% of unselected atopic sera analyzed during routine allergy workup might show significant nonspecific background binding in ImmunoCAP because of interference with cellulose glycans. Accordingly, the magnitude of the problem,

although real and relevant, seems to be limited, at least when sticking to the long-established cutoff of 0.35 kU_A/L. The situation might change considerably when the new cutoff suggestion of 0.10 kU_A/L is applied, which could be valuable in certain conditions to gain sensitivity[22] and is analytically reasonable because this value is still above the background noise of the ImmunoCAP.[23] Actually, ImmunoCAP is one of 3 commercial *in vitro* assays for the assessment of human IgE cleared by the US Food and Drug Administration with a limit of quantification of 0.10 kU_A/L.[23] Because mean binding to the ImmunoCAP cellulose matrix accounts for 2% to 3% of the bromelain reactivity, also anti-CCD IgE levels of around 4 kU_A/L will commonly induce binding scores of greater than 0.10 kU_A/L. In single sera we observed background binding of greater than 0.10 kU_A/L, even with anti-CCD IgE concentrations as low as 2.35 kU_A/L. Accordingly, a detection limit of 0.10 kU_A/L will aggravate the issue of CCD-dependent background binding, with more than 7% of all atopic sera being potentially affected. Regarding possible elimination of the 0.1 kU_A/L threshold in favor of the old 0.35 kU_A/L cutoff, we believe that our data do not justify a general recommendation to replace the 0.10 kU_A/L threshold because this cutoff is reliable in CCD-negative samples and often helpful to confirm the diagnosis, especially in subjects with low total IgE levels. Our findings might caution allergists to uncritically overinterpret such weakly positive results and advise them to carefully review test results for plausibility and consistency.

Although interference with cellulose glycans is a concern for any ImmunoCAP test, its clinical effect is especially important with respect to CRD. The crucial advantage of CRD over extract-based diagnosis is that it might clarify, with the help of well-defined marker allergens, individual sensitization patterns and thereby, for example, can prove the presence of IgE against high-risk food allergens[24,25] or discriminate in respiratory allergy between genuine sensitization and cross-reactivity.[26]

Another field in which CRD proved extremely helpful is venom allergy. Double sensitization to honeybee and wasp is a well-known diagnostic obstacle and often caused by CCD cross-reactivity.[11,12,27] Insect stings themselves appear to be potent inducers of carbohydrate-specific IgE,[12] explaining why CCD reactivity is strikingly widespread among patients with venom allergy and often associated with remarkably high serum levels of such antibodies.[11,18] In the last few years, recombinant venom marker allergens have substantially simplified diagnosis by providing a means to directly prove (or exclude) genuine sensitization to a particular insect.[28,29] Our present findings now caution against a too self-evident and uncritical interpretation of weakly positive test results of up to 2 kU_A/L with any of these marker allergens because quite a lot of them can be false positive and should be interpreted with caution. Six of 7 strongly CCD-positive patients with venom allergy investigated in this study would have been erroneously classified as truly double sensitized to bee and wasp venom and thus would have received immunotherapy with both venoms when the culprit insect was not identified. Also, the 2 case reports presented in this article illustrate that the proper use of CRD provided by the ImmunoCAP system can be misleading for the clinician and lead to wrong or unnecessary recommendations if the patient's serum contains large amounts of anti-CCD IgE antibodies.

Industrial extraction of cellulose from source materials (eg, wood and cotton) includes boiling in strong liquors and sodium sulfate for many hours to separate cellulose from lignin and hemicelluloses. The detection of allergenic $MMXF^3$ glycans, even in strongly processed cotton and wood cellulose samples, by means of MS proves that glycoprotein-derived N-glycans are remarkably resistant and can survive unscathed, even after such violent treatment, if only in low amounts. Although we did not have the opportunity to examine cellulose used for the manufacture of ImmunoCAPs, it appears reasonable to assume that it will have similar glycan composition.

Our data also suggest that the amount of residual N-glycans can vary to some extent between different cellulose batches. This can be caused by differences in primary materials or be due to the unpredictable degree of destruction/extraction of glycoproteins during the manufacturing process. Overall, we were able to perform experiments with 6 different ImmunoCAP cellulose preparations (2 streptavidin conjugated and 4 MBP conjugated). The amount of background binding was quite similar in 5 of them, whereas 1 sample (SA-CAP-1) yielded much higher scores. Background binding observed with currently sold customary recombinant allergen-coupled ImmunoCAPs (presumably representing different cellulose batches) was largely comparable with that of the 5 low-level background cellulose preparations. Accordingly, the SA-CAP-1 sample, which was purchased from Phadia in 2005, represents a peculiar outlier not reflecting the average CCD contamination of current marketed ImmunoCAPs. In the future, it is to be expected that single ImmunoCAP cellulose batches with exceedingly high CCD amounts will be eliminated readily from further processing because Phadia is aware of the problem.

