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Improved allergy diagnosis by depletion of clinically irrelevant IgE

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Zusammenfassung

CCDs (Cross-reactive Carbohydrate Determinants) sind häufig der Grund für falsch positive Ergebnisse in *in vitro* Allergietests wie RAST oder CAP Test. Diese Strukturen sind in N-Glykanen von Pflanzen, Insekten oder parasitischen Helminthen weit verbreitet und können deswegen Kreuzreaktionen von IgE Antikörpern auslösen. Die zwei Hauptstrukturen die zur Bildung kreuzreaktiver Anti-CCD IgEs führen sind eine α 1,3- gebundene Fucose, welche an das innerste N-Acetylglucosamin (GlcNAc) gebunden ist, und eine an Mannose gebundene β 1,2-Xylose.

Ungefähr 15 % - 30 % der Pollenallergiker weisen gegen CCDs gerichtete IgE Antikörper im Blut auf. Da diese Antikörper aber meist keine allergischen Reaktionen in den betroffenen Patienten auslösen können sie als klinisch irrelevant angesehen werden. Daher wäre es wünschenswert diese Anti-CCD IgE Antikörper in Blut zu detektieren und zu entfernen bevor das Serum zu weiteren *in vitro* Allergiediagnosen herangezogen wird.

In der vorliegenden Arbeit sollte eine Affinitätsmatrix hergestellt werden mit der es möglich ist gegen CCDs gerichtete IgE Antikörper aus Patientenseren zu entfernen ohne dabei die Menge allergiespezifischer IgE Antikörper zu verändern. Als Anti-CCD IgE Absorber wurde MUXF verwendet welches aus Bromelain gewonnen wurde. MUXF wurde in verschiedene Mengen an diverse Matrizen (z.B. Agarose – Gele, Eupergit) gekoppelt und die Bindungskapazität für IgE wurde mittels ELISA ermittelt. Matrizen die sich bewähren konnten wurden weiters mit CAP evaluiert.

Anti-CCD IgE Antikörper konnten erfolgreich aus den getesteten Patientenseren entfernt werden. Die Affinitätsmatrizen mit der besten Effektivität waren MUXF-BSA gekoppelte ECH Sepharose 4B und Prosep 9-CHO glass beads und MUXF-Lysozym gekoppelte ECH Sepharose 4B. Bis zu 96,7% der gegen CCDs gerichteten IgE Antikörper konnten aus den Seren entfernt werden während durchschnittlich über 90 % der allergenspezifischen IgEs erhalten blieben.

Abstract

Cross-reactive carbohydrate determinants (CCDs) are a frequent cause of false positive allergy diagnosis in *in vitro* allergy tests. These structures are widely spread among N-glycans from plants, insects and also parasitic worms. The two main motifs eliciting anti-CCD IgE production are an α 1,3-linked fucose linked to innermost N-acetylglucosamine (GlcNAc) and a β 1,2-xylose linked to the core mannose. About 15-30 % of pollen-allergic patients generate CCD directed IgE antibodies. However CCDs do not seem to cause any allergic reaction in most of the patients and can therefore be considered as clinically irrelevant. Therefore an important task would be to determine and remove anti-CCD IgE from patients' sera before they undergo *in vitro* allergy diagnosis.

In this work a solid affinity matrix was designed, which can remove anti-CCD IgE from patients' sera without influencing the amount of allergen specific IgE. For this cartridge, the so-called "MUXF" glycopeptide from bromelain was used as an anti-CCD IgE absorbent and coupled in different amounts to various solid supports. The binding capacity of each affinity matrix was evaluated by determining by ELISA the content of anti-CCD IgE in the sera after incubation with the matrix. Matrices which proved to be successful in ELISA tests were further evaluated by CAP test.

CCD directed IgE antibodies could successfully be depleted from patients' sera. The matrices with highest efficiency and binding capacity were MUXF-BSA coupled ECH Sepharose 4B and Prosep 9-CHO glass beads and MUXF-Lysozyme coupled ECH Sepharose 4B. Up to 96,7% CCD-specific IgE could be removed from patients' sera while over 90 % of allergen-specific IgE retained in the

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Introduction

1 Introduction

The immune system is the defence mechanism of humans and all other animals to protect their organism from disease by preventing invasion and recognition of pathogens.

Our immune system can be divided into two subclasses, the adaptive and the innate immune system. Innate immunity consists of biochemical, physical and cellular defence mechanisms and stands for fast and early defence against microbes. It reacts on common structures of pathogens and therefore is able to respond to infections rapidly.

Adaptive immunity on the other hand develops as a response to an infection and recognizes specific structures (antigens) what leads to the production of antibodies.

Although the immune system exists to protect an organism, it sometimes can cause damage and diseases by itself. Such diseases are termed hypersensitivity reactions and are mostly mediated by antibodies.

According to Gell and Coombs four different types of hypersensitivity reactions can be distinguished [1].

Tab. 1: Classification of immune mechanism of tissue injury by Gell and Coombs [1]

Type of hypersensitivity reaction	Mediators	Mechanism of damage
Type I (immediate hypersensitivity)	IgE antibody	Release of histamine and other mediators from mast cells
Type II (cytotoxic or antibody mediated hypersensitivity)	IgM and IgG antibodies bound to cell surface or extracellular matrix antigens	Complement mediated recruitment and activation of leukocytes, opsonization and phagocytosis of cells, abnormalities in cellular functions
Type III (immune complex mediated hypersensitivity)	Circulating immune complexes of antigens with IgM or IgG antibodies	Complement mediated recruitment and activation of leukocytes
Type IV (delayed or T cell mediated hypersensitivity)	CD4 ⁺ and CD8 ⁺ T cells	Activation of macrophages , direct killing of target cells and cytokine mediated inflammation

Of all four hypersensitivity types immediate hypersensitivity (type I), which is a classical immunoglobulin E (IgE) mediated allergy, is the most frequent one. It is caused by an immediate immune response of an individual to an environmental antigen, which leads to differentiation of naive T cells into CD4⁺ T-helper cells (T_H2 cells). T_H2 cells further activate the production of IgE antibodies, directed against the presented structure, in B cells (for details see Fig. 1). These antibodies bind to FcεRI receptors on mast cells and basophile granulocytes and when coming in contact with the antigen again this leads to cross-linking of these receptors by allergen bound IgEs and activation of the cells which immediately release a variety of cytokines and other mediators.

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These mediators further cause effects like vasodilatation, contraction of bronchial and visceral smooth muscles and increase of vascular permeability, reactions commonly known as allergies [2-3].

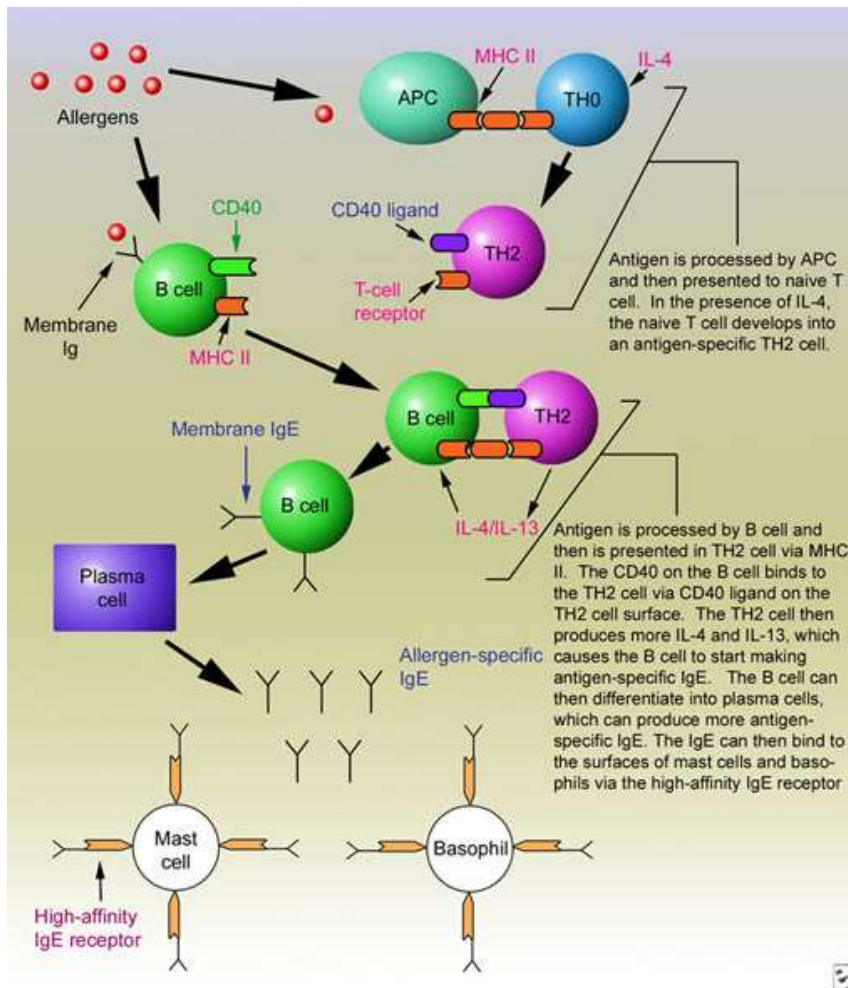


Fig. 1: Steps of the formation of a hypersensitivity type I reaction: after the first contact with the allergen TH2 cells are activated which stimulate allergen-specific IgE production of B cells. Secreted IgE binds to mast cells or basophile granulocytes via the FcεRI receptor (high-affinity receptor) and repeated contact with the allergen leads to activation of the mast cell or basophile and subsequent release of its mediators (Figure copied from [4])

In general allergic reactions can occur as immediate reactions (minutes after repeated contact with the allergen), late phase reactions (2-4 hours after anew exposure to the allergen) or as chronic allergic inflammations.

1.1 Allergens and allergy tests

Allergens are usually environmental proteins or chemicals bound to proteins to which an allergic individual is frequently exposed and which then lead to immediate hypersensitivity reactions. Common and typical allergens are several proteins in pollen, animal dander, house dust mites, foods or chemicals (e.g. like penicillin).

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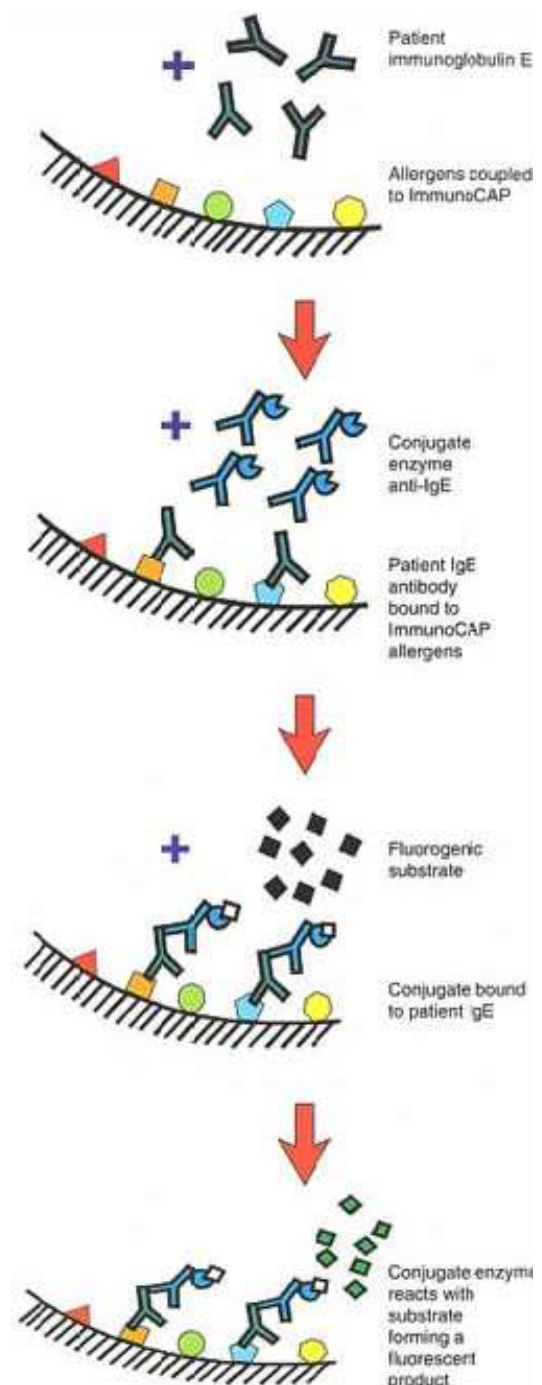
There are no structural characteristics that can definitely tell if a protein may act allergenic or not. Nevertheless there are some features that are typical for many of the most prevalent allergens, like low molecular weight, glycosylation and high solubility in fluids of the body [2].

There are several ways to screen for the existence of allergies in a patient.

The most common and also cheapest form of allergy diagnosis might be by skin prick testing (SPT). In this test small amounts of several allergens are introduced to the skin of a patient by pricking it with a needle. Depending on the reactions of the skin (if the patient is allergic to a certain substance he or she will develop a rash or urticaria where the allergen was placed) conclusions about the existence and severity of an allergy can be made.

Another form of testing for immediate hypersensitivity reactions would be an *in vitro* test like radioallergosorbent test (RAST) or the CAP FEIA (fluoroenzyme-immunoassay) method (Fig. 2). Here allergies are determined by the amount of allergen specific IgE found in a patients' serum.

Fig. 2: Detection of allergen specific IgE by CAP FEIA method. Allergen specific IgE in patients' serum recognizes its epitope. Secondary antibody binds to IgE and is visualized by a fluorescent reaction (Figure copied from [35]).



Advantages of these *in vitro* methods are that a lot of different allergens can be tested at once and that it is less unpleasant for the patient.

A big disadvantage would be that the concentration of IgE in a serum poorly correlates with the severity of clinical symptoms of a patient and sometimes allergen specific IgE can not even be detected despite sensitization.

So the best way to diagnose a type I hypersensitivity would be the combination of these allergy tests.

An interesting matter that was observed in this case was the fact that binding of IgE to allergens in *in vitro* tests did not always correlate with observations made in SPT.

Two discrepancies between *in vitro* tests and SPT could be observed: either a positive SPT and a negative RAST test or the other way around. In general a positive skin prick test with a negative IgE detection for the same allergen in RAST is more frequent than a negative SPT with a positive result in *in vitro* testing [5]. For the latter case it has been discovered that IgE antibodies,

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responsible for binding allergens in CAP/RAST tests but not eliciting a clinical reaction, are directed against so called cross-reactive carbohydrate determinants (CCDs) [6].

1.2 CCDs

The term CCD was first introduced by Alberse et al. who observed cross-reactions of IgE antibodies between vegetable foods, insect venoms and pollen and further found indications that the responsible antigen for this cross-reactivity should be a carbohydrate [6].

Subsequent investigations confirmed that CCDs are N-linked carbohydrate moieties of glycoproteins which exhibit two main motifs, namely an α 1,3-linked fucose linked to innermost GlcNAc and a β 1,2-xylose linked to the core mannose [7]. Both epitopes can not be found in mammalian glycoproteins and therefore can be considered as highly immunogenic structures [7-8].

N-linked glycosylation is one of the most important posttranslational processes for eukaryotic proteins and takes place in the endoplasmatic reticulum (ER) and Golgi apparatus. Glycosylation is a site specific enzyme directed process and needed for correct protein folding, stability of proteins and membrane interactions.

In the case of N-glycosylation glycans are always linked to the backbone of a protein through the free amino group of asparagine (Asn or N) in the sequence context Asn-X-Ser/Thr.

Until now it can be stated that most plants investigated so far exhibit the same spectrum of N-glycosylated structures [9]. The main differences to human N-glycans (see Fig. 3) are, as mentioned above, α 1,3-fucosylation of the inner most GlcNAc, β 1,2-xylose bound to the core mannose and the termination of the antennae with GlcNAc, mannose or galactose residues instead of sialic acid.

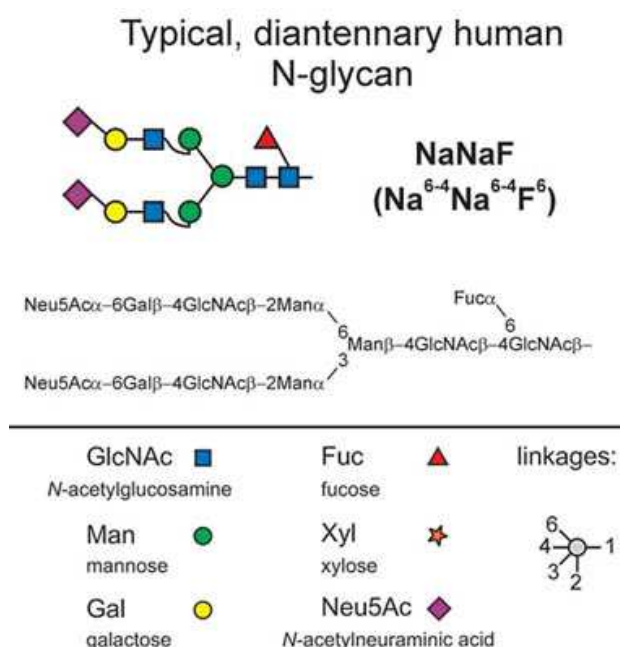


Fig. 3: Human N-glycan as example for mammalian glycoproteins. NaNaF is a typical complex-type N-glycan containing sialic acids [9].

As these carbohydrate structures not only occur in many allergens isolated from plants but also in allergens from insects and molluscs, IgE antibody production against CCDs results in a broad cross-reactivity between antigens from pollen, food and also insect venoms [6, 10-11].

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While plants possess epitopes, with α 1,3-linked fucose and β 1,2-xylose, insect glycoproteins lack core-linked xylose (Fig. 4) and hence only exhibit the fucose structure [7, 9, 12]. It also should be noted that xylose and fucose are parts of two different epitopes leading to two distinct antibody populations against these structures [10, 13]. It seems that human anti-CCD IgE is mainly directed against the fucose residue rather than xylose [14]. Although an Italian group found xylose-specific human IgE, they have not published this result yet.

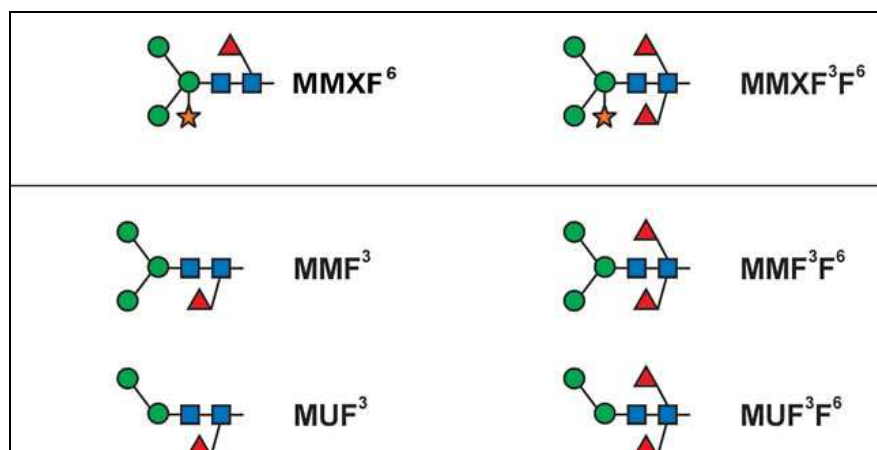


Fig. 4: Some typical snail, helminth and insect N-glycans. $MMXF^6$ and $MMXF^3F^6$ can be found in snails and helminths while MMF^3 is found in Bermuda grass (notably it contains no xylose). The remaining glycans without xylosilation are from insect venoms [9].

$MMXF^3$ glycan structures from horseradish peroxidase (HRP) and $MUXF^3$ from the pineapple stem protease bromelain seem to be the most common IgE binding structures among plant glycoproteins (Fig. 5) [7, 9].

Two complex-type plant N-glycan structures

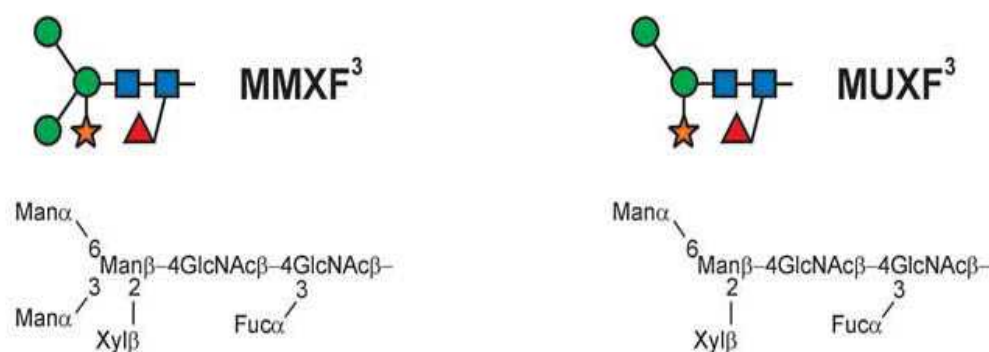


Fig. 5: Two of the most common CCD IgE inducing structures among plant glycans. $MMXF^3$ can be found in horseradish peroxidase (HRP) while $MUXF^3$ (MUXF) is derived from bromelain, a protease from pineapple stem. It should be noted that MUXF lacks a third mannose residue which is found in many plant glycoproteins like on HRP [9].

Up to now IgE against CCD structures can be found in 15%-30 % of sera gathered from patients predominantly allergic to pollen and insect venom [14-16]. It is a wide spread opinion that IgE against carbohydrates is primarily induced by contact with pollen or also insect stings [8].

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However anti-CCD derived IgE antibodies seem not to be able to elicit a clinical reaction in most of allergic patients. This low clinical significance was thought to be due to low binding affinity of anti-CCD IgE and/or poor biologic activity of these antibodies.