Why do sera with similar bromelain reactivity not always show comparable background binding? Background binding to the cellulose matrix accounted, on average, for 2% to 3% of the reactivity seen with bromelain, but there were considerable differences between subjects, ranging between less than 1% and 10%. One important reason for this variability might be differences in the antigen specificity of anti-CCD IgE antibodies. It is well known that the binding affinity of carbohydrate-specific IgE, although essentially linked to the presence of either α1,3 fucose or β1,2 xylose, can vary to some extent between different N-glycans, depending on what other substitutions these glycans carry.[18,30-32] Our MS analysis of cellulose samples mainly identified N-glycans of the $MMXF^3$ type, representing a ubiquitous glycan structure of plant tissues but different from the $MUXF^3$ glycans used in CCD screening tests offered by ImmunoCAP (bromelain k202, $MUXF^3$-CCD o214). Patients sensitized to CCDs through insect stings might generate antibodies with high affinity for typical insect glycans, such as MMF^3 and MUF^3, and variable affinity for xylosylated plant-derived glycans, such as $MUXF^3$ and $MMXF^3$. Although we currently do not have experimental evidence from our patients, such differences in antibody specificity can contribute to the overall modest correlation seen between binding to cellulose and binding to bromelain. In addition, absolute serum concentrations of anti-CCD antibodies appear to play a role. Samples with very high levels generally showed a lower percentage of background binding than samples with low or medium levels. This is possibly because of a limited number of glycan epitopes present on the cellulose matrix and cumulative saturation of binding sites. Also, high levels of anti-carbohydrate IgG antibodies, including CCD-specific antibodies, which can be commonly found in human serum samples,[33] might compete with IgE and generally interfere with the correct measurement of CCD-specific IgE in serum samples.

Are the observations made by us completely new? Nonspecific background binding to solid-phase matrices has been addressed repeatedly in early studies dealing with the sensitivity and specificity of current or upcoming *in vitro* IgE assays.[34,35] It has been recognized that rabbit and human serum samples frequently contain natural IgG antibodies directed against common assay matrices, such as cellulose and agarose, indicating carbohydrate-specific antibodies as a possible factor interfering with proper *in vitro* diagnosis.[36,37]

An interesting observation strongly reminiscent of our present findings was made by van Toorenenbergen et al in 1987.[35] They described 2 patients with strong IgE binding to virtually all tested allergens when allergens were coupled to cellulose but no reactivity when the same allergens were coupled to sepharose beads, making the authors conclude that a yet unknown structure of the cellulose discs was responsible for the irrelevant binding. However, antibody binding to the cellulose discs could not be inhibited by a (CCD-containing) peanut extract in this study, which in turn contradicts the involvement of CCDs in these cases.

Another observation possibly related to our findings comes from a Japanese group comparing a novel polystyrene-based IgE test with Phadezym RAST and ImmunoCAP.[38] Among 3004 sera tested, the authors encountered 96 (3.2%) samples displaying blank reactions to uncoated paper discs from the Phadezym RAST, with many of them also binding to uncoated ImmunoCAP cellulose. It appears very plausible that this was caused by CCDs on the cellulose.

How to deal with the problem in everyday practice? In case of a suspect false-positive result, CCD inhibition is probably the most elegant way to resolve the problem. However, although easy to perform in principle, inhibition is time-consuming and costly and unlikely a realistic approach for routine laboratories. Screening for anti-CCD serum antibodies is much easier to implement and will reliably identify those highly CCD-positive sera, but apart from additional costs, this will not always lead to a conclusive answer, irrespective of whether a weakly positive test result is specific. From our point of view, allergen-free dummy CAPs might be a promising, easy-to-perform, and presumably also economically sensible strategy to identify sera with nonspecific background binding. Considering the presumably low frequency of affected sera, such CCD screening might not be required *a priori* for all patients. Instead, defining characteristic traits to recognize suspect cases (and how to exclude them) will help keep additional testing to a minimum.