Clinical significance of anti-CCD IgE:

Concerning the first hypothesis, low binding affinity could be excluded by experiments from Jin et al. where binding affinities of anti-CCD IgE and –IgG were determined and turned out to be quite high [14]. It was also shown that anti-CCD IgG exhibited a stronger binding affinity than IgG directed against peptides. As the anti-CCD IgE titers in the sera of most patients were low it was assumed that IgG anti-CCD in allergic patients may act as blocking antibody by competing with IgE for allergens and hence prevent the development of clinical symptoms.

Poor biologic activity of cross-reactive CCD specific IgE was confirmed by van der Veen et al. by inhibition experiments and basophile histamine release assays (BHRA) [16]. In this study about 1/3 of patients allergic to grass pollen showed significant IgE levels against peanut allergens in RAST tests, without having a positive SPT or any allergic symptoms directed to peanuts. In 91 % of screened patients' sera (with positive RAST and negative SPT for peanut allergens) anti-CCD IgE could be detected and only a poor allergic reactivity could be observed in BHRAs in some patients after treatment with high doses of peanut allergens. It was suggested that detection of anti-CCD IgE in RAST is due to monovalent binding of IgE to parts of the glycan epitopes which in vivo would not be sufficient to activate mast cells or basophiles and lead to clinical symptoms.

Another study showed that even the polyvalent glycoprotein HRP, which exhibits seven N-glycans with CCD structures[17], was not able to elicit a positive SPT in 80 % of patients showing anti-CCD IgE reactivity in RAST tests [15].

However there are some studies demonstrating that IgE antibodies directed against plant glycan structures are able to trigger histamine release from mast cells and basophile granulocytes [18-20].

Although these experiments prove the biologic activity of anti-CCD IgE this does not mean that mediator release of effector cells automatically results in the occurrence of clinical symptoms.

If this was true than more or less all patients suffering from pollen allergy must also develop allergies to all plant derived foods, which obviously is not the case.

In a more recent trial clinical irrelevance of IgE antibodies against CCDs from plants could be indicated by a double-blind placebo-controlled oral challenge (DBPCOC) [21]. None of the allergic patients exhibiting significant biological activity of anti-CCD IgE *in vitro*, showed any clinical symptoms in SPT and DBPCOC after treatment with the allergen.

Therefore IgE antibodies directed solely against CCDs can be rated as clinically irrelevant for the majority of patients.

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1.3 Aim of this work

As CCD induced IgE antibodies frequently lead to false positive diagnosis in *in vitro* allergy diagnosis (when natural allergens are used) an important task would be the discrimination between anti-CCD and anti-peptide IgE. This would prevent unnecessary avoidance of “allergens” and inappropriate immunotherapy of atopic individuals which could cause even new real allergies.

To achieve this goal anti-CCD IgE has to be detected and/or removed from patients’ sera which undergo *in vitro* screening for allergen-specific IgE antibodies. Allergen specific IgE levels on the other hand should not have changed after the treatment to further assure accurate allergy diagnosis.

The two main steps of this work are first to create a proper affinity matrix for efficient removal of anti-CCD IgE antibodies from the serum of allergic patients and second to optimize the working process and amounts of material for optimal binding affinity and capacity of the, in our case, solid support.

To develop an anti-CCD IgE absorbing device, different matrices have to be coupled with various amounts of typical CCD glycans. By incubating the matrices with sera of allergic patients, only CCD specific IgE antibodies occurring in these sera should be removed and hence lead to more accurate results in subsequently performed *in vitro* allergy tests. The whole incubation and binding procedure is performed in small filter tubes where the matrix is added, settled and then incubated with the serum. Afterwards the serum can easily be removed from the gel by centrifugation.

In this work ELISA and CAP test (Phadia Diagnostics, Uppsala, Sweden) are chosen for determination of anti-CCD and allergen specific IgE before and after incubation of sera with the matrix.

In several trials the adequate amount of coupled glycopeptide for efficient removal of anti-CCD IgE should be determined.

For CCD glycan structures many sources would be available like various plant or insect glycoproteins. The limiting factor in this case is accessibility of the structures and costs of the different sources.

For our purpose we chose the MUXF³-glycopeptide (MUXF) which was gained by protease digestion of bromelain from pineapple stem.

A maybe weak point of MUXF could be that it does not exhibit α 1,3-mannose residue as it is the case for the majority of plant N-glycan structures like in HRP, several lectins or the grass pollen allergen Phl p1 [9]. In absence of α 1,3-mannose, fucose and xylose residues are more flexible exposing to the space. Previous studies by Jin showed that glycoconjugates with the MUXF structure inhibited anti-CCD IgE binding more efficiently than conjugates with the MMXF structure (see Fig. 9). Additionally, more common CCD structures could be derived from HRP or allergens from insect venoms but these are rather expensive sources and were therefore not chosen for our experiments.

Furthermore MUXF has been shown to successfully inhibit binding of CCD derived IgE antibodies to other glycan structures or detecting anti-CCD IgE in CAP test in several experiments [15, 18, 22].

After an appropriate combination of matrix and glycopeptide (-conjugate) has been determined the working process should be optimized and optimal ratios of solid support and serum for efficient anti-CCD IgE removal should be found.

2 Materials and Methods

2.1 Preparation of MUXF³ - Glycopeptide from pineapple stem bromelain

Bromelain extraction

30 g crude bromelain (Sigma No. B4882) are dissolved in 200 ml 0,05 M potassium phosphate buffer at pH 6.1 and stirred 1 h in cold room at 4°C. The mixture is centrifuged at 4°C, 10000 rpm for 20 minutes (in a Du Pont SLA 1500 rotor). The supernatant is used for further preparation and the pellet is discarded.

Cation exchanger chromatography

Batch-process: The gel (SP-Sephadex C50, Pharmacia) is equilibrated in starting buffer (0,05 M KH₂PO₄, pH 6,1) in a suction filter. The supernatant from 2.2.1 is mixed with the gel and sucked off. To remove non-binding particles the gel is washed several times with starting buffer. The column (5 x 17 cm) is filled with this gel.

Elution is done at 4°C (cold room) with elution buffer (0,3 M NaCl in starting buffer). 60 fractions with 12 ml (= 400 drops) are collected. The fractions are tested by orcinol-method and fractions containing the desired product are pooled and lyophilised overnight.

Acetone precipitation

50% acetone precipitation: After lyophilisation the product is dissolved in 50 ml cold dH₂O and while stirring (magnetic stirrer) 50 ml ice-cold acetone (for analysis) is added. The mixture is put on -80°C for 15 min. After centrifugation (centrifuge: RC5C Sorvall Instruments, Du Pont SN 8800066, SS34 rotor) at 8000 rpm, 15 min the supernatant is again precipitated. Pellet is not discarded!

70% acetone precipitation: 40 ml of acetone are added to the supernatant (while stirring) and put on -80°C for 15min. Centrifugation with 8000 rpm, 15min

The pellets from both precipitations are dissolved in dH₂O.

A flask is weighed before putting the dissolved pellets in (for pronase digestion afterwards), then the solution is lyophilised over night.

Pronase digestion and gel filtration

The lyophilised product is dissolved in 100 ml buffer (0,15 M Tris/HCl pH 7,8 with 1mM CaCl₂) per gram and denatured 30min at 80°C in water bath. Then 2% Pronase (Protease from *Streptomyces griseus*, Sigma) is added and the mixture is incubated at 37°C over night. Before gel filtration the digestion is put on a rotary evaporator until a volume of about 20ml is reached. The product is applied to the gel (Sephadex G50 superfine, Pharmacia) of the column (2,5 x 120 cm) and 60 fractions with 12 ml (= 400 drops) are collected over night (running buffer: 1% acetic acid)

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Fractions are tested by orcinol method and UV absorbance is measured at 280 nm. Fractions containing the desired product are pooled and lyophilised overnight (flask is weighed before filling the fractions in). Then pronase digestion and gel filtration are repeated once.

Glycopeptide purification by anion exchanger chromatography

The lyophilised pool is dissolved in 50-60 ml starting buffer (0,05 M Ammoniumacetate pH 8,8) and the pH is adjusted to 8,8 with ammoniac. The gel (Sephadex DEAE A25, Pharmacia) is equilibrated in a suction filter and filled into the column (2,5 cm x 20 cm). The sample is applied to the gel and the gel is washed with at least one column volume of starting buffer. Fractions are collected as soon as the sample is put on the gel. Afterwards 300 ml starting buffer and 300 ml elution buffer (0,4 M NaCl in starting buffer) are prepared in a gradient mixer. Elution is done by applying a linear gradient, 60 fractions (12ml) are collected over night.

The fractions are tested by orcinol-method and UV absorbance at 235 nm, fractions containing the desired product are pooled and lyophilised over night.

To remove salts the lyophilised product is dissolved in a few ml of 1% acetic acid and applied to the gel (Sephadex G25 fine, Pharmacia, Column: 1,5 x 50 cm). 30-40 fractions (6 ml) are collected (running buffer: 1% acetic acid). The protein is determined by orcinol-method and UV absorbance (235 nm). Fractions containing MUXF are pooled and lyophilised overnight (flask is weighed before adding the probe for lyophilisation).

As a final step the product should be controlled via mass spectrometry.

2.2 Coupling of MUXF-glycopeptide to proteins

Material:

- Buffer A: 0,1 M KH_2PO_4 , pH 7,2
- Buffer B: 0,4 M sodium tetraborate, pH 10,0
- Elution buffer: 50 mM NH_4Ac , pH 5,5

2.2.1 MUXF-BSA

MUXF (1,36 μmol) is dried in a Speed-Vac. The sample is mixed with 100 μl of Buffer A on ice. 500 μl DFDNB (6% 1,5-difluoro-2,4-dinitrobenzene in methanol, w/v) and 70 μl 7 M guanidine-HCl are added and incubated at room temperature for 15 min. The reaction mixture is extracted by 1ml of diethylether three times by vortexing the tube rigorously. The mixture is spun down shortly and the organic upper phase is discarded. The residue is dried in the Speed-Vac again.

If the sample is not pure, the activated glycopeptides are separated by Sephadex G25 fine (1 x 50 cm, 30% methanol, 100 drops/fraction). The first eluted orcinol positive peak should be the activated glycopeptides.

100 μl 10% BSA (about 150 nmols, w/v) in Buffer B is added and the mixture is incubated in the dark at room temperature over night. The BSA conjugates are then purified by gel filtration using BioGel P 30 (1,5 x 50 cm, medium $130 \pm 40 \mu\text{m}$, BioRad). First the column is washed with one column volume of elution buffer. After applying the sample (diluted with elution buffer up to

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500 µl) the column is eluted with elution buffer (40 drops/fraction, ca. 2 ml fraction size). Then the protein content is determined with the Micro BCA™ protein assay kit and sugar is determined by orcinol method.

2.2.2 MUXF-Lysozyme

5 mg MUXF are dissolved in 100 µl of Buffer A on ice. 500 µl DFDNB (6% 1,5-difluoro-2,4-dinitrobenzene in methanol, w/v) and 70 µl 7 M guanidine-HCl are added and incubated at room temperature for 15 min. The reaction mixture is extracted with 1 ml of diethyl ether three times by vortexing the tube rigorously. The mixture is spun down briefly and the organic upper phase is discarded. The residue is dried in the Speed-Vac or lyophilised.

2,5 mg Lysozyme are dissolved in Buffer B and 100 µl are added to the MUXF. The mixture is incubated in the dark at room temperature over night. The conjugates are then purified by gel filtration using BioGel P 30 (1,5 x 50 cm, medium 130 ± 40 µm, BioRad). First the column is washed with one column volume of elution buffer. After applying the sample (diluted with elution buffer up to 500 µl) the column is eluted with elution buffer (40 drops/fraction, ca. 2 ml fraction size). Then the protein content is determined with the Micro BCA™ protein assay kit and sugar is determined with the orcinol method.

2.2.3 Quantitation of total protein by Micro BCA™ protein assay kit

The quantitation is done with the Micro BCA™ Protein Assay Kit (Pierce).

A fresh set of protein standards is prepared by diluting the 2.0 mg/ml BSA stock standard, preferably in the same diluent as the sample.

Tab. 2: Preparation of standards for BCA protein determination

Volume of BSA to add (µl)	Volume of diluent to add (µl)	Final BSA concentration (µg/ml)
50 (STOCK)	450	200 (A)
268.8 (A)	89.6	150 (B)
198.4 (B)	99.2	100 (C)
137.6 (C)	137.6	50 (D)
115.2 (D)	172.8	20 (E)
128 (E)	128	10 (F)
96 (F)	96	5 (G)
32 (G)	128	1 (H)

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Preparation of the BCA working reagent: 25 parts of Micro BCA™ reagent MA (75 µl/sample) and 24 parts of Micro BCA™ reagent MB (72 µl/sample) are mixed with 1 part of Micro BCA™ reagent MC (3 µl/sample).

When the Micro BCA™ reagent MC is initially added to the solution of the other two reagents, a turbidity is observed that quickly disappears upon mixing, yielding a clear green working reagent.

Then 150 µl of each standard or unknown sample are pipetted into the appropriate wells of the microtiter plate. 150 µl of the diluent are used for the blank wells.

150 µl of the working reagent are added to each well and then the plate is mixed well on a plate shaker for 30s. The plate is covered and incubated at 37°C for 1h. After incubation the plate is cooled to room temperature. Then the absorbance should be measured at or near 562 nm on a plate reader. For evaluation the average 562 nm reading for each standard or unknown sample is calculated.

A standard curve is prepared by plotting the average blank corrected 562 nm reading for each BSA standard versus its concentration in µg/ml.

2.2.4 Orcinol Test

5 µl of each sample are transferred into a single well of a 96-well microtiter plate (e.g. Sarstedt* 96-Well Microtest Plate, PS, Flat). 200 µl of orcinol reagent (200 mg orcinol in 75 ml H₂SO₄ and 25 ml H₂O) is added to each well containing a sample. Afterwards the plate is covered and put on 80°C for 45 min (or on 90°C for 30 min). The optical density (OD) is measured at 405 nm with a SLT Spectra plate reader.

2.3 Coupling glycopeptides to matrices

2.3.1 Poly-L-Lysine Agarose

Material:

- Poly-L-Lysine Agarose, Sigma-Aldrich, P1666
- EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride), Pierce
- Sulfo NHS, Pierce

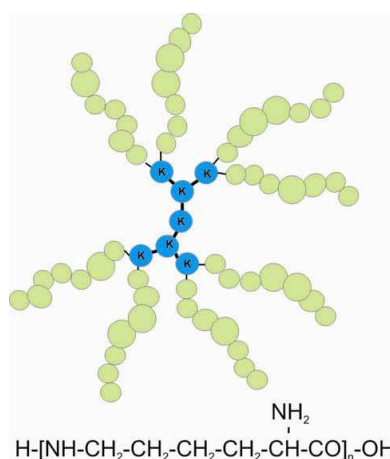


Fig. 6: Structure of Poly-L-lysine

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Coupling MUXF to Poly-L-lysine-Agarose via EDC and Sulfo-NHS

20 mg of MUXF are dissolved in 1 ml 0,15 PBS, pH 7.2. In the meantime, 0,4 ml of Poly-L-Lysine-Agarose are washed with PBS three times (500 µl each). MUXF-bromelain solution is added to the gel cake (depleted of PBS buffer) and 5 µl are taken out for orcinol test.

EDC (MW 191.7) is dissolved in water to give a concentration of 1 M (191.7 mg/ml). Sulfo-NHS (MW 217.14 g/mol) is dissolved in water to give a concentration of 50 mM (10.859 mg/ml). Then 100 µl of EDC and sulfo-NHS are added to the mixture, respectively. 5 µl are taken out for orcinol test. The mixture is incubated at room temperature for 2 h. The residual reagent is removed by centrifugation using a centrifugal filter (10 000 g for 1 min) and the gel is washed 3 times with 500 µl PBS buffer. 5 µl from each washout are taken for orcinol test to control the coupling efficiency.

Coupling MUXF-BSA to Poly-L-lysine-Agarose via EDC and Sulfo NHS

1 mg of MUXF-BSA is dissolved in 1 ml 0,15M PBS, pH 7.2. In the meantime, 0,4 mL of Poly-Lys-Agarose are washed three times with 500µl PBS. Then the MUXF-BSA solution is added to the gel cake (depleted of PBS buffer). 5 µl are taken out for orcinol test. EDC (MW 191.7) is dissolved in water to give a concentration of 1 M (191,7 mg/ml). Sulfo-NHS (MW 217.14 g/mol) is dissolved in water to give a concentration of 50 mM (10,859 mg/ml). 100 µl of EDC and sulfo-NHS are added to the mixture, respectively. 5 µl are taken out for orcinol test. Then the mixture is incubated at room temperature for 2 h. The residual reagent is removed by centrifugation using a centrifugal filter (10,000 g for 1 min) and the gel is washed with 500 µl PBS buffer 3 times. 5 µl from each washout are taken for orcinol test.

2.3.2 EDA / EPOXY disk

EDA disk:

Before working the buffers and ligand solution are filtered through a 0,45 µM or 0,22 µM filter.

The CIM® Disk Monolithic Column (BIA Separations, Austria) is assembled with the appropriate disk (Catalogue No.: 210.5116) and put on the HPLC. The column is washed with ca. 10 ml of a 50 mM Na-phosphate buffer, pH 7,5 at a flow rate of 1-3 ml/min. The amino groups are activated by pumping at least 1 ml of the 50 mM Na-phosphate buffer containing 1 % glutaric dialdehyde through the column. Then the disk is removed from the housing and put into a beaker with 10 ml of the same activating buffer at room temperature over night.

The disk is put back into the column and, to remove the excess glutaric dialdehyde, washed with ca. 10 ml of 50 mM Na-phosphate buffer, pH 7,5, at the working flow rate.

The ligand solution is prepared by dissolving 2 mg/ml MUXF-BSA in a 50 mM Na-phosphate buffer. At least 8 ml of this ligand solution should be pumped through the column to completely fill the pores of the disk and the flow through is collected in a beaker. Afterwards the disk is removed from the column and incubated with the flow through for 24 hours at room temperature (beaker should be covered).

Then the disk is reinserted in the column and washed with ca. 10 ml of 50 mM Na-phosphate buffer at a flow rate of 1-3 ml/min. Subsequently the column is rinsed with 10 ml of 50 mM Na-

Materials and Methods

phosphate buffer containing 1 M NaCl at a flow rate of 1-3 ml/min. Afterwards the column is re-equilibrated with 10 ml of the 50 mM Na-phosphate buffer at the same flow rate as before.

For longer storage time the disk is removed from the column and kept in a sealed plastic bag in 0,02 % NaN₃ at 4° C.

EPOXY disk:

Before working the buffers and ligand solution are filtered through a 0,45 µM or 0,22 µM filter. The CIM® Disk Monolithic Column is assembled with the appropriate disk (Catalogue No.: 213.7175) and put on the HPLC.

The ligand solution is prepared by dissolving 3 mg/ml MUXF-BSA in a 0,5 M Na-phosphate buffer, pH 8. The column is equilibrated by washing it with ca. 5 ml of the 0,5 M Na-phosphate buffer at a flow rate of 1-3 ml/min. At least 1 ml of the ligand solution is pumped through the column to fill the monolithic pores and the flow through is collected in a beaker. The disk is removed from the column and put in the beaker with the flow through. The beaker is covered with parafilm and left on room temperature for 24 hours. Then the disk is reinserted in the column and washed with ca. 5 ml of 0,5 M Na-phosphate buffer, pH 8, at a flow rate of 1-3 ml/min. The excess EPOXY groups are blocked by incubating the disk with 1 ml of 1M ethanolamine at room temperature overnight.

Afterwards the column is rinsed with ca. 5 ml of 0,5 M Na-phosphate buffer containing 1 M NaCl at a flow rate of 1-3 ml/min. Subsequently the column is re-equilibrated with 5 ml of 0,5 M Na-phosphate buffer at the same flow rate as before. For longer storage time the disk is removed from the column and kept in a sealed plastic bag in 0,02 % NaN₃ at 4° C.

2.3.3 Chromatography with monolithic affinity columns

The samples are added to the fast monolithic chromatography supports equipped with the appropriate monolithic disk and the chromatography was evaluated with an UV-VIS detector at 280 nm (SPD-10A, UV-VIS detector, Shimadzu).

After the system was washed with buffer A (50 mM Na-Phosphate buffer, pH 7,5), the samples were added. Elution is done with buffer B (0,2 M Glycine, pH 2,2).

Programme:

Time (min)	Buffer A (%)
0,1	100
6	100
6,5	0
9	0
9,5	100
13	100

A flow rate of 1ml/min was chosen.

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2.3.4 Eupergit

Material:

- Eupergit ® C, Sigma-Aldrich
- Eupergit ® CM, Sigma-Aldrich
- Eupergit ® C250L, Sigma-Aldrich
- 1,2 M Potassium phosphate buffer, pH 7,0: 320 µl 2 M K₂HPO₄, 40,68 ml 2M KH₂PO₄, 100 ml dH₂O

Tab. 3: Characteristics of the different Eupergit matrices [23-24]:

Immobilization product	Pore size	Activated group	Density activated group
Eupergit ® C	10 nm	Oxirane	600 µmol/g
Eupergit ® CM	10-100 nm	Oxirane	300 µmol/g
Eupergit ® C250L	100 nm	Oxirane	300 µmol/g

Eupergit ® C/ CM/ C250L was kindly provided by Dr. Roland Ludwig (Research Centre Applied Bio Catalysis, Division of Food Biotechnology, BOKU).

The desired amount of MUXF-BSA is dissolved in 10 ml 1,2 M potassium phosphate buffer, pH 7. The solution is added to 1 g of dry Eupergit beads and put on a rotating wheel at room temperature for 48 hours. Then the beads are washed with 40 ml of PBS in a filter syringe. To determine the efficiency of the coupling process, the protein concentration in the washout is determined by BCA method. Blocking is carried out with 4 ml of 5 % (v/v) aqueous 2-mercaptoethanol or with 0,5 % BSA in TTBS at 4°C on a rotating wheel over night. Afterwards the Eupergit beads are washed again with 40 ml of PBS. The coupled Eupergit is stored in PBS at 4° C.

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2.3.5 ECH Sepharose™ 4B

Material:

- ECH Sepharose™ 4B, GE Healthcare

Tab. 4: Technical specifications of ECH Sepharose™ 4B [25]:

Group to be coupled	- NH ₂
Coupling conditions	pH 4.5–6, 4°C to 25°C, 1.5–24 h; organic solvents possible
Matrix	Agarose, 4 %
Average particle size	90 µm
Spacer arm	6-amino caproic acid, 10 atom
Substitution	12–16 µmol carboxyl/ml medium
Storage temperature	4°C to 8°C

Coupling of MUXF –BSA/Lysozyme to ECH Sepharose 4B

1. 5 ml of sedimented matrix are washed on a sintered glass filter with 200 ml (in aliquots) distilled water (pH adjusted to 4,5 with HCl) followed by 200 ml 0,5 M NaCl. The almost dry gel is transferred into 2 ml Eppendorf tubes to estimate the amount (you should end up with about 3 ml gel).
2. **Coupling:** the desired amount of MUXF conjugate is dissolved in water (pH adjusted to 4,5 with HCl). This ligand solution is added to the gel in a ratio of about 1:1 (3ml dry gel + 3 ml ligand solution). EDC is added to a final concentration of 0,1 M (from a 1 M stock solution with dH₂O pH 4,5) and the pH is adjusted to 4,5 again with HCl. Then the mixture is put on a rotating wheel at 4°C. During the first hour of coupling the pH to 4,5 is adjusted again with 0,1 M NaOH. The coupling reaction is left at 4° C over night.
3. **Blocking:** the gel is blocked with Ethanolamin in an end concentration of 0,1 M, over night (ligand solution eventually can be removed before adding Ethanolamin). For a 1 M solution 142 µl Ethanolamin-Hydrochloride 60% solution are dissolved in 858 µl dH₂O pH 4,5.
4. The product is then washed thoroughly with at least three cycles of alternating pH. Each cycle consists of: 0,1 M sodium acetate (or acetic acid) pH 4 containing 0,5 M NaCl, followed by a wash with 0,1 M Tris-HCl buffer pH 8 containing 0,5 M NaCl. In the end the gel is washed with distilled water.
5. ECH Sepharose is transferred to a 15 ml Falcon tube with 4 ml PBS, pH 8 and NaN₃ is added in an end concentration of 0,2 % for storage.

Materials and Methods

2.3.6 Agarose beads

Material:

- Glyoxal agarose beads, HISPANAGAR (Spain)
- Aminoethyl agarose beads, HISPANAGAR (Spain)

Glyoxal agarose beads:

Tab. 5: Technical specifications of Glyoxal agarose beads [26]

Immobilization product	Glyoxal agarose beads	
Bead (geometry, size)	Spherical, Standard: 40-180 μm (approx.)	
Cross-linked, autoclavable	Yes, 121°C, 30 min	
Storage temp. and antimicrobial agent	2-8°C, suspension in 0.5 M NaCl containing 0.02% thimerosal	
Matrix active groups	Agarose with some diols oxidized to aldehydes	
Product Name	Low density glyoxal agarose	
Agarose (%)	4 BCL (4BCL-GL0-X)	6 BCL (6 BCL-GLO-X)
Activation degree (μmol glyoxyl/ml gel)	15 – 25	15 – 25
Binding capacity (mg BSA/ml gel)	5 – 10	5 – 10

1 ml of glyoxal agarose beads is washed with 10 ml dH_2O using a glass filter or a filter tube (Millipore). The desired amount of MUXF-BSA is dissolved in a 50mM bicarbonate buffer, pH 10,05 and 9 ml of this ligand solution are mixed with 1 ml of agarose beads. The following steps should be carried out at 4°C if the ligands are not stable at room temperature.

The mixture is put on a rotating wheel at 4°C for 2 hours. A longer incubation time may lead to a stronger interaction of biomolecule and agarose beads but also could lead to unfavourable distortions.

After the coupling step 10 mg solid sodium borohydride are added to the suspension and stirred for 30 minutes in an open container at room temperature to allow hydrogen to escape. Then the suspension is washed with a 25 mM phosphate buffer, pH 7, using a vacuum filter to eliminate the excess of borohydride. Subsequently the coupled agarose beads are washed with dH_2O in a filter and stored in PBS, pH 7,4, with 0,2 % NaN_3 at 4° C.

Materials and Methods

Aminoethyl agarose beads:

Tab. 6: Technical specifications of Aminoethyl agarose beads [26]

Immobilization product	Aminoethyl agarose beads	
Bead (geometry, size)	Spherical, standard: 40-180 μm (approx.)	
Storage temp. antimicrobial agent	2-8°C, suspension in 0.5 M NaCl containing 0.02% thimerosal	
Cross-linked, Autoclavable	Yes, 121°C, 30 min	
Matrix active groups	Amino groups	
Product name	Low density Aminoethyl agarose 6 BCL (6BCL-AL0-X)	Very low density Aminoethyl agarose 4 BCL (4BCL-AVL4-X)
Agarose (%)	6	4
Activation degree (μmol aminoethyl/ml gel)	15-25	3-6
Binding capacity (mg BSA/ml gel)	2-6	2-4

1 ml of aminoethyl agarose beads is washed with 10 ml of dH_2O using a glass filter or a filter tube. A solution of 8,85 ml dH_2O containing 0,19 g EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) and the desired amount of MUXF-BSA is prepared. Hence the ligand is not stable at room temperature the following steps were carried out at 4° C.

1 ml (about 0,7 g) aminoethyl agarose beads are added to the ligand solution and put on a rotating wheel at 4° C for 2 hours.

Afterwards the beads are washed with dH_2O , to eliminate excessive reagents, then with 1 M NaCl and finally with dH_2O again. Subsequently the coupled aminoethyl agarose beads are stored in PBS, pH 7,4, with 0,2% NaN_3 at 4° C.

Materials and Methods

2.3.7 PROSEP 9-CHO glass beads

Material:

- Prosep-9 CHO glass beads, 1000 Å, Millipore

The Prosep-9 CHO matrix contains of porous glass with interconnected pores of the same size. It is chemically and mechanically stable and insoluble over a wide range of conditions such as low pH, exposure to detergents, differing pressure etc. The matrix does not swell or shrink in different solutions and is extremely durable and incompressible.

Proteins and other molecules can be covalently coupled to Prosep-9 CHO via their primary amino groups.[27]

Coupling process:

The desired amount of MUXF-conjugate is dissolved in PBS buffer pH 7,4. 150 mg Prosep-9CHO glass beads are mixed with 1 ml of this ligand solution. The beads and ligand solution are mixed on a roller mixer at 4° C for 4 hours. Afterwards glycine (final concentration: 0,2 M) and sodium cyanoborohydride (final concentration: 0,05 M) are dissolved in PBS, and added to the glass beads. The beads are mixed with this solution at room temperature for 1 hour (after 15 minutes the cap of the tube is opened to allow the exposure of gases from the mixture). The tube is then left in a fume with the top loose for about 10 hours at room temperature.

Then the glass beads are washed with PBS ten times. Polyethylene glycol 20 000 (1% w/v) is dissolved in PBS, and mixed with the beads for 1 hour at 4° C. If required the glass beads can be additionally blocked with 0,5 % BSA in PBS for at least 2 h at 4°C on a roller mixer. Finally the beads are washed with PBS and stored in PBS at 4° C.

2.4 Human Sera

Before applying to the column, all sera were centrifuged in filter tubes at 12000 x g for 1-2 minutes to remove lipids and dead cell contents which otherwise would block the matrix during incubation.

Most sera used were obtained and kindly provided by Univ.-Doz. Dr. Wolfgang Hemmer from the Allergy Centre Floridsdorf (FAZ).

CCD positive sera with negative SPT:

Serum No.	Bormelain CAP (kU/L)	Total IgE	Allergy types	CCD positivities
HE	8.4	375	ragweed: CAP 15.4 kU/l, skin test negative; birch: CAP 11.2 kU/l, skin test negative; wheat flour: CAP 11.2 kU/l, skin test negative	CCD positive
D01	1.7	15		CCD positive no allergy

Materials and Methods

Sera of patients with pollen allergies and eventually CCD IgE:

No.	Skin test	Total IgE	Birch	Bet v 1	grass	Phl p 1/5	hdm	Der p 1	Fel d 1	Others
D03	grass birch	78		7.00		5.60				alternaria 4,2
D04	grass birch	53		0.00		5.60				
D06	birch	444	>100		0.00					
D09	grass	44			25.60					
D10	grass	412		0.00		87.50			32.10	Phl p 7 22,4
D12	birch grass	368		7.00		1.96				
D20	grass	67	0.00		14.00					
D22, MG	gras	740	0.4		> 100					
P044	Birch			4.20						

Serum pools, all with anti-CCD IgE containing sera from FAZ:

- Old serum pool 3: pooled human anti-CCD sera, 1.6.06
- Serum pools 1-9, 29.1.09
- New serum pools 1-4, 24.4.09

Serum from the Division of Environmental Dermatology and Venereology, Medical University of Graz:

Serum no.	Culprit insect	IB CCDs	Skin test honeybee pos. at	Skin test wasp pos. at	IgE honeybee CAP	IgE wasp CAP	BAT honeybee	BAT wasp
G21	wasp	+++	neg	0,1	5.59	11.5	neg	pos

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2.5 Chromatography

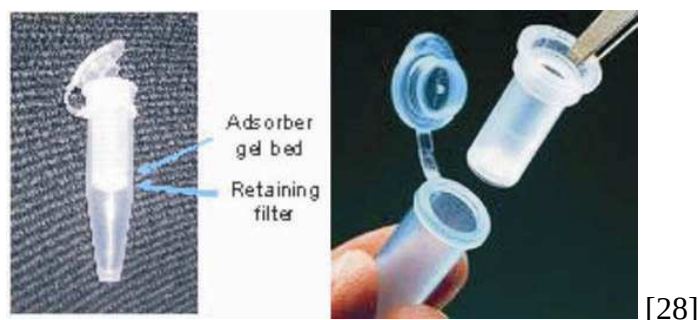


Fig. 7: Filter tubes used for incubation of matrix and serum. The serum was removed from the gel by centrifugation and the breakthrough was collected in the bottom of the tube. The capacity of the tube (beneath the filter) is up to 400 μ l.

50 μ l of affinity gel are transferred into a filter tube (Ultrafree MC, centrifugal filter units, 0,65 μ m pore size, Millipore, see Fig. 7) and washed 3 times with 200 μ l of 0,15 M PBS, pH 7,2. After centrifugation at $1000 \times g$, for 1 min (to settle down the gel), the desired amount of serum (previously centrifuged to remove lipids) is subjected to the matrix. The gel is incubated with the serum for 10 min at room temperature on a shaker at 300-350 rpm. After a short centrifugation (either $1000 \times g$ for 1 min or with the desk-centrifuge for a few seconds) the breakthrough is transferred to an 1,5 ml Eppendorf tube. The gel is washed twice with 50 μ l PBS and the washout is combined with the breakthrough. Afterwards the gel is treated with 100 μ l of 0,2 M glycine-HCl, pH 2,2, vortexed for a few seconds and centrifuged. The elution is collected in another Eppendorf tube and 50 μ l 0,5 M Tris-HCl, pH 7,8 are added to the tube to neutralize the elution.

In later trials the centrifugation speed before incubation was raised to $11000 \times g$ as the binding capacity of the matrix could be increased and dilution of the sample was decreased. Additionally the washout was collected separately and not combined with the breakthrough anymore.

2.6 ELISA

Material:

- ELISA microtiter plates: Immuno 96 MicroWell plates, MaxiSorpTM C96, NuncTM
- TTBS buffer: 0,1 M Tris-HCl, pH 7,2, 0,1 M NaCl, 2,5 mM MgCl₂, 0,05 % Tween 20 in dH₂O
- Coating buffer: 42 mM Na₂CO₃, 60 mM NaHCO₃ and 0,02% NaN₃, pH 9,6 in dH₂O
- Blocking buffer: 0,5 % BSA in TTBS
- AKP conjugated rabbit anti human IgE, BD PharmingenTM
- AKP conjugated rabbit anti human IgG, Jackson Immuno Research
- Anti-HRP IgG: Anti-Peroxidase antibody produced in rabbit (P7899), Sigma-Aldrich
- Alkaline Phosphatase anti rabbit IgG (H+L), made in goat, affinity purified, Vector Laboratories

Materials and Methods

2 µg/ml glycoprotein (HRP) or glycopeptide-conjugate (MUXF-BSA) are dissolved in coating buffer and coated onto a 96-well microtiter plate. After incubation at 37°C for 1 h, the plate is washed with TTBS (150 µl / well) 3 times. Afterwards the wells are blocked with TTBS buffer containing 0,5% BSA (50 µl / well) and incubated at 37°C for another 1 h. The plate is washed 3 times with TTBS buffer and 50 µl from each sample are added to an individual well. The plate is then incubated at 37°C for 1 h. After washing with TTBS buffer 3 times the appropriate antibody is added (50 µl/well) and incubated at 37°C for 1 h. After another washing step (3 times with TTBS), visualization is carried out with 50 µl of 0,1% PNP (p-nitrophenyl phosphate) in 0,1 M diethanolamine, pH 9,8.

The colour reaction is stopped after 60 min of incubation at room temperature when detecting IgE. For IgG detection a shorter developing time is necessary (check colour every 10-15 minutes). 10-15 µl of 70% ethanol are added to each well (containing a sample) to remove bubbles. The plate is read immediately at 405 nm using a SLT Sepctra plate reader.

For detection of anti-CCD IgE an AKP conjugated rabbit anti human IgE antibody was used in a dilution of 1:500. All breakthroughs were diluted 1:10.

For detection of IgG an AKP conjugated rabbit anti human IgG antibody or anti rabbit IgG (H+L) was added in a dilution of 1:2000.

2.7 SDS Page

Material:

- SDS Page Apparatus from Biorad
- 5x Electrophoresis Buffer: 15 g/l Tris, 72 g/l Glycine, 5 g/l SDS

12,5% SDS Page, 1 Gel:

- Resolving gel:
3x 833 µl 30% Acrylamide, 780 µl 1% Bis, 2x 750µl TGP (0,5 M Tris/HCl, pH 6,8), 2x 570µl dH₂O, 60 µl SDS, 36 µl APS, 3,6 µl Temed
- Stacking gel:
570 µl 30% Acrylamide, 390 µl 1% Bis, 750 µl SGP (0,5 M Tris/HCl, pH 6,8), 2x 640 µl dH₂O, 30 µl SDS, 24 µl APS, 2,4 µl Temed

Assembly of SDS Page apparatus:

After the glass cassette is fixed in the casting stand the resolving gel can be filled in with a Pasteur pipette or a 1000 µl pipette. After the gel was poured it is overlaid with 200 µl of isopropanol immediately. Polymerisation is taking about 45 minutes.

After that isopropanol is washed out with dH₂O, the rest of the water between the glass plates is removed with a kitchen towel and the stacking gel can be poured on top. The desired comb is inserted between the spacers and the gel is left for 30 – 40 minutes to polymerize.

Afterwards the comb is gently removed from the gel which then is prepared for sample loading.

Before the glass gel cassette can be fixed in the electrode assembly, the U-shaped gaskets have to be placed in the appropriate slots of the assembly.

Materials and Methods

The inner chamber of the apparatus is built and put into the tank.

Then the inner tank is filled with fresh 1x electrophoresis buffer, the outer tank with used 1 x electrophoresis buffer. The samples mixed with loading buffer (containing DTT) are put on a heating block for 5 – 10 minutes to denature the sample. Then the samples are centrifuged shortly and loaded into the appropriate wells with a 25 µl glass syringe.

When all samples are loaded, gel electrophoresis is done with 200 V constant for about 45 minutes.

After electrophoresis is done, the power supply is turned off, the lid is removed and the used electrophoresis buffer from the outer tank is discarded. The buffer from the inner tank is stored in a bottle for reuse.

2.8 Coomassie Staining

After electrophoresis the stacking gel part is discarded and the resolving gel is put into fixing buffer (500 ml Methanol, 70 ml HAc in 1 l dH₂O) for 15 minutes on a shaker. After the buffer was discarded, Coomassie Blue (3,8 ml Perchloric acid, 0,4 g Coomassie Blue G-250, 10 ml Methanol in 1 l dH₂O) is added to the gel which is stained for 30 minutes – 1 hour, shaking. The Coomassie Blue solution is put back into another bottle for reuse and the gel is decolourized with 5 % HAc for at least 30 minutes on the shaker. In the last step the gel is washed with dH₂O and scanned.

2.9 Silver Staining

Material:

- Fixation buffer: 40 % ethanol, 10 % HAc (v/v)
- Sensitive buffer: for 100 ml: 30 ml ethanol (30%), 500 µl 25% glutaraldehyde solution (0.125%), 314 mg Na₂S₂O₃ 5H₂O (0.2%), 11,3 g NaAc 3H₂O (6.8%)
- Silver buffer: for 100 ml: 250 mg AgNO₃ (0.25%), 40,5 µl of 37% formaldehyde solution (0.015%)
- Developing buffer: for 100 ml: 2,5 g Na₂CO₃ (2.5%), 20 µl of 37% formaldehyde solution (0.0074%)
- Stopping buffer: 1,46 % EDTA

The resolving gel is fixed with fixation buffer for 30 minutes on a shaker at room temperature. Subsequently the buffer is discarded and sensitive buffer is added for another 30 minutes.

The gel is washed with dH₂O for 5 minutes three times. Staining is done with silver buffer for 20 minutes, shaking. Afterwards the gel is washed twice with dH₂O, for exactly 30 seconds per wash.

Visualization is carried out with developing buffer for 2 – 30 minutes. When the desired colour is reached, the reaction is stopped by adding stopping buffer for 10 minutes. Finally 5 % HAc is added to end the reaction.

Materials and Methods

2.10 Western Blot

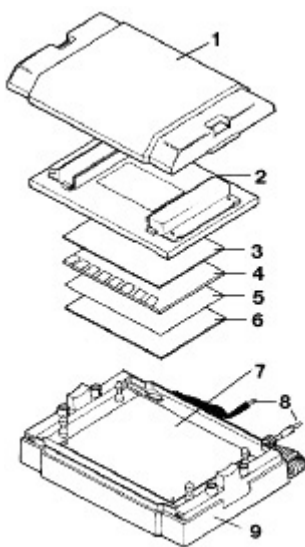
Material:

- Blotter: Trans-Blot SD, Semi Dry Transfer Cell, Biorad
- Filter Paper: extra thick filter paper, 15 x 20 cm, BIORAD
- Nitrocellulose: BioTrace™ NT, 30 cm x 3 m, Pure Nitrocellulose Blotting Membrane, Pall Corporation
- 10x Blotting Buffer: 250 M Tris, 1,93 M Glycine
- 1x Blotting Buffer: 10% 1x Blotting Buffer, 20% Methanol, 70% dH₂O
- Ponceau: 0,5 % Ponceau S (Sigma) in 1%-HAc
- Blocking Buffer: 0,5% BSA in TTBS
- Colour developing: Sigma FAST, BCIP/NBT, one tablet is dissolved in 10 ml dH₂O

The filter paper and nitrocellulose membrane are cut into the size of the gel and soaked in blotting buffer. The blotting cell is wiped with a wet kitchen towel soaked with blotting buffer to wet the surface of the blotter. Then the blotting sandwich is built and airbubbles are removed from it by carefully rolling a glass tube over the whole sandwich. The blotter is closed and the blot is left for 25 min at 15 V.

Blotting Sandwich:

- 1 layer of wet filter paper
- Nitrocellulose membrane soaked in blotting buffer
- Gel
- 1 layer of wet filter paper



[29]

Fig. 8: Assembly of the blotting sandwich and blotting apparatus. 1: lid of blotter, 2: cathode assembly, 3: filter paper, 4: gel, 5: nitrocellulose membrane, 6: filter paper, 7: spring-loaded anode platform, mounted on four guide posts, 8: cables for power supply, 9: base of apparatus

Materials and Methods

After blotting the nitrocellulose membrane is stained with Ponceau solution to control if the protein transfer worked. If the transfer was successful and enough protein is present, pink bands should appear on the membrane. Ponceau is then put back for reuse and the remaining solution is removed by washing the blot a few times with dH₂O. Subsequently the membrane is blocked with blocking buffer for at least 1 hour.

Afterwards the membrane is washed 3 times for 5 minutes with TTBS. The first antibody is added (diluted with TTBS or blocking buffer) and the blot is incubated for 1 h. The membrane is washed 3x for 5 minutes with TTBS again and if necessary the secondary antibody can be added for 1 h. After another washing step (3 x 5 minutes with TTBS) the colour is developed for a few minutes. When the desired intensity of the colour reaction is reached the reaction is stopped by adding 5 % HAc.

Results

3 Results

As the main aim was to find a combination of matrix and glycopeptide which successfully could deplete anti-CCD IgE antibodies from patients' sera, several matrices were coupled with MUXF (-conjugates) and tested for anti-CCD IgE removal.

Previous studies in this field with several glycopeptides and matrices like Sepharose or Poly-Lysine Agarose were done by Jin. These studies showed that glycoconjugates with the MUXF structure inhibited anti-CCD IgE binding more efficiently than conjugates with the MMXF structure (see Fig. 9).