In conclusion, we showed in this study that the allergen-carrying cellulose matrix of the ImmunoCAP contains small amounts of residual CCDs sufficient to cause nonspecific background binding in sera with high levels of anti-CCD IgE antibodies. This might misleadingly mimic positive test results, even with nonglycosylated recombinant allergens in approximately 2% to 3% of atopic sera. Irrespective of the diagnostic improvements offered by up-to-date CRD, it is important to alert clinicians about the possibility that weakly positive ImmunoCAP results of up to 2 kU_A/L might be false positive and thus should be interpreted with caution and critically reviewed for plausibility. Although such CCD-dependent reactivity with the cellulose matrix represents a troublesome limitation of the ImmunoCAP system to be considered, it should be kept in mind that the major untoward effect of CCDs on *in vitro* allergy diagnosis is still the widespread use of glycosylated allergen extracts and the lack of awareness of many allergists that such extracts are a common source of irrelevant test results. Although an estimated 2% to 3% of patients might be at risk for nonspecific background binding, as much as 20% to 25% will definitely show irrelevant test results with extracts from pollens, plant foods, latex, and insect venoms, irrespective of the test platform used.

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Key messages

- **Clinically irrelevant IgE antibodies against CCDs are found in 20% to 25% of atopic sera and are known to interfere with proper *in vitro* allergy diagnosis.**
- **Cellulose used as an allergen carrier in *in vitro* IgE assays might contain low amounts of intact residual CCDs.**
- **This can cause nonspecific background binding of up to 2 kU_A/L, even with unglycosylated recombinant allergens, in serum samples with high levels of anti-CCD IgE antibodies.**

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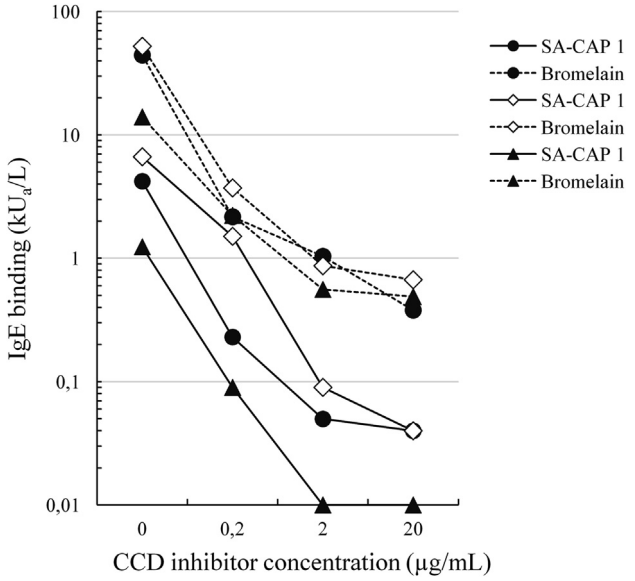

FIG E1. Dose-dependent inhibition of IgE binding to a streptavidin-coupled dummy CAP (SA-CAP-1; *black lines*) and bromelain *(dotted lines)* by a CCD blocker in 3 patients with high anti-CCD IgE levels.