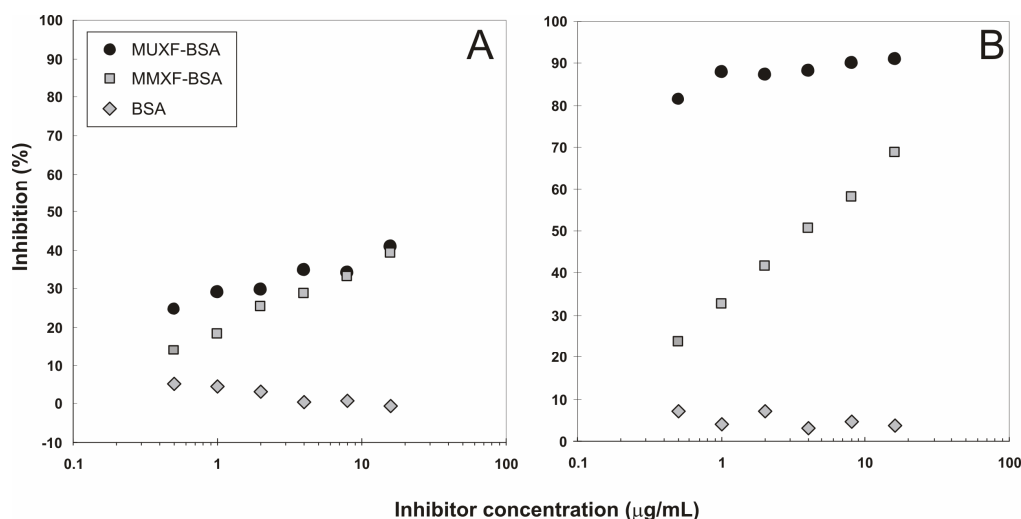


Fig. 9: Comparison of MUXF-BSA with MMXF-BSA. Rabbit anti-HRP antiserum (A) or human CCD-serum pool (B) was added to 96-well microtiter plate which was pre-coated with HRP (2 μg/ml). The bound antibody was detected either with AKP-conjugated goat anti-rabbit IgG or AKP-conjugated mouse anti-human IgE antibody pre-incubated with various amount of the indicated inhibitors. BSA was used as a negative control.

After every preparation from bromelain the occurrence of MUXF-glycopeptide was controlled via mass spectrometry (Fig. 10).

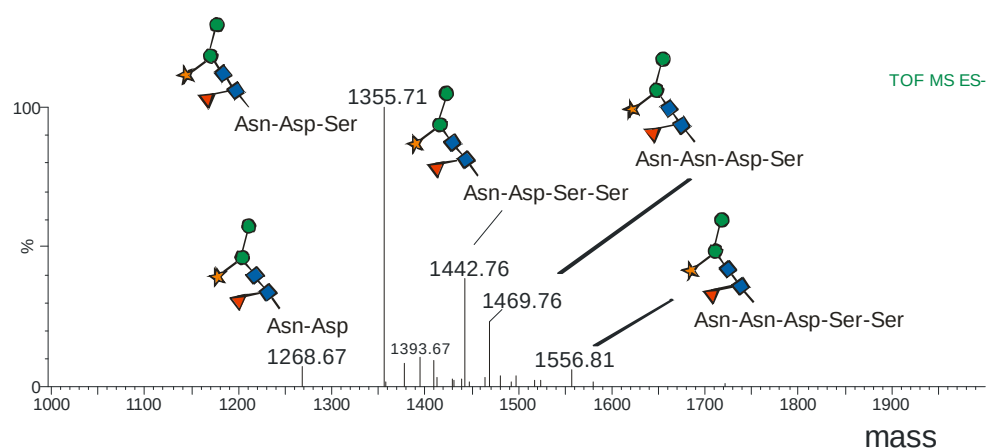


Fig. 10: Mass spectrum (here carried out with MALDI-TOF) of bromelain from pineapple stem containing MUXF.

Previous experiments performed by Jin (results not shown here) and trials in this work (see 3.4.1) showed that coupling solely the glycopeptide to a matrix often results in decreased removal of anti-CCD IgE. When linking MUXF to another protein, e.g. BSA, binding capacities for anti-CCD IgE could be increased. The difference in the removal of CCD-specific IgE probably was caused by

Results

different coupling abilities of the glycopeptide and the glycopeptide-conjugate. Most coupling procedures would need an amino or carboxyl group of the ligand for cross-linking. Unlike the MUXF-BSA conjugate, each MUXF-glycopeptide molecule only provides one amino/carboxyl group. Once one MUXF-BSA molecule is coupled to the porous support, it will present 5-6 MUXF structures on the surface. Furthermore, multiple covalently immobilized BSA molecules should become much more stable than coupled MUXF alone. The conjugation of MUXF with another protein should also increase accessibility of MUXF for CCD derived IgE antibodies and hence enhance the binding capacity of the cartridge.

Therefore in the beginning new trials with Poly-L-Lysine Agarose coupled with a MUXF-BSA conjugate were performed to test these assumptions.

3.1 Poly-L-Lysine Agarose

To create a proper affinity matrix, different amounts of MUXF-BSA were coupled to Poly-L-lysine agarose. Previous results (not shown here) showed that coupling via EDC and Sulfo-NHS lead to the best binding of MUXF-BSA to the agarose in comparison with EDC alone.

1 mg, 10 mg, 20 mg or 40 mg MUXF-BSA were coupled to 400 μ l Polylysine gel, and the effectiveness was tested by monitoring free MUXF-BSA in coating buffer using the BCA method (Fig. 11). Because of self-ligation the coupled ligand did not increase with the amount of ligand added. It was obvious that 10 mg MUXF-BSA did not saturate the Polylysine gel, while 40 mg MUXF-BSA did.

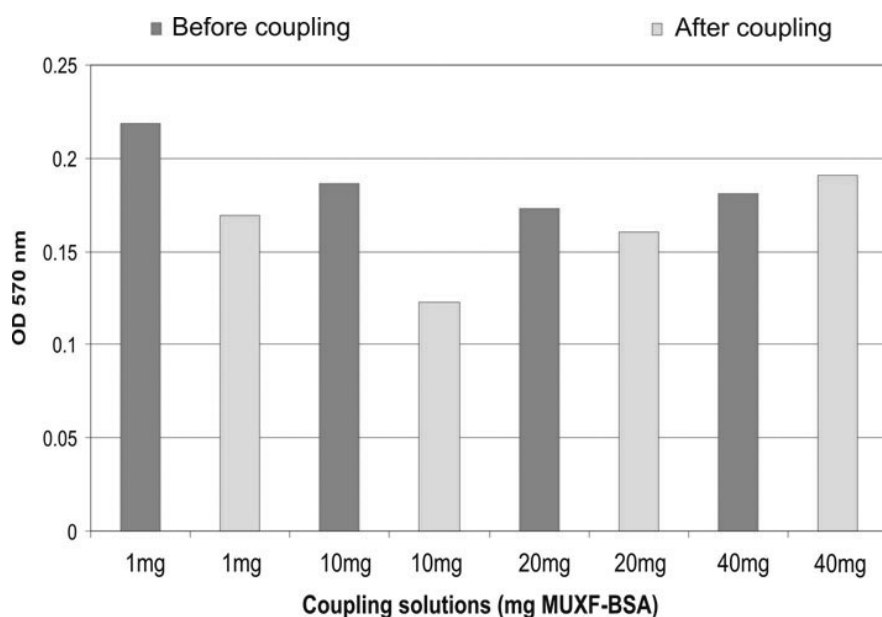


Fig. 11: Results of BCA test after coupling of different amounts of MUXF-BSA to Poly-L-lysine agarose. Aliquots of 10 μ l before and after coupling of each coupling approach were evaluated. The amount of conjugated protein was measured at an OD of 570_{nm} by BCA method.

10 mg and 40 mg MUXF-BSA gels were chosen for further trials.

To test the anti-CCD IgE binding capacities of the two coupled Polylysine gels, 10 μ l (+ 40 μ l TTBS) and 50 μ l of serum G21, a serum from a wasp allergic patient, were applied to 50 μ l of coupled gel, respectively. The breakthrough was collected and anti-CCD IgE occurrence was tested via ELISA.

Results

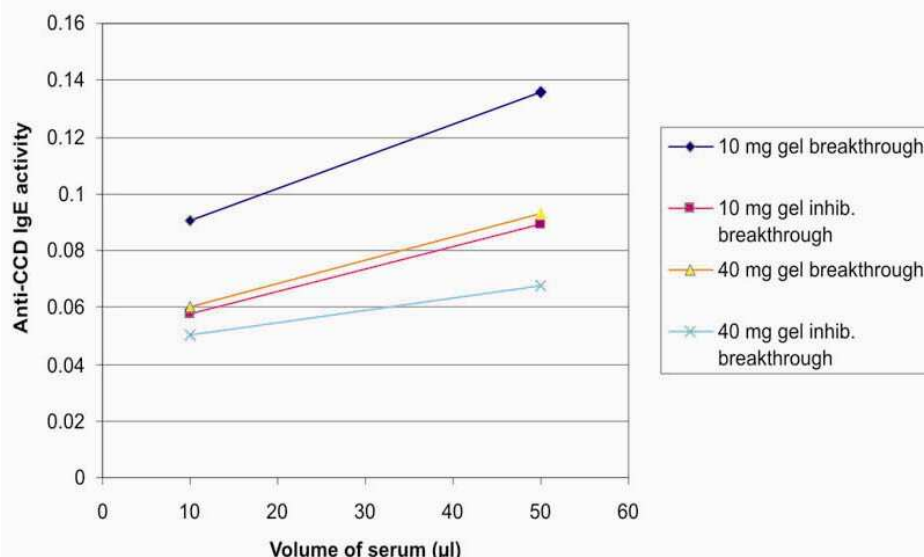


Fig. 12: Serum after 10 mg and 40 mg MUXF-BSA Poly-L-lysine gels. The serum before column (not shown in this figure) in a dilution of 1:10 showed a reading of $OD_{405nm} = 0,544$ and when inhibited with $5 \mu\text{g/ml}$ MUXF-BSA a reading of $OD_{405nm} = 0,156$. All breakthroughs were diluted 1:10 for ELISA evaluation. The plate was coated with $2 \mu\text{g/ml}$ HRP.

The breakthrough after the 10 mg MUXF-BSA column showed higher values in ELISA for anti-CCD IgE than after the 40 mg column. The ratio with 1:5 (serum/gel, v/v) was more efficient in removing anti-CCD IgE from the serum than the ratio of 1:1. To test if the breakthrough still contained anti-CCD IgE after the column, an aliquot was treated with $5 \mu\text{g/ml}$ MUXF-BSA, which should inhibit anti-CCD-IgE left in the breakthrough. The inhibition showed that the occurring IgE was directed against CCDs as the values could be reduced to background level.

As the 40mg MUXF-BSA Poly-L-lysine agarose was more efficient in binding anti-CCD IgE, this gel was used for further testing.

To determine the binding capacity of the 40 mg MUXF-BSA Poly-L-lysine agarose the column was treated with different dilutions of rabbit anti-HRP IgG antibody. The capacity was calculated from the resulting ELISA values before and after incubation of the gel with the rabbit antibody.

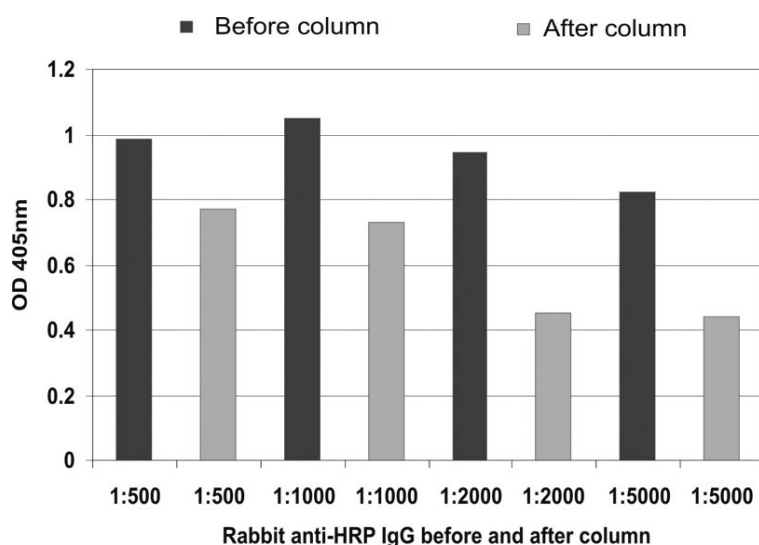


Fig. 13: Determination of binding capacity of 40 mg MUXF-BSA Polylysine agarose. ELISA plate was coated with $2\mu\text{g/ml}$ MUXF-BSA. $50 \mu\text{l}$ of gel were incubated with $200 \mu\text{l}$ of rabbit anti-HRP IgG in concentrations from 1:500 – 1:5000. Anti rabbit IgG was used as secondary antibody in a dilution of 1:2000. The colour reaction was left at room temperature for 10 min.

Results

1 ml of this gel was able to bind about 150 µg rabbit anti-CCD IgG as judged from the ELISA results. In detail when 7,2 µg (1:500, 200 µl) passed through the column, 0,89 µg of rabbit IgG bound to the MUXF-BSA gel; when 3,6 µg (1:1,000, 200 µl) passed through, 0,64 µg of rabbit IgG bound to the gel and when 1,8 µg (1:2,000, 200 µl) were incubated with the agarose, 0,63 µg of rabbit IgG bound to the column.

The next step was to find the ratio of gel to serum that yields satisfying results concerning the removal of anti-CCD antibodies from the serum.

50, 100, 200 and 400 µl of serum pool 3 were added to 50 µl of gel, to test at which ratio serum:gel the anti-CCD IgE start to leak. Each breakthrough and elution was evaluated via ELISA without and with inhibition by MUXF-BSA.

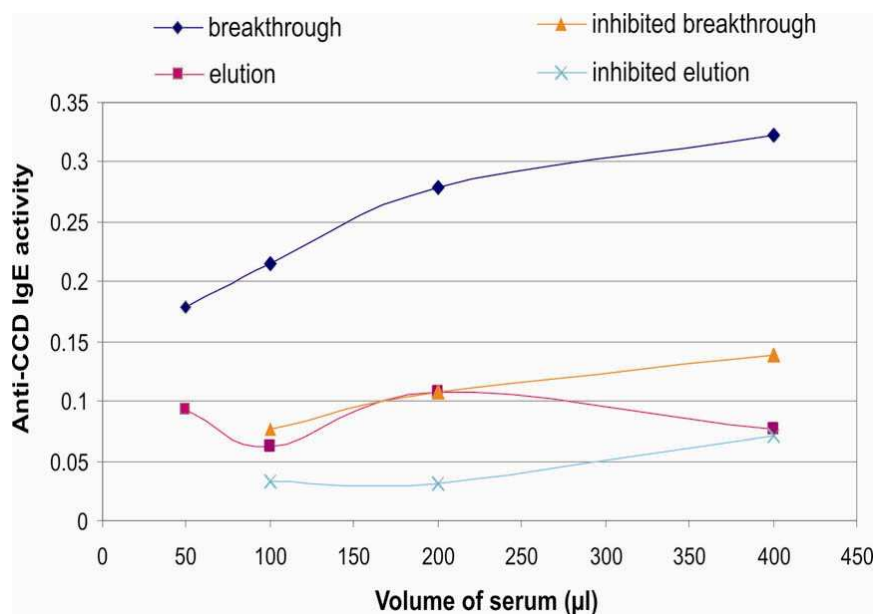


Fig. 14: Determination of binding capacity of 40 mg MUXF-BSA Polylysine agarose. Inhibition was carried out with 10 µg/ml MUXF-BSA. Coating was done with 2 µg/ml HRP.

As can be seen in Fig. 14 the amount of anti-CCD IgE after column (breakthrough) increased with an increasing amount of serum.

The pure serum pool in a dilution of 1:10 gave a reading of $OD_{405nm} = 0,429$ and when inhibited with 20 µg/ml MUXF-BSA the reading was at 0,137. Therefore the values for the serum pool after incubation with the gel in a ratio of 1:1 could be considered as at background level.

When comparing the results for the 100 µl breakthrough with its inhibition it could be seen that the reading for the serum after column could be reduced to background when inhibited. This indicated that a considerable amount of anti-CCD IgE was already escaping from the column at a ratio of 1:2. For all other ratios a clear overloading of the gel was detectable.

A possible reason for leaking of anti-CCD IgE from the column could be the binding of anti-CCD IgG to the gel. The concentration of IgG in the serum of a healthy patient is supposed to be around 13,5 mg/ml and IgE concentration should be about 0,05 mg/ml. [2] Although IgE levels rise in individuals with type I hypersensitivities, the IgG concentration is still 100-1000 times higher than for IgE and this could lead to a displacement and leaking of anti-CCD IgE from the column.

To test this assumption a serum pool rich in anti-CCD IgE (old serum pool 3) was evaluated by ELISA for anti-CCD IgE and IgG before and after removal of IgG and after incubation with 40 mg MUXF-BSA Polylysine agarose. 50 µl of gel were incubated with 100 µl and 200 µl of serum pool. To remove IgG antibodies, some samples were previously treated with Protein G Plus- Agarose gel before applying them to the MUXF-BSA-Poly-L-Lysine Agarose.

Results

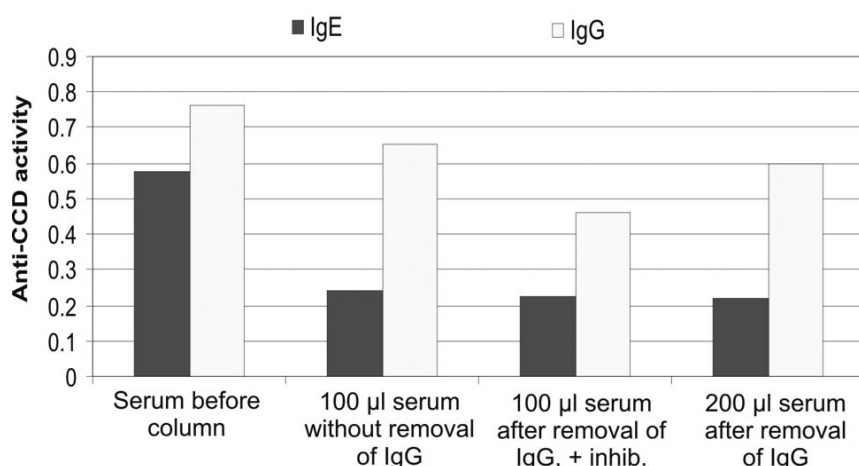


Fig. 15: Determination of anti-CCD IgE and IgG levels after treatment of the serum pool with MUXF-BSA Polylysine Agarose (the 40 mg version) and after removal of IgG with Protein G plus Agarose gel. All breakthroughs were diluted 1:10 for IgE measurement and 1:1000 for IgG measurement. Inhibition was carried out with 10µg/ml MUXF-BSA.

To test if the reading in ELISA was due to anti-CCD IgE/IgG antibodies, some breakthroughs were treated with 10 µg/ml MUXF-BSA to inhibit eventually occurring anti-CCD antibodies in the samples. As can be seen in Fig. 15 anti-CCD IgE values did not change after inhibition, which means the IgE antibodies could be removed from the serum pool. For CCD specific IgG antibodies inhibition could be observed suggesting that not all anti-CCD IgG could be removed by Protein G and CCD-polylysine columns. The preferential binding of anti-CCD IgE over IgG is in line with the higher affinity of IgE as determined recently [14].

Therefore we concluded that the presence of anti-CCD IgG in the serum did not affect the binding of anti-CCD IgE to the MUXF-BSA Poly-L-lysine gel.

Further tests on anti-CCD IgE and IgG were performed with MUXF-BSA Polylysine agarose (40 mg version), using a new serum pool with higher anti-CCD IgE levels, and evaluated with ELISA.

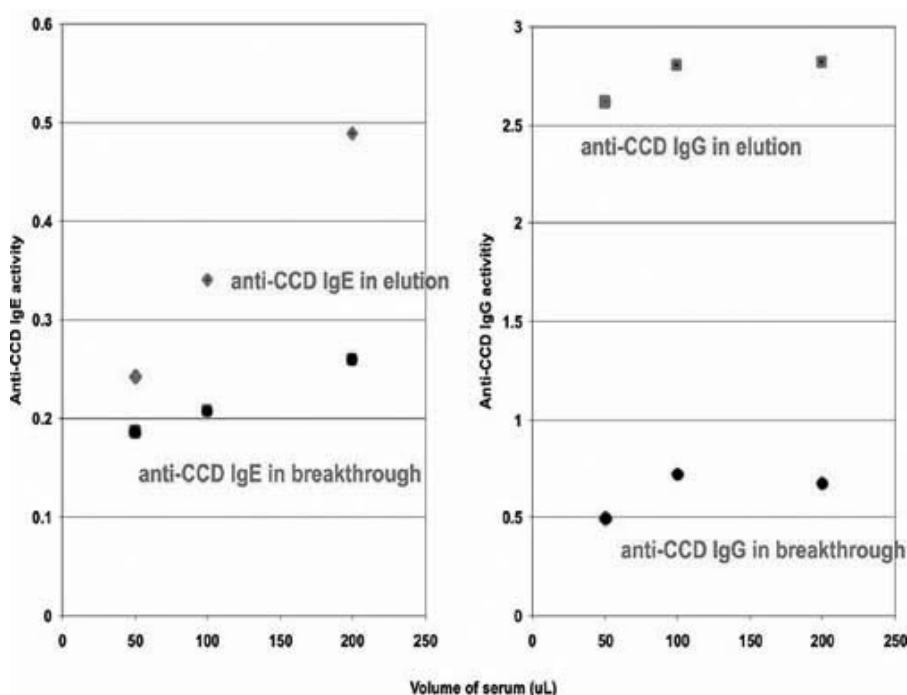


Fig. 16: 50 µl of MUXF-BSA Polylysine agarose (40 mg version) tested with 50, 100 and 200 µl of a new serum pool. Breakthroughs for IgE determination were diluted 1:10, samples for IgG determination were diluted 1:1000. Anti-CCD IgE before column: OD_{405nm} = 0,946

Results

With an increasing amount of serum (and a fixed amount of the coupled Poly-L-Lysine Agarose), there was an increase of anti-CCD IgE in the serum after incubation with the gel. Nevertheless at a ratio of gel to serum of 1:2 a quite high depletion of anti-CCD IgE could be reached (see Fig. 16). Anti-CCD IgG was also bound by the column but did not seem to affect the binding efficiency and capacity of anti-CCD IgE up to a ratio of gel to serum of 1:2.

To test if the gel is effective in removing anti-CCD IgE without influencing the amount of allergen specific antibodies a CAP test was done. Therefore the 40 mg MUXF-BSA Poly-L-lysine Agarose was incubated with different amounts of an anti-CCD serum pool and the anti-CCD and allergy-specific IgE levels were measured before and after treatment by CAP method. For this serum pool serum P044, a serum of a birch allergic patient, and serum G21 were mixed in a ratio of 1:1. 130 µl of the 40 mg/ml MUXF-BSA Polylysine Agarose were incubated with 130 µl of the serum pool and the breakthrough was collected (and not combined with the washout). The CAP test was performed at the FAZ.

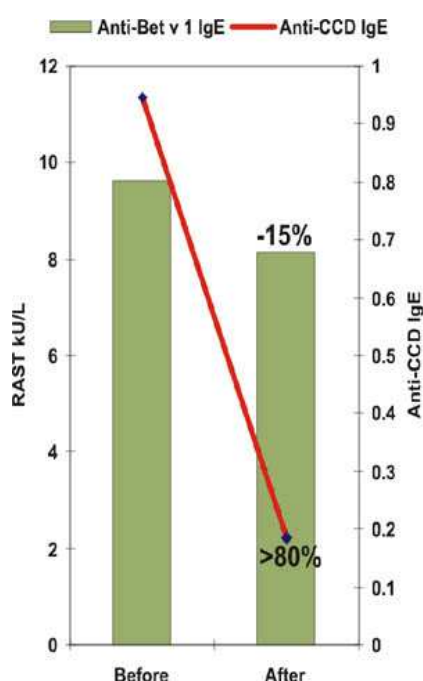


Fig. 17: CAP results for the second serum pool after treatment with 40 mg MUXF-BSA Poly-L-lysine agarose. Cut off for allergy detection is at 0,34 kU/L.

The CAP test revealed results similar to the previous ELISA tests. At a ratio of gel to serum of 1:1 more than 80 % of anti-CCD IgE (before cartridge 0,946 kU/l; after cartridge 0,185 kU/l) could be removed from the serum while allergen specific IgE (in this case Anti-Bet v1 IgE) was slightly decreased (15 %, before cartridge 9,64 kU/l, after cartridge 8,15 kU/l) after incubation with the Poly-L-lysine Agarose. In the CAP test system the cut-off for positive allergy detection is 0,34 kU/l. Hence after incubation with the MUXF-BSA coupled polylysine matrix, the serum became CCD-negative.

Compared to other matrices a lot of MUXF-BSA (more than 30 mg/ml matrix) had to be coupled to the polylysine agarose to get to a satisfying removal of anti-CCD IgE from the sera.

In later trials new MUXF-BSA coupled polylysine gels were prepared (0, 1, 3, 6 mg/ml MUXF-BSA) in order to be compared with equally coupled ECH Sepharose 4B gels.

Results

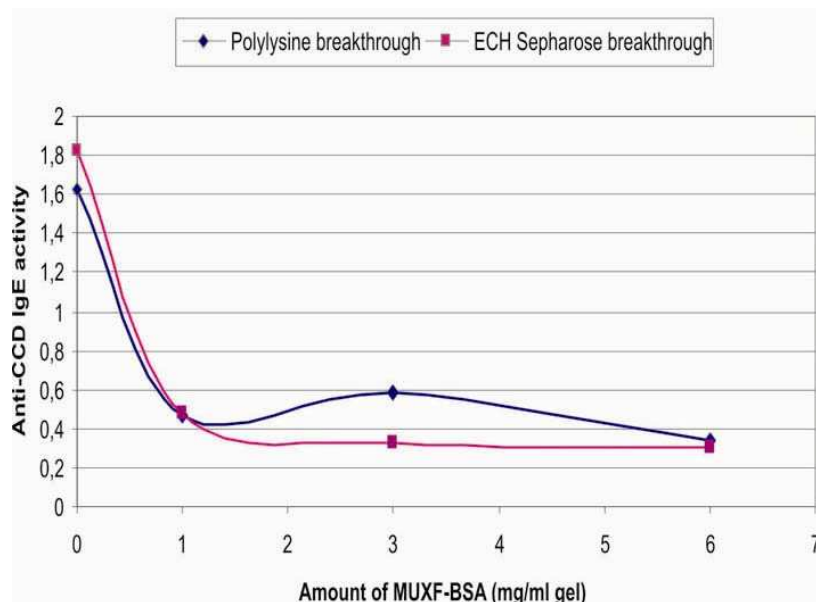


Fig. 18: Comparing binding capacities of various MUXF-BSA coupled ECH Sepharose and Poly-L-lysine Agarose gels. Breakthroughs were analysed for anti-CCD IgE by ELISA. Gels without coupled glycopeptide (0mg/ml) were used as negative control to determine unspecific binding of antibodies to the matrix. OD_{405nm} of serum before column = 1,9.

Although the binding capacity for anti-CCD IgE of both gels was quite similar in ELISA tests it was decided to work on with ECH Sepharose instead of Poly-L-lysine Agarose as the agarose showed a higher unspecific binding of antibodies to the matrix itself and an unexplainable higher anti-CCD IgE value in the breakthrough of the 3mg/ml MUXF-BSA coupled Polylysine.

Repeated use of the MUXF-polylysine agarose:

To test if the MUXF-BSA coupled Poly-L-lysine agarose is reusable after eluting previously bound antibodies, an already used and a fresh gel were treated with the same amount of serum under the same conditions. The anti-CCD IgE content in the sera after column and after elution of the antibody was measured by ELISA.

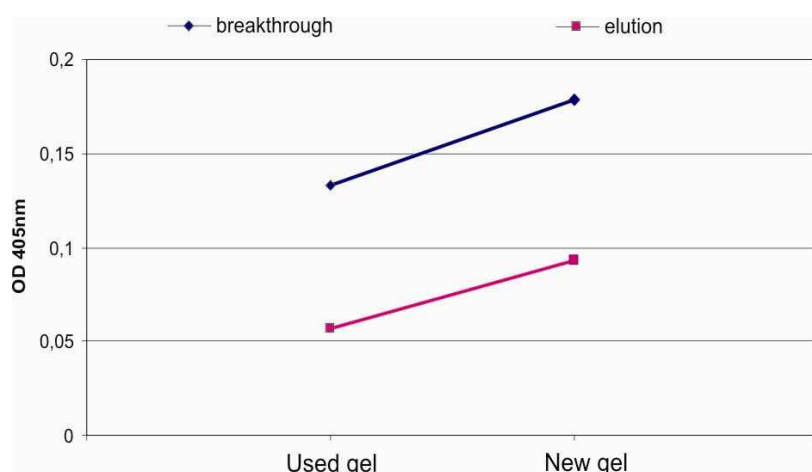


Fig. 19: Testing reusability of 40 mg MUXF-BSA coupled Poly-L-lysine agarose. 50 µl of regenerated gel were incubated with 50 µl serum pool 3 and compared to results with fresh gel.

ELISA results suggested that the gel can be reused without decreasing the binding capacity and effectiveness of anti-CCD IgE to the MUXF-BSA coupled Poly-L-Lysine Agarose.

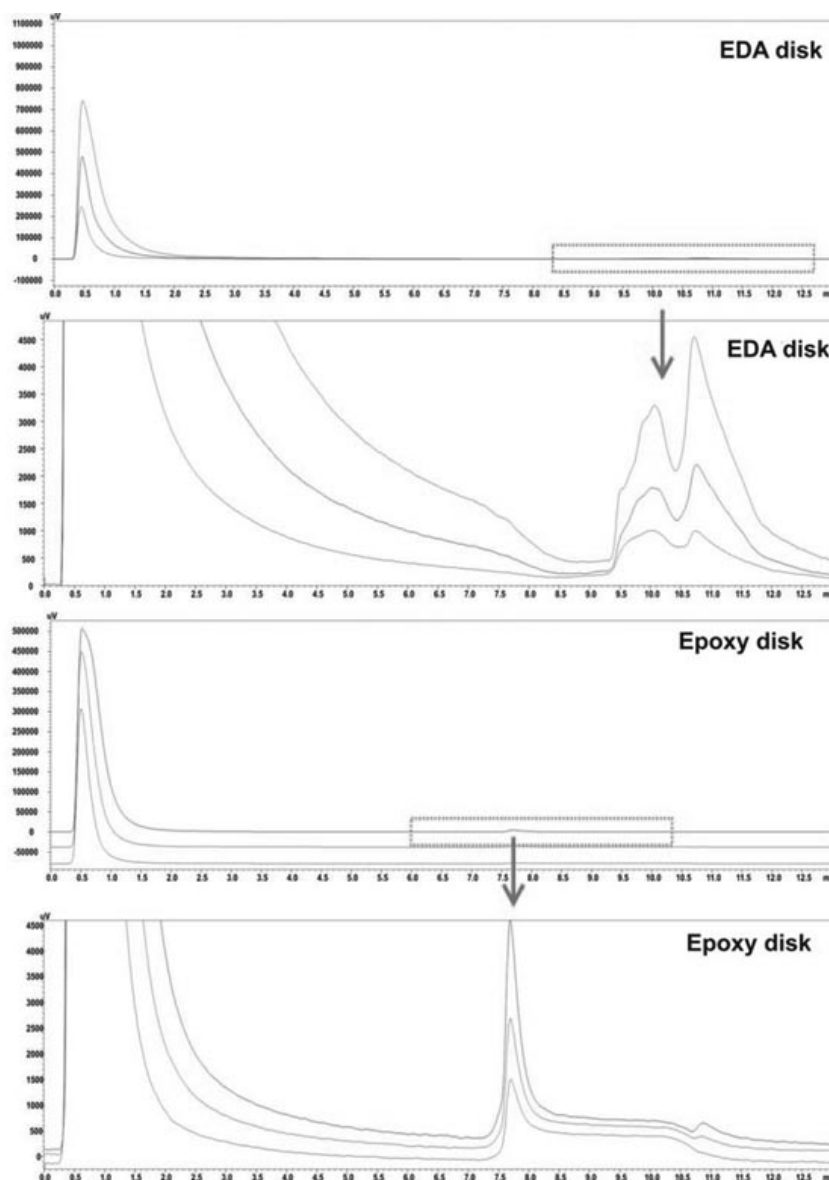
Results

3.2 EDA / EPOXY disk

Unlike agarose based matrices, CIM disk uses polyglycidyl methacrylate-co-ethylene dimethacrylate as solid support which is very stable at higher pressure and features a more rigid structure. Both disks have a volume of 340 μ l and were coupled with 2 mg (EDA) or 3 mg (Epoxy) MUXF-BSA.

50 μ l, 100 μ l and 200 μ l of new CCD serum pool 3 were injected and breakthrough and elution were collected manually according to the peaks detected in HPLC.

Fig. 20: Chromatography of human anti-CCD antibodies with EDA and Epoxy disk. Various amounts of serum pool were injected and breakthrough and elution were collected separately by hand according to the displayed peaks.



All breakthroughs of both disks had a volume of 1 – 2 ml. However the elution from EDA disk occurred about 2 minutes later than the elution from Epoxy disk. It also gave a broader peak and hence a bigger volume (ca. 2 ml elution from EDA disk and 0,8 ml elution from Epoxy disk). It is not clear why the antibodies from EDA disk eluted later than from Epoxy disk. Noteworthy, with this device, the loaded serum was diluted 8 - 10 times.

The peaks observed during HPLC analysis were further used to calculate the amount of antibody bound to the disk. The results suggested that EDA disk on average had a 3,3-fold higher binding capacity than Epoxy disk.

Results

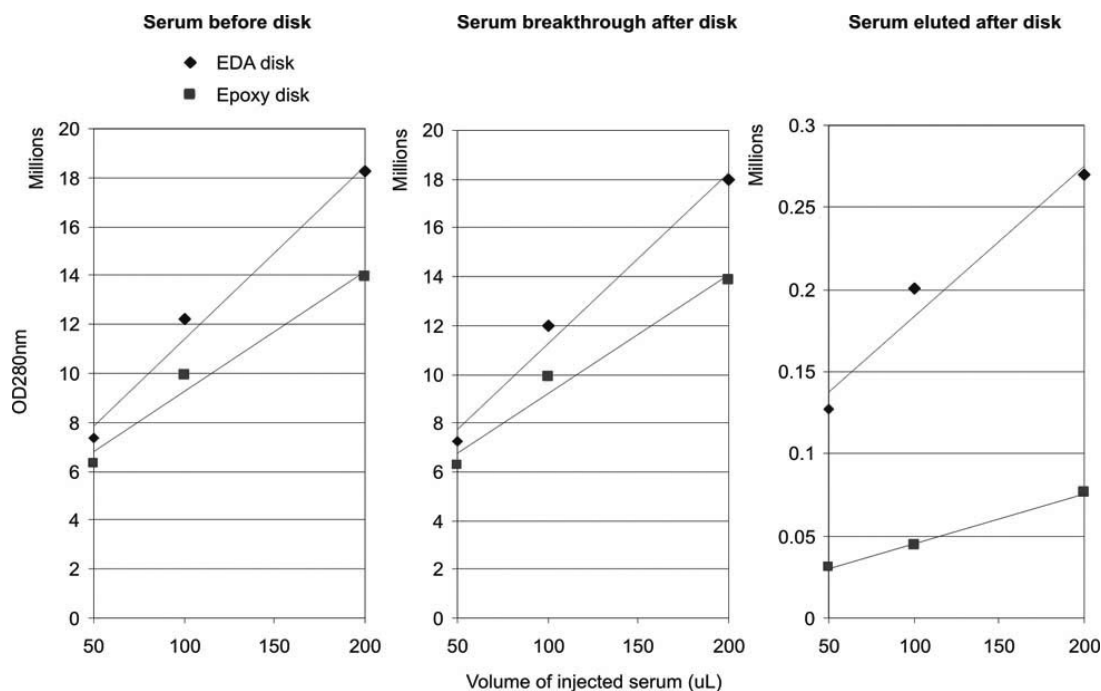


Fig. 21: Comparison of untreated serum, breakthrough and elution. The peak area was used to indicate the amount of protein in each sample.

In the next step the activity of anti-CCD IgE and IgG in collected breakthrough and elution was analyzed by ELISA.

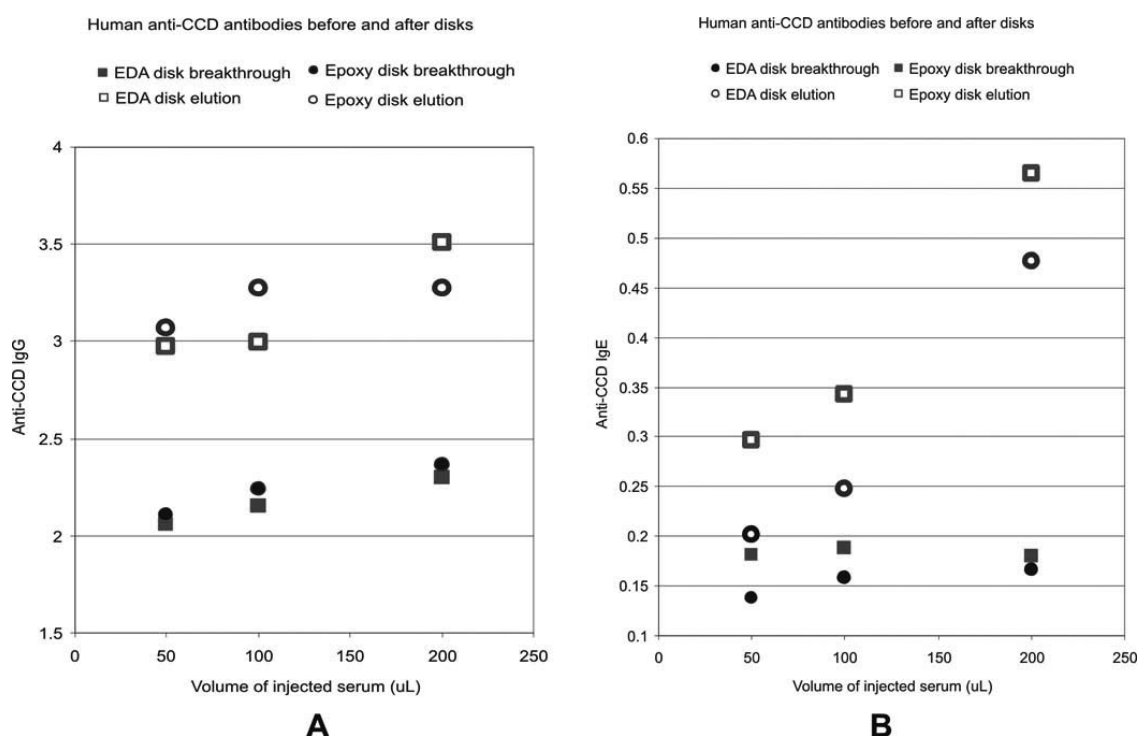


Fig. 22: Breakthrough and elution from EDA and Epoxy disk tested via ELISA. The samples were not further diluted. Plates were coated with 2µg/ml HRP. For anti-CCD IgE the ELISA value for untreated serum (1:10) was 1,7.

In case of anti-CCD IgG the breakthrough of both disks showed an increase of antibodies with increasing serum volume. This indicates that IgG could not be removed efficiently from the serum.

The ELISA values for anti-CCD IgE in breakthrough remained almost stable with an increasing amount of serum injected, which indicates that it could be bound efficiently to both disks and hence

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removed from the patients sera. With an increasing volume of serum an increase of anti-CCD IgE in the elution was detectable but still the anti-CCD activities in the elution were quite low compared to the activities before disk (even when considering the dilution effect and anti-CCD IgE in breakthrough).

It is not clear where the rest of anti-CCD IgE went. A possible reason for this loss could be degradation of IgE during the elution from the column by acidic elution buffer. Another reason could be that anti-CCD IgE was bound to the disk so tight that it could not be eluted under the used conditions.

As the anti-CCD IgE concentration in the used serum was about 0,5 µg/ml and there were almost no IgE antibodies observed in the breakthrough of 200 µl injected serum (0,59 ml serum/ml matrix) the binding capacity of both disks was calculated with about 0,3 µg/ml.

In order to find out if the eluted proteins were human antibodies, a Western blot was done. 200 µl from the EDA disk elution and 100 µl of the Epoxy disk elution were precipitated, mixed with loading buffer (without DTT) and separated on a non-reducing SDS page. For Western blot anti-human IgG was used in a dilution of 1:2000. As blotting failed the first time it had to be repeated. That may have caused the loss of low molecular weight bands near 40 – 50 kDa.

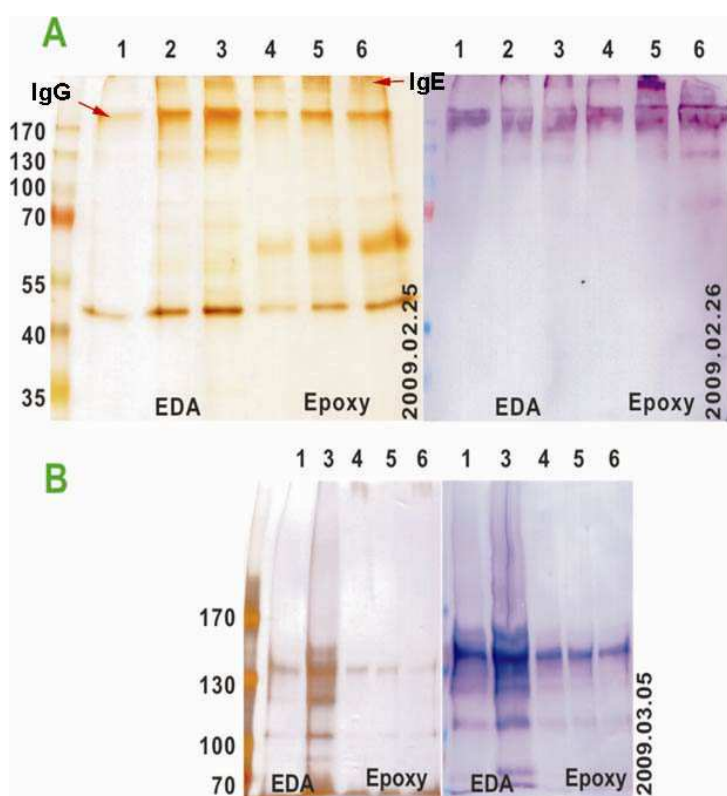


Fig. 23: Analysis of sera after monolithic EDA or Epoxy column by SDS page (silver staining) and Western blot. A: elutions of 50 (1), 100 (2) and 200 (3) µl serum pool from EDA disk and elutions of 50 (4), 100 (5) and 200 (6) µl serum pool from Epoxy disk. B: repeated silver staining and Western blot with same samples as in A. Blotting worked out, but lower bands got lost. For Western blotting anti-human IgG antibody was used in a concentration of 1:2000.

As can be seen in Fig. 23 the band at about 170 kDa was considered to be IgG and Western blot showed that the eluted IgG was a human antibody.