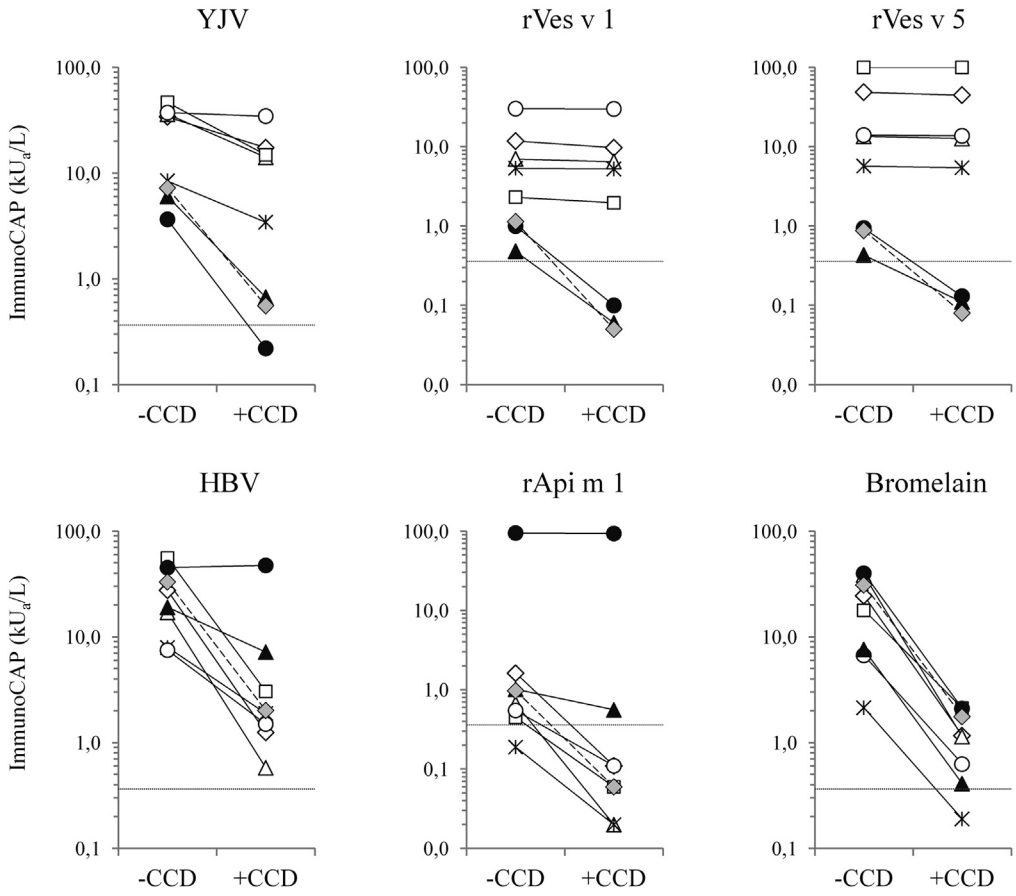

FIG E2. ImmunoCAP IgE binding to Hymenoptera venoms and recombinant venom allergens before and after CCD inhibition in 7 CCD-positive patients with either bee venom allergy (*black symbols*, n = 2) or yellow jacket venom allergy (*white symbols*, n = 5) and in a CCD-positive control subject without venom hypersensitivity *(gray symbols)*. *+CCD*, After CCD inhibition; *−CCD*, without inhibition; *HBV*, honeybee venom; *YJV*, yellow jacket venom.

TABLE E1. IgE binding of 15 CCD-positive sera to 6 samples of allergen-free ImmunoCAPs coupled with either streptavidin (SA-CAP-1 and SA-CAP-2) or MBP (MBP-CAP 1-4) and CCD-dependent antibody binding to recombinant allergens

	Bromelain	SA-CAP-1		SA-CAP-2*		MBP-CAP-1		MBP-CAP-2		MBP-CAP-3		MBP-CAP-4		Mean binding with recombinant allergens	
Patient no.	kU/L	kU/L	Bromelain (%)	kU/L	Bromelain (%)	kU/L	Bromelain (%)	kU/L	Bromelain (%)	kU/L	Bromelain (%)	kU/L	Bromelain (%)	kU/L	Bromelain (%)
1	44.6	4.22	9.46	1.25	2.80	0.96	2.15	1.22	2.74	1.09	2.44	0.79	1.77	0.95	2.13
2	24.6	2.55	10.37	0.76	2.80	0.62	2.52	0.74	3.01	0.63	2.56	0.63	2.56	0.99	4.03
3	17.2	1.79	10.41	0.43	2.50	0.65	3.78	0.58	3.37	0.61	3.55	0.44	2.56	0.48	2.76
4	32.1	1.33	4.14	0.27	0.84	0.54	1.68	0.36	1.12	0.39	1.21	0.36	1.12	0.35	1.08
5	14.0	1.24	8.86	0.18	1.29	0.30	2.14	0.30	2.14	0.28	2.00	0.21	1.50	0.40	2.84
6	40.1	2.44	6.08	0.74	1.85	0.55	1.37	0.68	1.70	0.65	1.62	0.55	1.37	0.97	2.42
7	16.7	2.36	14.13	0.57	3.14	0.55	3.29	0.66	3.95	0.64	3.83	0.54	3.23	0.68	4.05
8	2.35	0.33	14.04	—	—	0.24	10.2	0.14	5.96	0.15	6.38	0.14	5.96	0.08	3.19
9	23.8	0.89	3.74	—	—	0.20	0.84	0.24	1.01	0.23	0.97	0.17	0.71	0.19	0.82
10	40.9	0.71	1.74	—	—	0.20	0.49	0.23	0.56	0.22	0.54	0.21	0.51	0.21	0.51
11	5.74	0.62	10.80	0.12	2.09	0.13	2.26	0.17	2.96	0.15	2.61	0.13	2.26	—	—
12	1.02	0.11	10.78	0.04	5.88	0.06	5.88	0.06	5.88	0.04	3.92	0.05	4.90	—	—
13	16.8	1.87	11.13	—	—	0.42	2.50	0.54	3.21	0.45	2.68	0.34	2.02	—	—
14	19.2	1.74	9.06	—	—	0.37	1.93	0.56	2.92	0.43	2.24	0.32	1.67	—	—
15	3.44	0.50	14.53	—	—	0.16	4.65	0.18	5.23	0.15	4.36	0.11	3.20	—	—
Mean			9.3 ± 3.9		2.4 ± 1.0		3.1 ± 2.4		3.1 ± 1.7		2.7 ± 1.5		2.4 ± 1.5		2.4 ± 1.3

*Only 10 of the sera were tested on SA-CAP-2.