Although anti-CCD IgE could be removed from the sera with EDA and EPOXY disks this method showed some weak points. The samples could not be further used for CAP testing because the high dilution of the sera after column would also dilute the allergen specific antibodies and hence lead to

Results

a more complicated and maybe incorrect interpretation of results in CAP test. Also the final dilution of the samples could hardly be determined as they were collected by hand according to the peaks in HPLC and the exact amount could only be estimated.

Conclusively these monolithic affinity matrices did not show clearly superior binding capacity to warrant further efforts in this direction.

3.3 Eupergit

Eupergit C and its derivatives consists of macroporous beads made by copolymerization of N,N'-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide [23].

At first about 5 mg and 20 mg of MUXF-BSA were coupled to 1 g dry Eupergit C. During the coupling process the gel swell to ca. 5 ml which lead to 1 mg/ml and 4 mg/ml MUXF-BSA Eupergit C coupled gels. Coupling efficiency was checked via BCA test and revealed that for the 1 mg/ml gel 96,8 % of MUXF-BSA bound to Eupergit C, while for the 4 mg/ml gel it was 90,2 %.

The matrices were blocked with 5 % 2-mercaptoethanol.

In the next step, different amounts of a serum pool rich in anti-CCD IgE were added to a fixed amount of coupled MUXF-BSA Eupergit C.

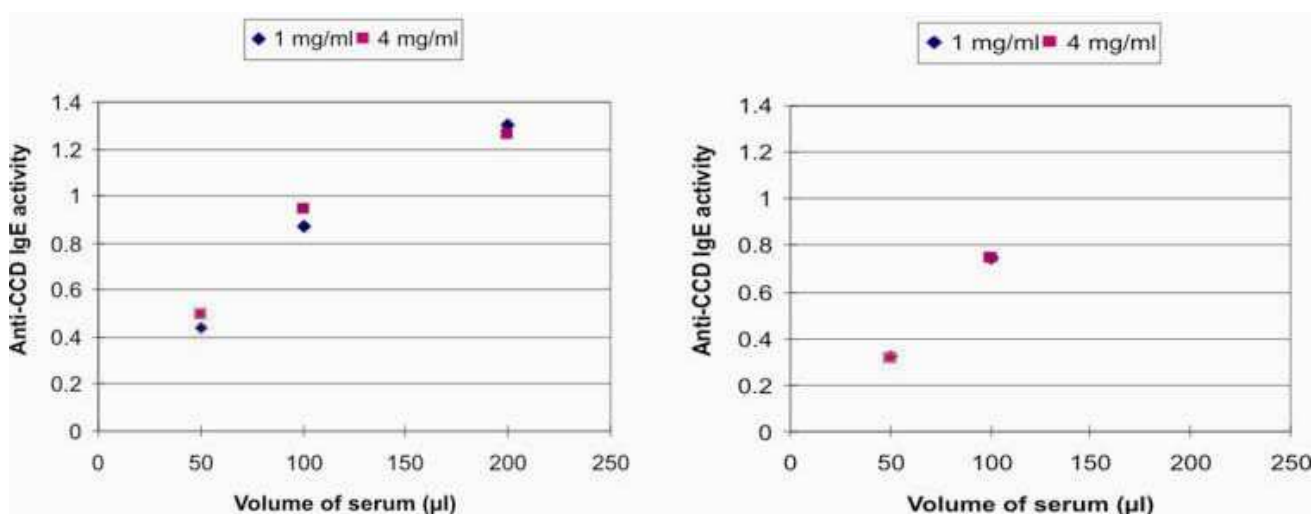


Fig. 24: 50 µl and 100 µl of 1 mg/ml and 4 mg/ml MUXF-BSA Eupergit C matrix were incubated with 50 µl, 100 µl and 200 µl of serum pool. The anti-CCD IgE content in breakthrough and elution was determined by ELISA. Plate was coated with 2µg/ml HRP.

The best results were obtained for 50 µl MUXF-BSA coupled Eupergit C incubated with 50 µl of serum pool. However the 1:1 ratio of gel:serum in case of 100 µl coupled gel (incubated with 100 µl serum pool) showed more anti-CCD IgE in the breakthrough.

A possible reason for leaking of the antibody could be that Eupergit C is just suitable for molecules up to 100 kDa and IgE has a size of about 190 kDa [30]. If that was the case, most of anti-CCD IgE would not be able to enter the matrix. This also could explain the slightly higher IgE levels for the breakthrough of the 4 mg/ml MUXF-BSA Eupergit, where the higher MUXF-BSA concentration would lead to clogging of the pores and leave even less space for the antibodies.

Results

To test these assumptions new gels were prepared.

2 mg and 40 mg MUXF-BSA were coupled to 0,5 g Eupergit C (leading to a 0,8 mg/ml and 16 mg/ml MUXF-BSA gel). This time the matrices were blocked with 0,5 % BSA in TTBS instead of mercaptoethanol.

50 μ l of coupled Eupergit C were tested with 25, 50 and 100 μ l of an anti-CCD IgE rich serum pool.

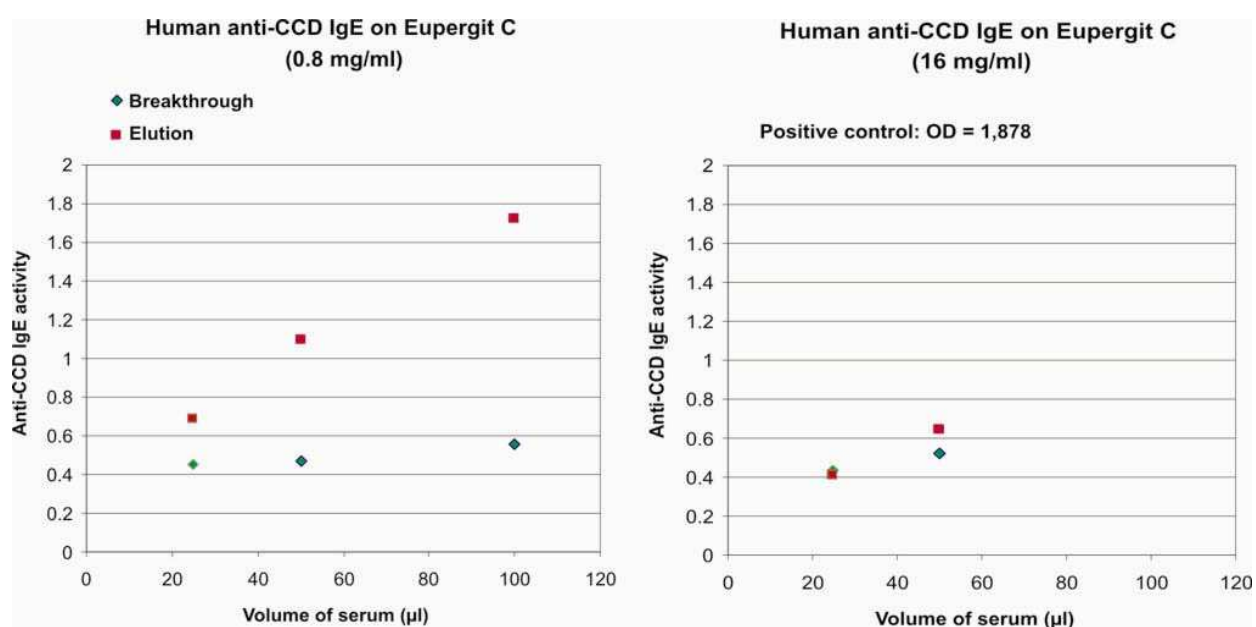


Fig. 25: New Eupergit MUXF-BSA couplings tested with various amounts of serum pool.

As can be seen in Fig. 25 the 0,8 mg/ml MUXF-BSA Eupergit matrix showed a higher binding capacity than the 16 mg/ml MUXF-BSA gel and also a ratio of gel to serum of 1:2 exhibited lower amounts of anti-CCD IgE than in previous tests. Unlike previous tests with Poly-L-lysine agarose and monolithic disks, a high level of anti-CCD IgE could be detected in the elution from 0,8 mg/ml MUXF-BSA Eupergit C.

In another step 0,5 g of Eupergit CM and -C250L were coupled with 2 mg MUXF-BSA to see whether the pore size plays a role in binding of anti-CCD IgE. 50 μ l of each gel were then tested with 25 μ l, 50 μ l and 100 μ l of serum pool.

Results

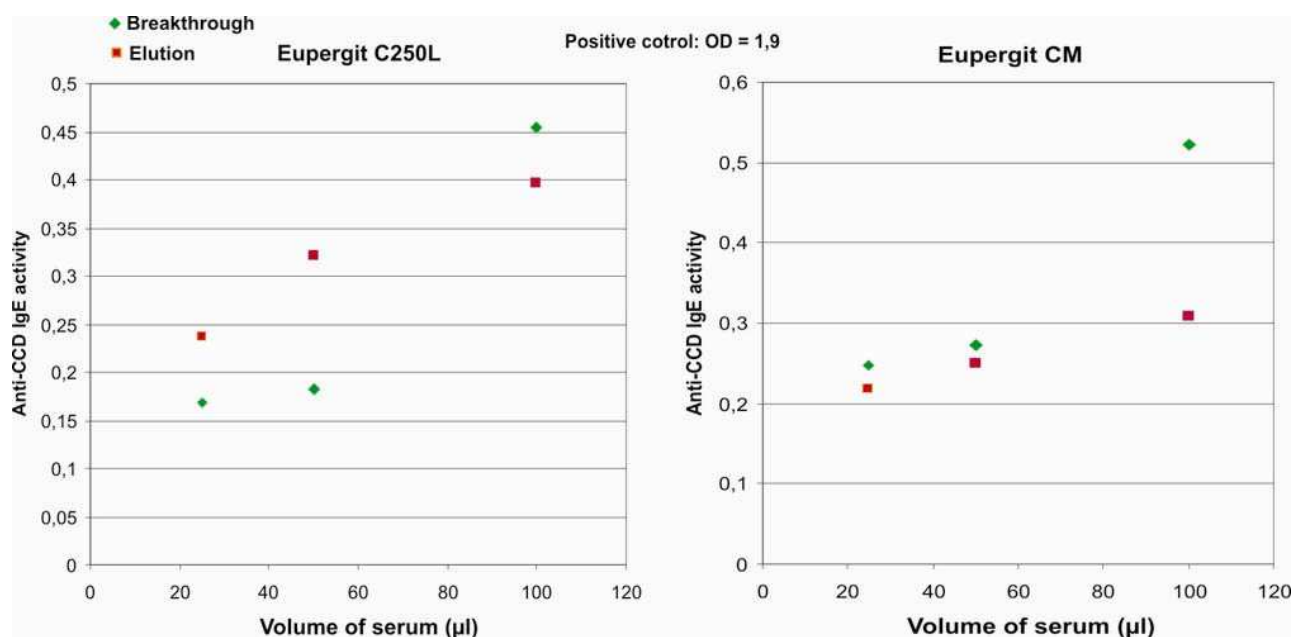


Fig. 26: Eupergit CM and C250L coupled with 0,8 mg/ml glycopeptide conjugate. Breakthroughs and elutions were evaluated with ELISA. Plates were coated with 2µg/ml HRP.

For both gels the reading for anti-CCD IgE was lower compared to Eupergit C, which means that the pores of Eupergit C probably were too small for IgE to enter the matrix. For Eupergit CM and C250L a ratio gel:serum of 1:1 showed a low anti-CCD IgE activity. When raising the ratio to 1:2 a lot of anti-CCD IgE could be found in the breakthrough.

A final comparison of the three different Eupergit matrices coupled with 0,8 mg/ml MUXF-BSA glycopeptide conjugate suggested Eupergit C250L as the affinity matrix with the best binding capacity for anti-CCD IgE.

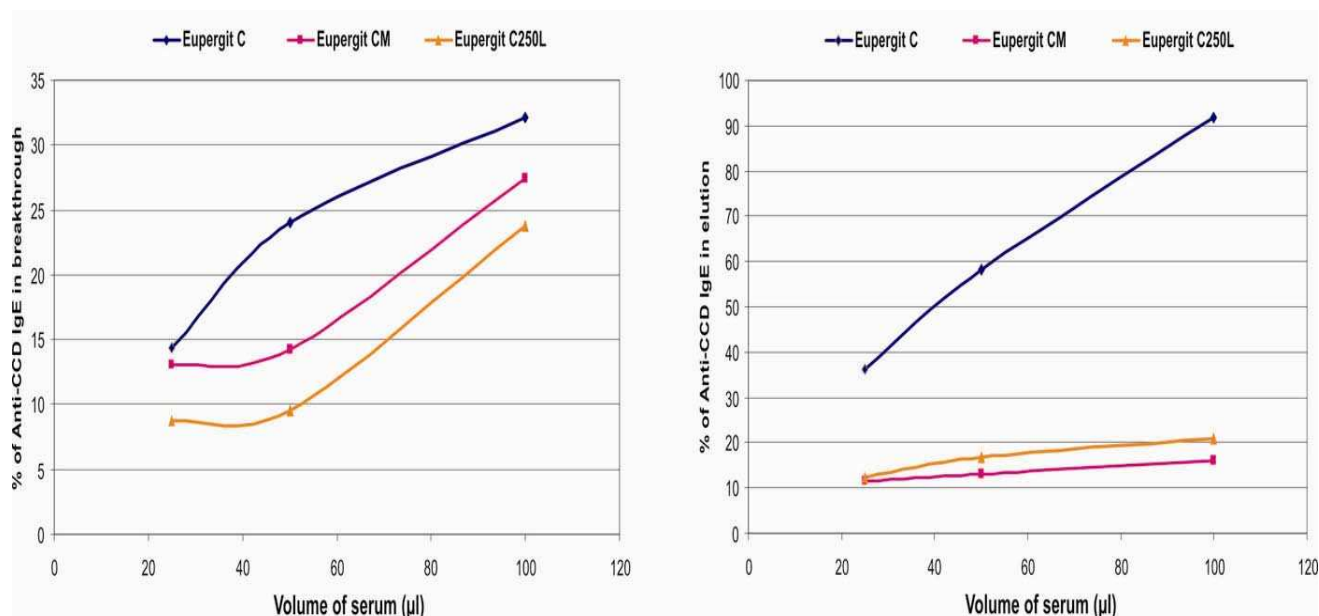


Fig. 27: Final comparison of the three different Eupergit matrices all coupled with 0,8mg/ml MUXF-BSA. The percentage of anti-CCD IgE was calculated with values determined by ELISA.

0,8 mg/ml MUXF-BSA Eupergit C250L showed the lowest anti-CCD IgE amount in the breakthrough. When comparing the antibody amount in the elution, Eupergit C showed the highest antibody level, but this may be due to the fact that IgE could not enter the pores of Eupergit C

Results

anyway and thus could be removed from the gel easily. In case of Eupergit CM and C250L the amount of eluted anti-CCD IgE was as low as with previously tested matrices (see Poly-L-lysine and monolithic disks).

Although Eupergit C250L showed very promising results in removing anti-CCD IgE from patients' sera, further testing with this matrix was stopped as it came to our notice that Eupergit is no longer produced and commercially available.

3.4 ECH Sepharose 4B

Like poly-lysine agarose, ECH Sepharose 4B is an agarose based support. Different coupling strategies were tried with this matrix: coupling the MUXF-glycopeptide alone, coupling a MUXF-BSA and MUXF-Lysozyme conjugate.

3.4.1 Coupling MUXF-glycopeptide

In order to define the coupling capacity of this matrix, various amounts of MUXF were coupled to the ECH Sepharose matrix.

The coupling capacity was determined by the amount of glycopeptide in the solutions before and after coupling by orcinol test.

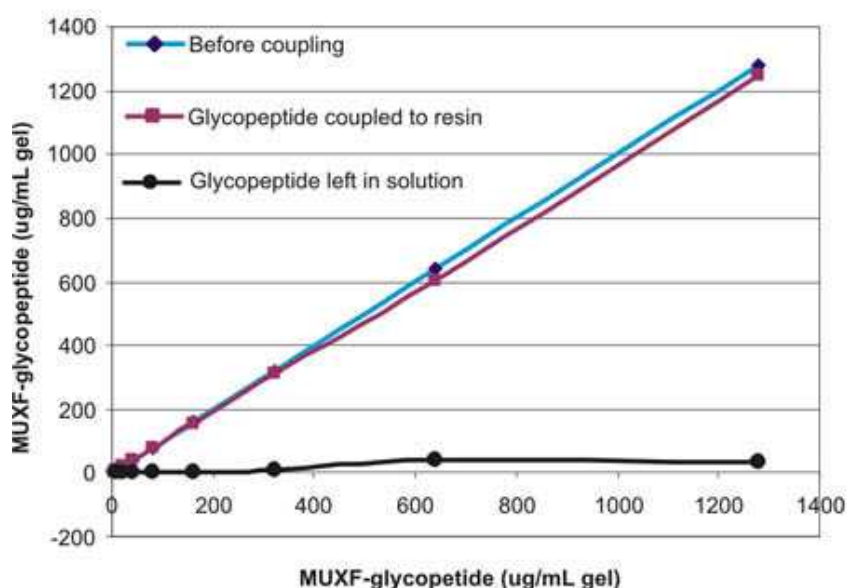


Fig. 28: Determination of the coupling capacity of ECH Sepharose 4B for MUXF. Aliquots of the coupling solution before and after coupling were taken and the different amount of glycopeptide was evaluated by orcinol test.

According to results from orcinol test the coupling capacity of ECH Sepharose 4B for the MUXF-glycopeptide was higher than 1246 $\mu\text{g/ml}$ gel (see Fig. 28).

To determine the gels binding capacity for anti-CCD IgE, 50 μl of each coupled MUXF-Sepharose (5, 10, 20, 40, 80, 160, 315, 629 and 1258 $\mu\text{g/ml}$ MUXF) were incubated with 50 μl serum pool.

Results

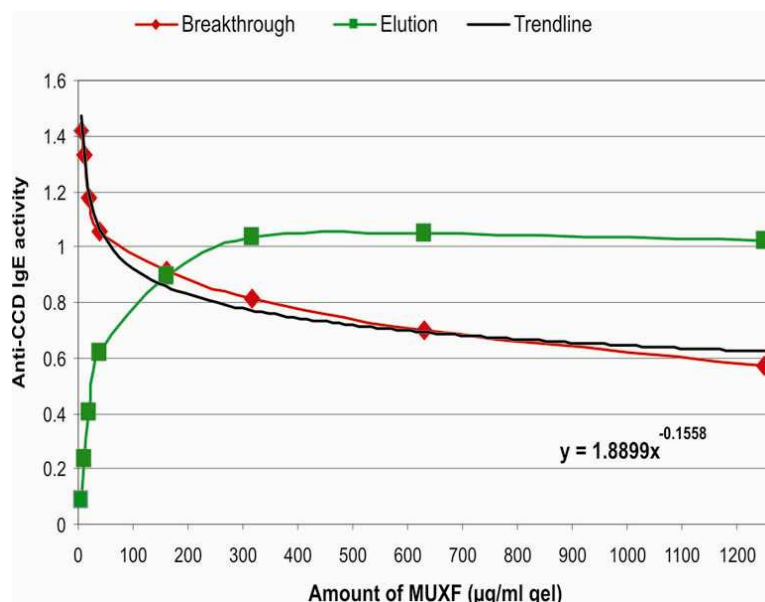
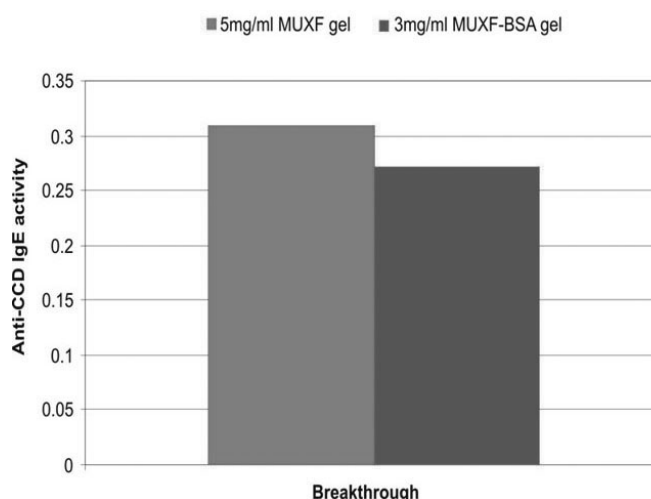


Fig. 29: ECH Sepharose coupled with various amounts of MUXF glycopeptide. Gel and serum were incubated in a ratio of 1:1. Breakthroughs and elutions were tested for anti-CCD IgE via ELISA.

As can be seen in Fig. 29 the amount of anti-CCD IgE in breakthrough decreased with an increasing amount of coupled glycopeptide. At the highest MUXF concentration (1258 µg/ml gel) the ELISA still detected a quite high amount of antibody in the serum after column. The reading was at $OD_{405nm} = 0,57$ while the background was supposed to be at $OD_{405nm} = 0,2$. This indicated that a lot more MUXF glycopeptide had to be coupled to the ECH Sepharose to remove most of anti-CCD antibodies from the serum.

According to the fitting curve and derived formula from the graph in Fig. 29, the amount of glycopeptide to get to an $OD_{405nm} = 0,2$ in the serum after column, should be more than 700 mg/ml gel. To test this hypothesis another coupling was done with 5 mg/ml MUXF to ECH Sepharose. 50 µl coupled gel were incubated with 50 µl of serum pool. To compare binding capacities an ECH Sepharose 4B matrix, coupled with 3 mg/ml MUXF-BSA, was tested under the same conditions.

Fig. 30: Comparison of ECH Sepharose coupled with MUXF (MUXF-glycopeptide) and with the glycopeptide conjugate MUXF-BSA. The anti-CCD IgE content in the serum after the column was determined by ELISA.



As shown in Fig. 30 the breakthrough after the MUXF-BSA Sepharose column contained about 13 % less anti-CCD IgE as the serum after the MUXF coupled gel. MUXF has a mass of 1500 Dalton which leads to a concentration of 3,3 µmol/ml in the 5 mg/ml coupled gel. MUXF-BSA has an MUXF concentration of 0,3 µmol/ml gel in the 3 mg/ml MUXF-BSA ECH Sepharose, when BSA has a size of 65 kDa and 5-6 molecules MUXF are supposed to bind to one BSA molecule.

This means that although the glycopeptide was 10 times more concentrated than in the MUXF-BSA Sepharose, the MUXF-Sepharose still had less binding capacity for anti-CCD IgE than the gel

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modified with the MUXF-BSA. A possible reason could be that MUXF is a small and short molecule and when coupled to the gel alone it is not as good accessible for the antibodies as when conjugated with an extra peptide.

The results suggested that coupling solely the glycopeptide to the gel would mean that a huge amount of MUXF has to be used to get to a satisfying depletion of anti-CCD IgE from the serum. Hence it was decided to end trials with these gels.

3.4.2 Coupling MUXF-BSA

Various amounts (0,1 mg/ml – 25,6 mg/ml) of the MUXF-BSA conjugated glycopeptide were coupled to ECH Sepharose 4B. The resulting gels were incubated with a CCD serum pool in a ratio of 1:1 (gel:serum).

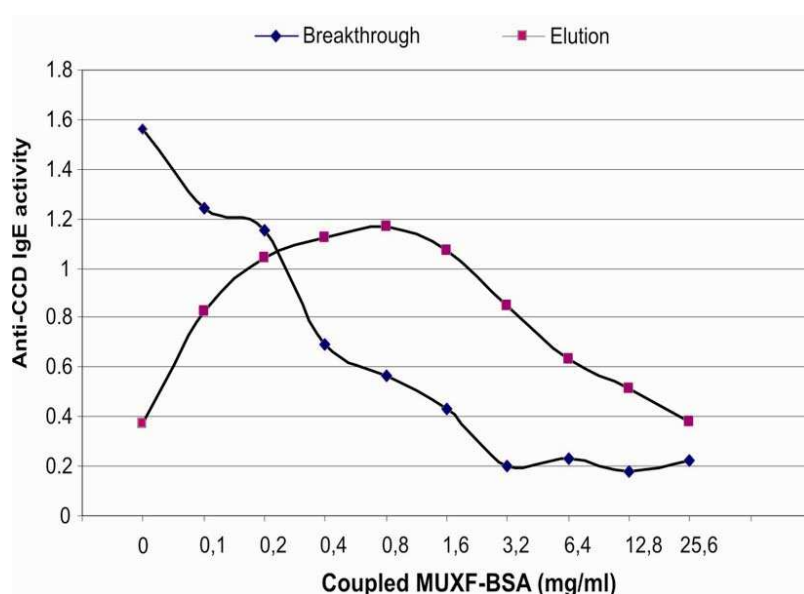


Fig. 31: Various amounts of MUXF-BSA coupled to ECH Sepharose 4B. Binding capacity and efficiency for anti-CCD IgE was determined by evaluation of anti-CCD IgE content in the sera after column by ELISA. Plate was coated with 2µg/ml HRP.

With an increasing amount of MUXF-BSA coupled to the Sepharose, there was a decrease of anti-CCD IgE detectable in the serum after column. When the binding capacity of the gel was increasing the amount of eluted antibodies became less. This may indicate a very strong interaction between MUXF and anti-CCD IgE which could not be terminated by the elution method used. At a concentration of 3,2 mg/ml MUXF-BSA the amount of anti-CCD IgE in the serum after column reached the lowest level which did not improve when more protein was coupled. At the highest glycopeptide-conjugate concentration of 25,6 mg/ml results even showed a slight decrease of binding capacity. The results could be repeated (not shown here) and two more Sepharose gels with a concentration of 1 mg/ml and 3 mg/ml MUXF-BSA were prepared for further trials.

50 µl of the 1 mg/ml and 3 mg/ml MUXF-BSA ECH Sepharose were incubated with 50 µl, 75 µl and 100 µl of serum pool. The sera after column and elution were evaluated via ELISA.

According to the ELISA results (Fig. 32), the 3 mg/ml gel showed a slightly better binding capacity for anti-CCD antibodies, while elution efficiencies did not vary a lot. Encouraged by these results 150 µl of the 3 mg/ml MUXF-BSA coupled gel were treated with 150 µl sera from two grass pollen allergic patients (MG, BA). Breakthroughs were sent to the FAZ for determination of the anti-CCD IgE and allergen specific IgE amounts via CAP.

Results

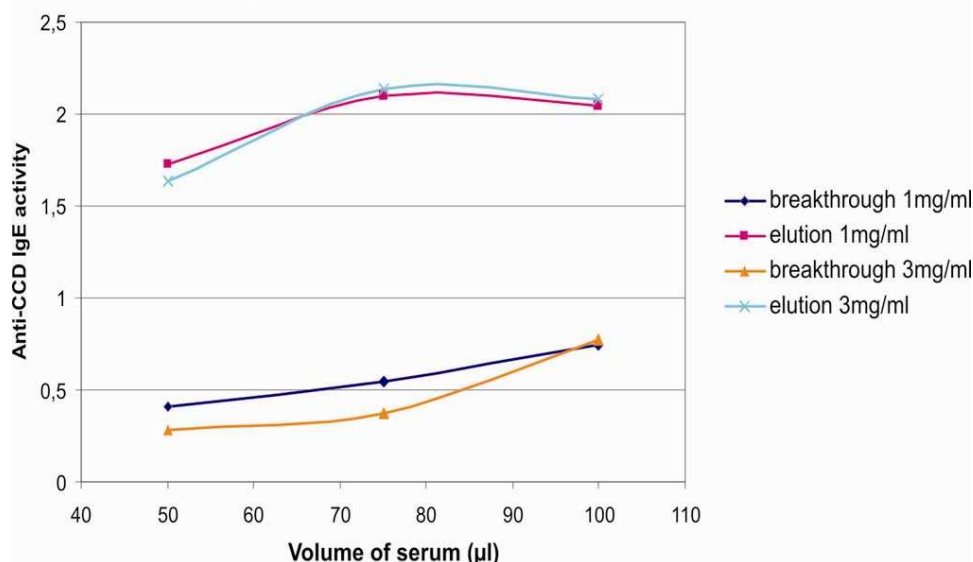


Fig. 32: Comparison of anti-CCD IgE binding efficiency of 1 mg/ml and 3 mg/ml MUXF-BSA sepharose. A fixed amount of gel was incubated with various amounts of sera. Breakthrough and elution were checked for the presence of anti-CCD IgE by ELISA testing. ELISA plate was coated with 2 µg/ml HRP.

Fig. 33: CAP results for sera after treatment with a 3 mg/ml MUXF-BSA ECH Sepharose 4B. The sera were checked for anti-CCD IgE and allergen specific IgE (grass pollen, rPhl p1+5) before and after cartridge.

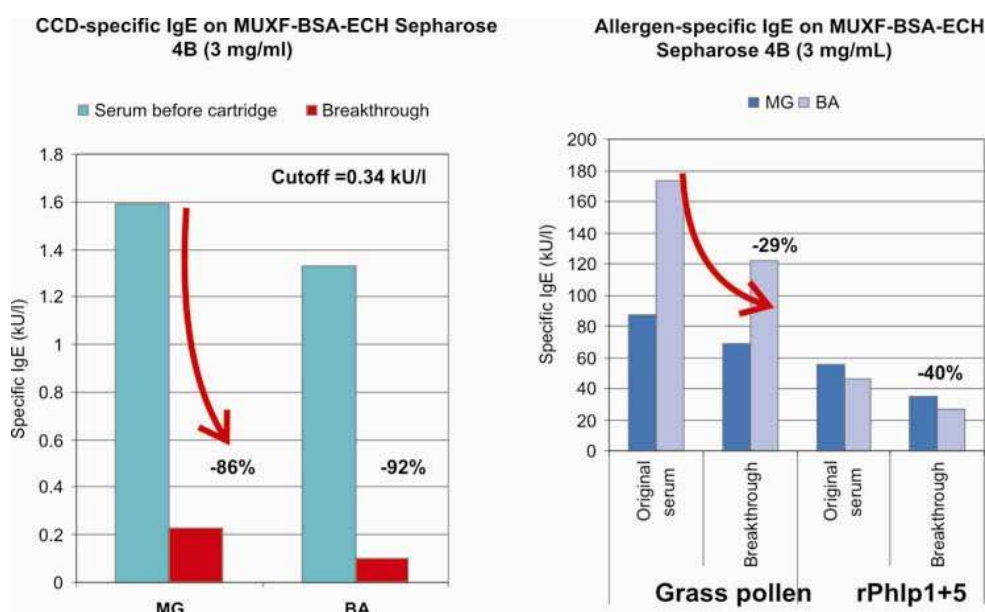


Fig. 33 shows that anti-CCD IgE could be removed from the patients sera with high efficiency (cut-off for allergy detection is at 0,34 kU/l). Unfortunately the allergen specific IgE antibodies were also removed from the serum at quite a high level. It is possible that allergen-specific IgE bound to the ECH Sepharose in an unspecific way or that the antibodies somehow were stuck in the pores of the gel and hence could not be detected in the breakthrough. It also has to be considered that the pollen allergic sera used did not have very high anti-CCD antibody levels so when working with a higher anti-CCD IgE content the binding capacity of the gel may decrease.

To test these possibilities a 0mg/ml, 3mg/ml and 6mg/ml MUXF-BSA coupled ECH Sepharose 4B was tested in different approaches with mixed serum (ratio 1:1).

Results

Tab. 7: Samples for CAP test. 100 µl of matrix were incubated with 100 µl of mixed serum.

Mixed serum:	serum MG 1:16 (+ PBS(1:8) + anti-CCD serum pool (1:2))
b1:	serum after 0mg/ml column + 50 µl washout with TTBS
b2:	serum after 0mg/ml column + 50 µl TTBS
b3:	serum after 3mg/ml MUXF-BSA column + 50 µl washout with TTBS
b4:	serum after 3mg/ml MUXF-BSA column + 50 µl TTBS
b5:	serum after 3mg/ml MUXF-BSA column + 50 µl TTBS, after extra blocking with 0,5 % BSA in TTBS
b6:	serum after 6mg/ml MUXF-BSA column + 50 µl TTBS
w1:	washout 0mg/ml column with 150 µl TTBS
w2:	washout 3mg/ml column with 150 µl TTBS
w3:	washout 6mg/ml column with 150 µl TTBS

The samples were analysed in the FAZ by CAP.

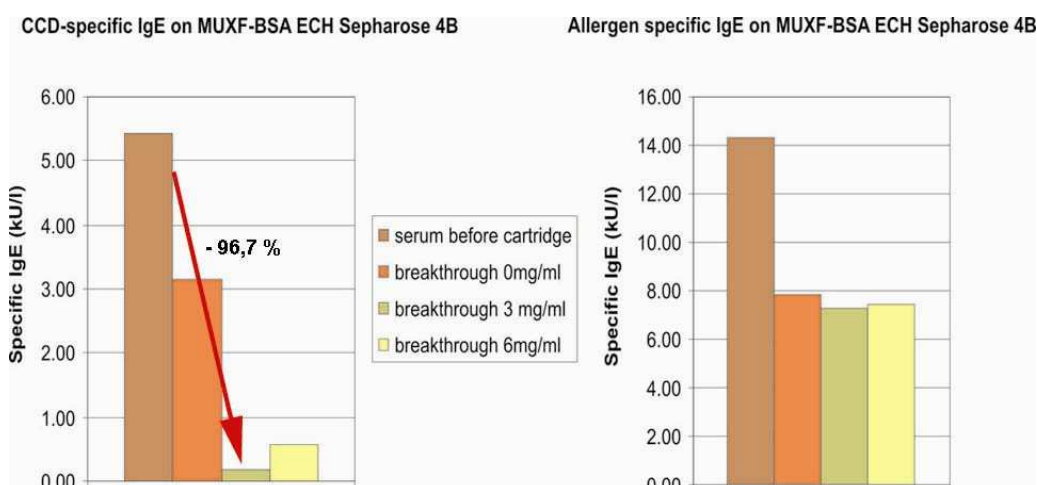


Fig. 34: CAP results for mixed serum after incubation with different MUXF-BSA sepharose gels. For allergen specific IgE rPhl p1+5 levels were measured.

For anti-CCD specific IgE the amount in the serum after column could be reduced for 96,7 % with the 3 mg/ml MUXF-BSA ECH Sepharose, while it was only 89,5 % for the 6 mg/ml gel. As can be seen in Fig. 34 a significant amount of CCD directed IgE bound to the negative control (0mg/ml MUXF-BSA), indicating unspecific binding of antibodies to the ECH Sepharose itself.

Again a lot of allergen specific IgE bound to the column but could partly be recovered by washing the column with TTBS (see Fig. 35). When comparing the negative control with 3mg/ml and 6 mg/ml MUXF-BSA Sepharose it can be seen that most of the binding of allergen specific IgE is unspecific.

Results

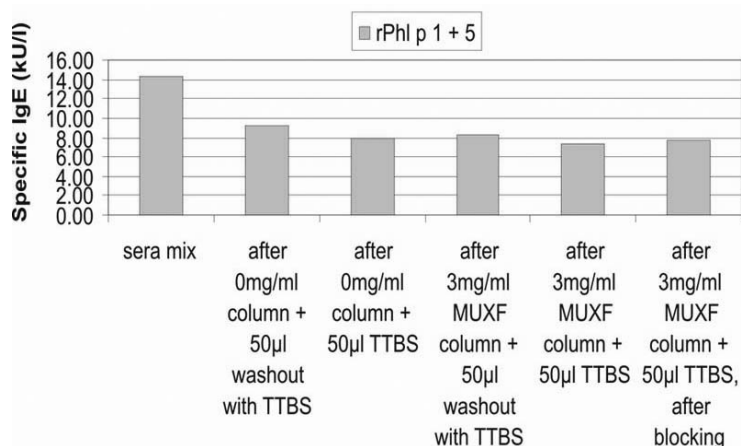


Fig. 35: CAP results from allergy centre. The content of allergen specific IgE in breakthroughs after different approaches was measured.

Extra blocking with 0,5 % BSA in TTBS of the 3 mg/ml MUXF-BSA ECH Sepharose gel barely influenced unspecific binding of allergen specific IgE to the column. As the previous results suggested human IgE antibodies trapped in the pores of the beads, which could lead to poor recovery of allergen specific IgE, it was tried to overcome this problem by changing the centrifugation speed. When increasing the speed up to 11000 x g before incubation with the serum, about 14 µl of PBS buffer could be additionally removed from the gel which otherwise would lead to a dilution of the sample when just centrifuging with 1000 x g.

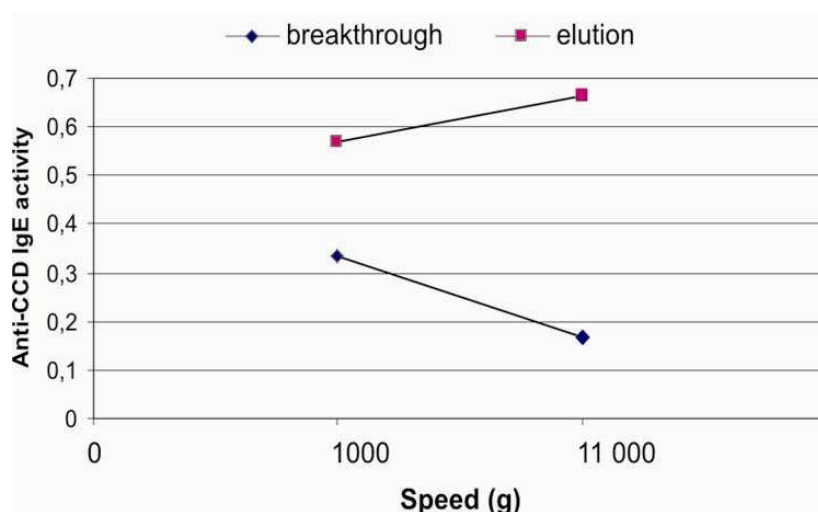


Fig. 36: 50 µl of the 3 mg/ml MUXF-BSA coupled ECH Sepharose were added into the filter tubes. One tube was centrifuged with 1000 x g and the other one with 11000 x g before incubation with the serum. Both approaches were incubated with 50 µl of serum pool under the same conditions. Samples were checked for anti-CCD IgE by ELISA.

When testing breakthrough and elution via ELISA, a clear decrease of anti-CCD IgE in the serum after column could be observed for the samples centrifuged with 11000 x g. In total ECH Sepharose seemed to be stable under higher speed conditions, the dilution of the sample after column was decreased and the anti-CCD IgE binding capacity of the MUXF-BSA coupled Sepharose could be increased. Later on a 3mg/ml MUXF-BSA coupled ECH Sepharose was tested again on anti-CCD IgE and compared with MUXF-BSA Prosep-9 CHO glass beads. 150 µl of a 0 mg/ml (negative control) and 3 mg/ml MUXF-BSA ECH Sepharose 4B were incubated with 150 µl of a mixed serum (MG 1:16 with PBS and CCD serum pool). The centrifugation speed before

Results

incubation was 11000 x g. Breakthrough, elution and washout were analyzed at the FAZ for allergen specific IgE (rPhl p1) and anti-CCD IgE by CAP.

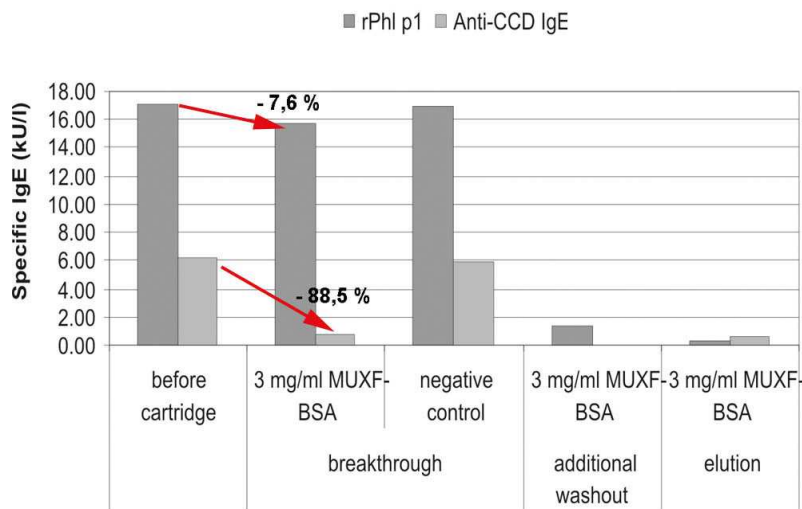


Fig. 37: CAP analysis from the FAZ. Amounts of anti-CCD and allergen specific IgE (rPhl p1) in the mixed serum were determined before and after treatment of serum with a 3 mg/ml MUXF-BSA ECH Sepharose gel.

The CAP results revealed an decrease of anti-CCD IgE in the breakthrough of 88,5 %, while rPhl p1 antibodies showed only weak binding to the MUXF-BSA column (7,6 %). There also was almost no binding of antibodies to the matrix itself as could be observed with the negative control.

When comparing these CAP results with the results from 4 mg/ml MUXF-BSA Prosep-9 CHO glass beads (see Fig. 52), ECH Sepharose showed slightly better binding capacity for anti-CCD IgE and lower binding of allergen specific antibodies.

The removal of anti-CCD IgE after 3mg/ml MUXF-BSA ECH Sepharose and 4 mg/ml MUXF-BSA Prosep glass beads was further compared in ELISA tests. 50 μ l of each coupled matrix were centrifuged with 11000 x g and incubated in ratios of 1:1 and 1:2 with CCD serum pool.

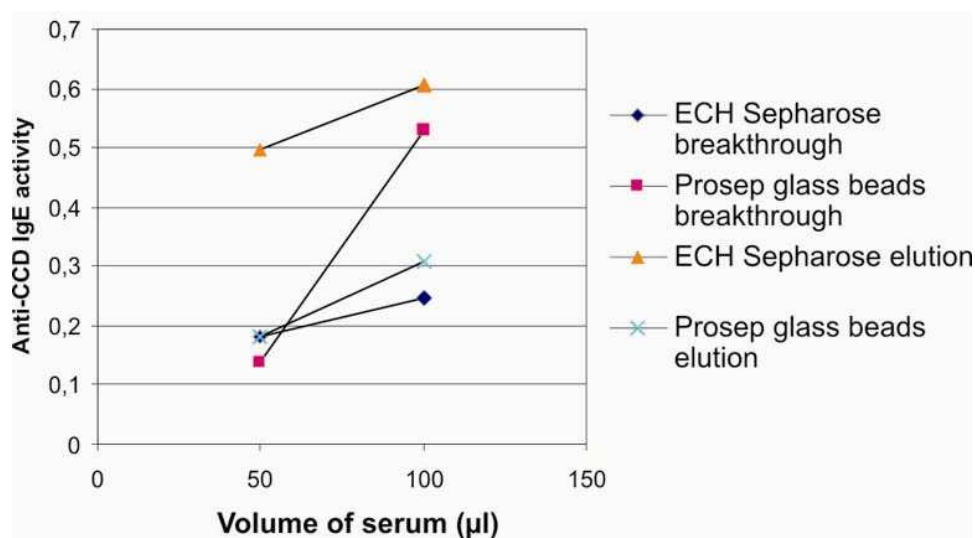


Fig. 38: Comparison of 3mg/ml MUXF-BSA ECH Sepharose and 4mg/ml MUXF-BSA Prosep-9 CHO glass beads. Matrices were incubated with 50 μ l and 100 μ l of serum pool. Anti-CCD IgE content in the sera after column and elutions was detected by ELISA.

At a ratio gel:serum of 1:1, coupled glass beads showed a slightly better binding capacity for anti-CCD IgE than ECH Sepharose. Nevertheless when increasing the serum volume, 3 mg/ml MUXF-

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BSA ECH Sepharose was clearly able to bind more IgE than 4 mg/ml MUXF-BSA glass beads. For the Sepharose at a ratio of 1:2 the ELISA read out increased slightly while in case of glass beads a clear increase was detectable. It also has to be considered that in case of ECH Sepharose a fewer amount of MUXF-BSA was coupled (3mg/ml) while for glass beads it was 4 mg/ml. So with ECH Sepharose 4B a better anti-CCD IgE binding capacity could be achieved and less material had to be used.

According to the previous results, a 3 mg/ml MUXF-BSA ECH Sepharose was tested with 3 different types of sera: sera which only contained anti-CCD IgE (HE, D01), sera which showed anti-CCD and allergen specific (rPhl p1+5) IgE (D03, D09, D10, D22) and sera which contained only allergen specific (rPhl p1+5, Bet v1) IgE and no anti-CCD antibodies (D04, D06, D12, D20). 150 µl of gel were tested with 150 µl of serum (ratio 1:1). Breakthroughs were collected and transferred to the FAZ for evaluation by CAP.

As shown in Fig. 39 up to 95 % of anti-CCD IgE could be removed from the sera (on average 91,7 % were removed). All anti-CCD IgE levels detected in the sera after treatment with the MUXF-BSA sepharose lay under the positive cut-off level of 0,34 kU/l, which means they could be considered as negative in the CAP allergy test. In case of allergen specific IgE on average 90 % of antibodies were recovered from the column.

To test if environmental factors affect the ECH Sepharose, 50 µl samples of the 3 mg/ml MUXF-BSA coupled gel were dried at room temperature and left in buffer at room temperature for 4 days. Afterwards the dried gel lost half of its volume and was not swelling when stored in PBS buffer again. Also heating at 70°C and cooking at 95°C did not influence the consistence. All prepared gels were incubated with CCD serum pool in a ratio of 1:1 and breakthrough and elution were collected.

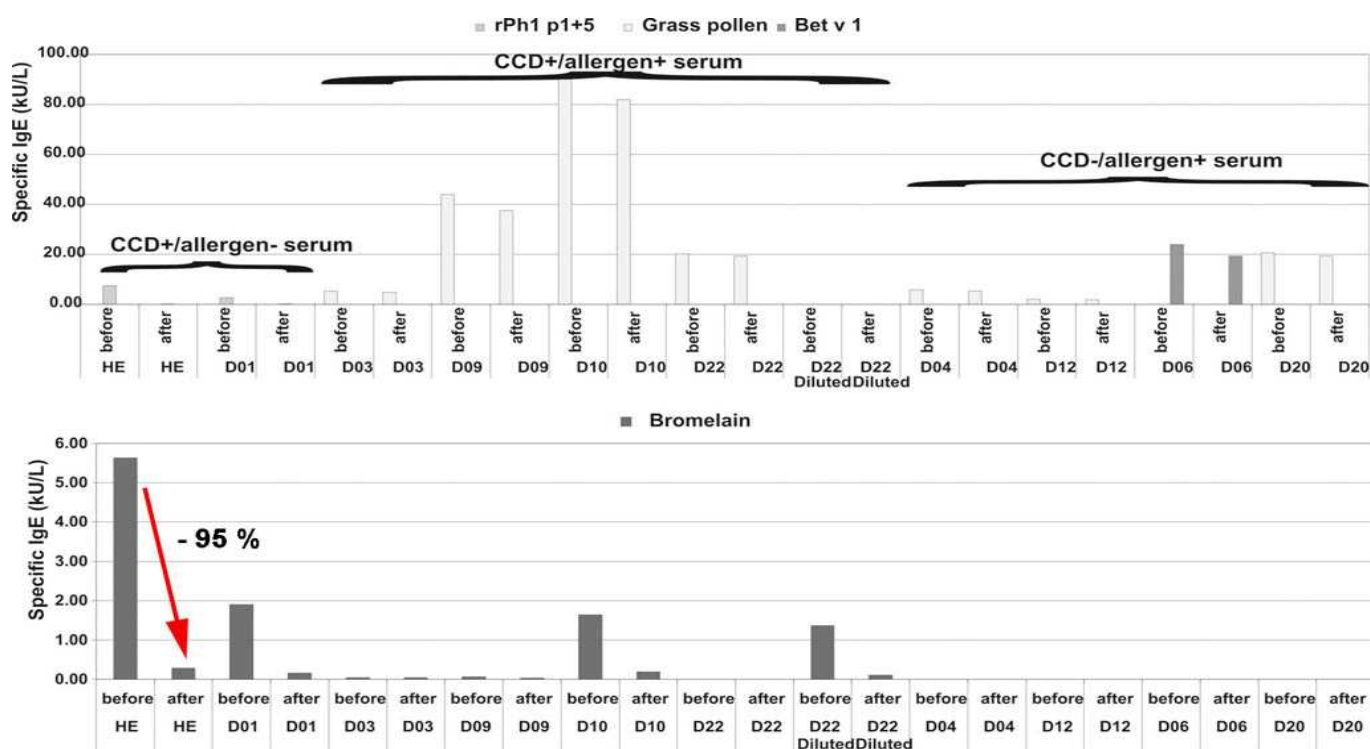


Fig. 39: Final CAP results for 3 mg/ml MUXF-BSA ECH Sepharose. Several Patients sera were picked and tested for anti-CCD and allergen specific IgE before and after incubation with the affinity matrix. Sera containing only CCD specific IgE: HE, D01. Sera containing CCD- and allergen specific IgE: D03, D09, D10, D22. Sera containing only allergen specific IgE: D04, D06, D12, D20.

Results

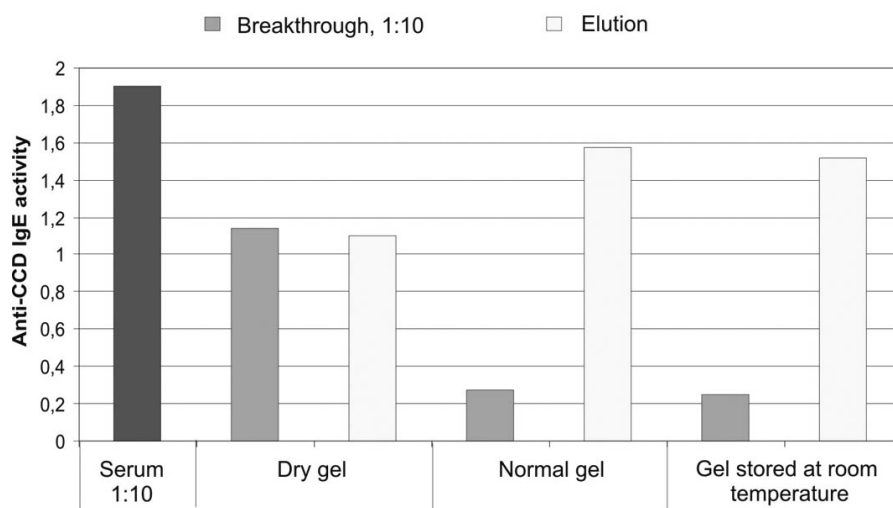


Fig. 40: Stability and functionality of 3mg/ml MUXF-BSA coupled ECH Sepharose 4B tested under various environmental conditions: completely dried gel, gel left in buffer at room temperature for several days, normally treated gel (stored in buffer at 4°C). Anti-CCD IgE levels before and after treatments with matrices were measured with ELISA.

The dried gel gave poor results as more than half of all anti-CCD antibodies could be detected in the serum after incubation with the dried column. The breakthrough from the gel left on room temperature showed no difference in anti-CCD IgE levels compared to a normally (in PBS buffer, at 4°C) stored ECH Sepharose. So the coupled ECH Sepharose is stable at room temperature but should not be left without buffer as this leads to destruction of the matrix.

3.4.3 Coupling MUXF-Lysozyme

The conjugation of MUXF to lysozyme was controlled by Western blot, Coomassie staining and silver staining.

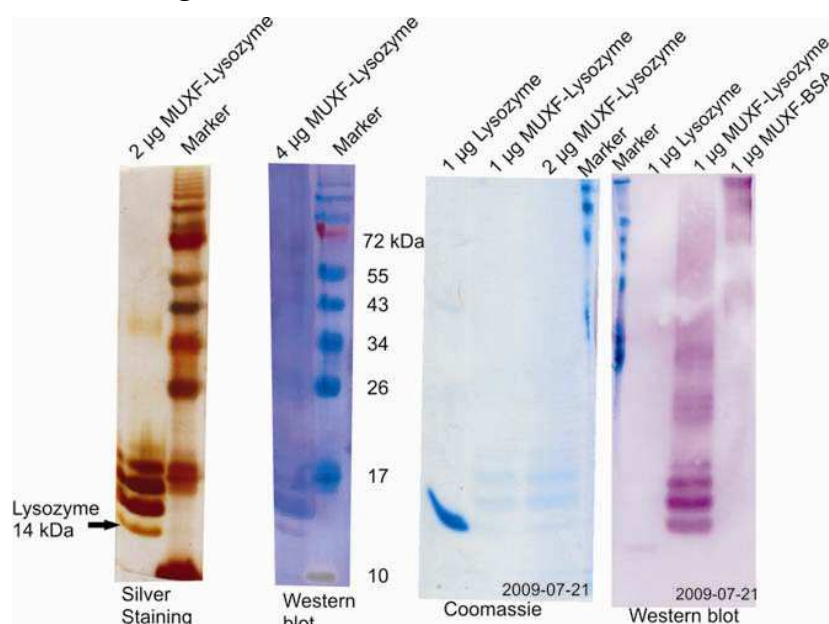


Fig. 41: Coupling of MUXF to Lysozyme detected by different methods. Marker: Page Ruler™ Prestained Protein Ladder, Fermentas.

Results

As shown in Fig. 41 different numbers of MUXF molecules bound one lysozyme molecule. In fact about 4-5 glycans per lysozyme could be coupled. 2 mg/ml and 6 mg/ml MUXF-Lysozyme (MUXF-Lys) were coupled to ECH Sepharose 4B. 50 μ l of each coupling were incubated with 50 μ l of an anti-CCD IgE rich serum pool and breakthrough and elution were collected and evaluated via ELISA.

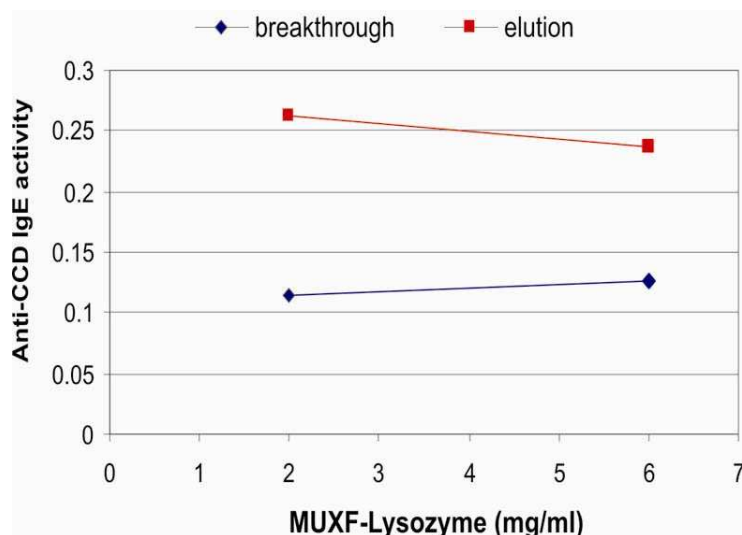


Fig. 42: First ELISA test with serum pool after treatment with 2mg/ml and 6mg/ml MUXF-Lysozyme coupled Sepharose. Reading for serum pool before column: $OD_{405nm} = 1,22$.

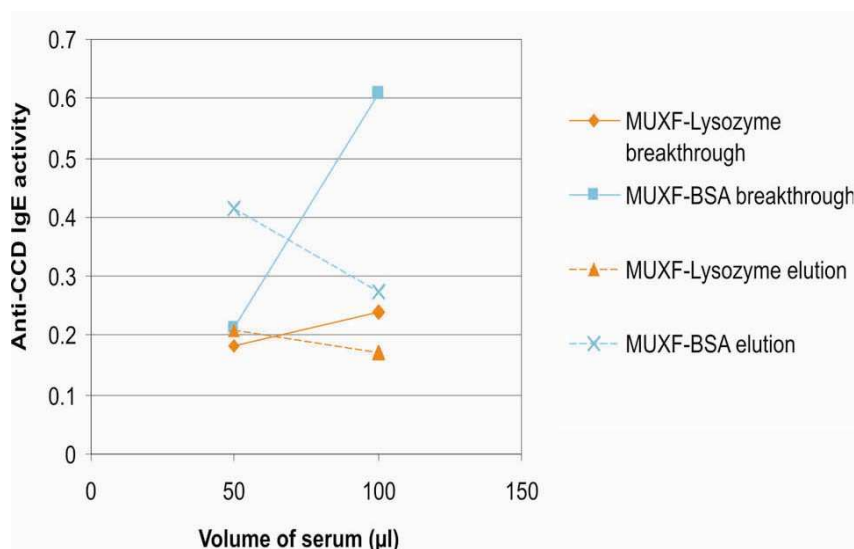
Both matrices were successful in removing anti-CCD IgE from the sera as the untreated serum (diluted 1:10) had a reading of $OD_{405nm} = 1,22$.

A slight increase of anti-CCD IgE could be observed in the breakthrough of the 6 mg/ml MUXF-Lys Sepharose ($OD_{405nm} = 0,13$) compared to the serum after column with 2 mg/ml MUXF-Lys ($OD_{405nm} = 0,11$).

According to above results, the 2 mg/ml MUXF-Lys coupled ECH Sepharose was further tested with an increasing amount of serum. In order to compare MUXF-BSA with MUXF-Lys coupling a 3 mg/ml MUXF-BSA coupled ECH Sepharose was also tested under the same conditions.

Each affinity gel was incubated with serum pool in the ratios (gel slurry to serum) 1:1 and 1:2.

Fig. 43: Comparison of binding efficiencies of 3 mg/ml MUXF-BSA and 2 mg/ml MUXF-Lys coupled ECH Sepharose. 50 μ l of both gels were incubated with 50 μ l and 100 μ l of serum pool. Breakthroughs and elutions were analysed via ELISA. Readout for serum (diluted 1:10) before column: $OD_{405nm} = 1,11$.



Results

At a ratio of gel to serum of 1:1, the binding capacities of both affinity matrices, MUXF-BSA and MUXF-Lys, were very similar. When increasing the serum amount up to 100 μ l (with 50 μ l of gel), the MUXF-BSA column was leaking much more anti-CCD IgE than the MUXF-Lysozyme coupled Sepharose. When calculating from the pure serum (1:10) $OD_{405nm} = 1,106$, for the MUXF-BSA column the CCD specific IgE was reduced 45 % while in serum after MUXF-Lys column there was still a 78,4 % reduction with a ratio gel to serum of 1:2. These results suggested that the BSA protein may have been too big (about 66 kDa) and hence blocked the pores of the Sepharose which limited the number of IgE antibodies that could enter the matrix. As lysozyme has a molecular weight of about 14 kDa it was much smaller than BSA and so the binding capacity of the column could be increased.

In the next trial the 2 mg/ml MUXF-Lys coupled ECH Sepharose was compared to Prosep-9 CHO glass beads coupled with the same amount of glycopeptide-conjugate. 75 μ l and 100 μ l of serum pool were added to 50 μ l of each gel.

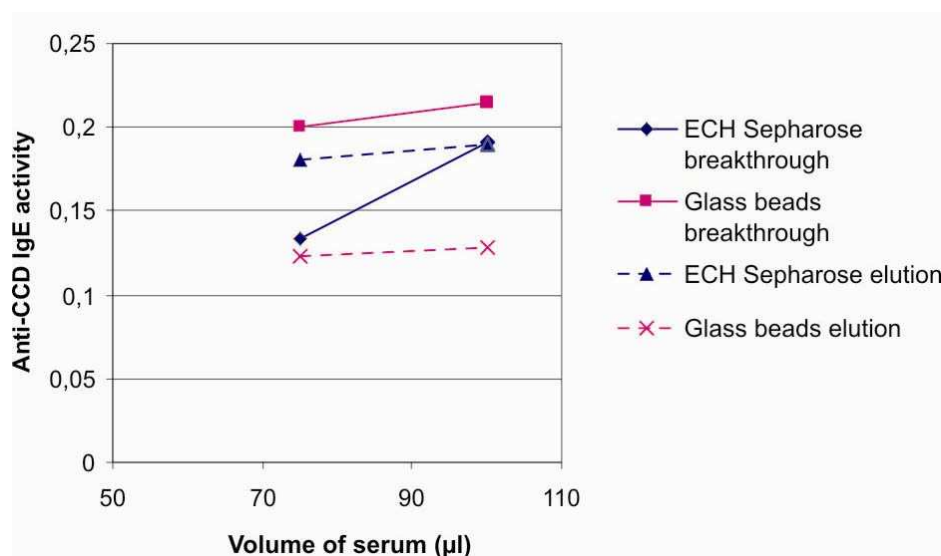


Fig. 44: Comparison of binding efficiencies of 2 mg/ml MUXF-Lys coupled ECH Sepharose and Prosep-9 CHO glass beads. Gels were incubated with serum pool in the ratios 2:3 and 1:2 (50 μ l gel with 75 μ l and 100 μ l serum) and anti-CCD IgE occurrence in the samples after column was evaluated via ELISA. OD_{405nm} for the untreated serum pool (1:10) was at 1,12.

Both affinity matrices showed a very good binding capacity for anti-CCD IgE. For each serum volume used, a lower IgE amount in breakthrough could be achieved with the 2 mg/ml MUXF-Lys ECH Sepharose matrix compared to glass beads. For the 2:3 ratio gel:serum about 88,7 % of anti-CCD IgE could be removed from the serum with the MUXF-Lys coupled Sepharose, while it was about 83,1 % for glass beads. But still even in the 1:2 ratio gel to serum both matrices were able to remove more than 80 % of anti-CCD IgE from the serum pool.

Due to these encouraging previous results another test was performed with a 2 mg/ml MUXF-Lys ECH Sepharose 4B. The gel was incubated with a CCD positive mixed serum (MG, 1:16 with PBS and CCD serum pool) and a CCD negative, allergen specific IgE positive serum (D 20) in the ratios gel : serum of 1:1 and 2:3. The breakthroughs were collected and sent to the FAZ for further analysis by CAP.

Results

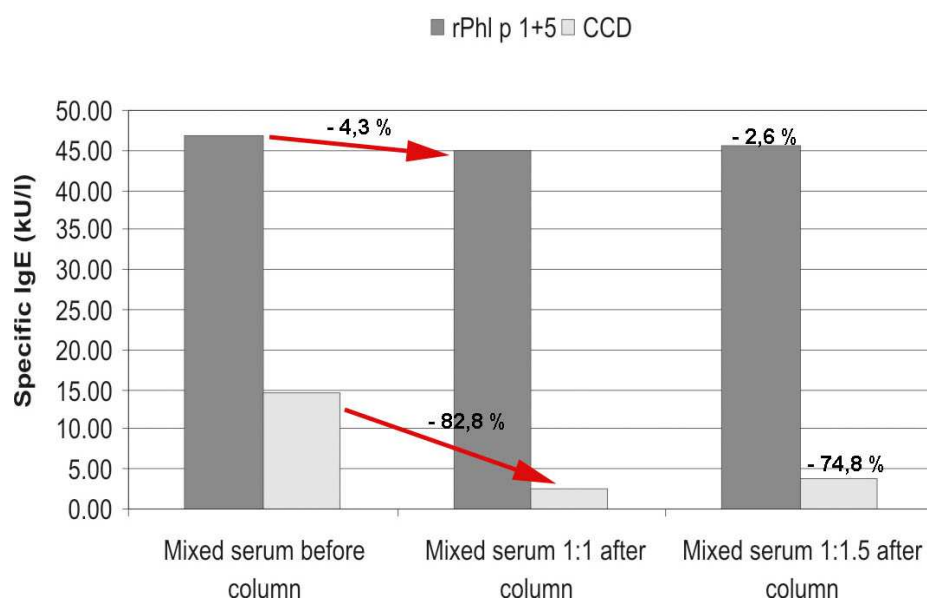


Fig. 45: CAP results for 2mg/ml MUXF-Lys coupled ECH Sepharose 4B. Gel was treated with a mixed serum pool in the ratios 1:1 and 2:3. Anti-CCD and allergen specific (rPhl p1+5) IgE content in the sera was measured before and after incubation with the column.

Although the ELISA tests for this matrix looked very promising, CAP results showed more anti-CCD in the breakthrough than previously for MUXF-BSA coupled Sepharose. 82,8 % anti-CCD IgE could be removed from the serum while it was up to 96,7 % when using a MUXF-BSA coupled ECH Sepharose (see Fig. 34). Only 4,3 % of allergen specific IgE antibodies were lost after treatment when using a ratio of 1:1. For the 2:3 ratio the amount was even less (2,6 %). For serum D20, containing only allergen specific IgE, the loss of IgE after column was 6,7 % for the 1:1 ratio and 0 % for the 2:3 ratio gel to serum (not shown in Fig. 45).

Finally trials with a 2 mg/ml MUXF-Lys ECH Sepharose incubated with 40 sera of patients, containing anti-CCD and allergen-specific IgE, and 20 sera of patients, exhibiting only allergen-specific IgE, were performed (for full sera list see Appendix). The sera were applied to the matrices in a ratio of 1:1 and breakthroughs were evaluated at the FAZ by CAP test.

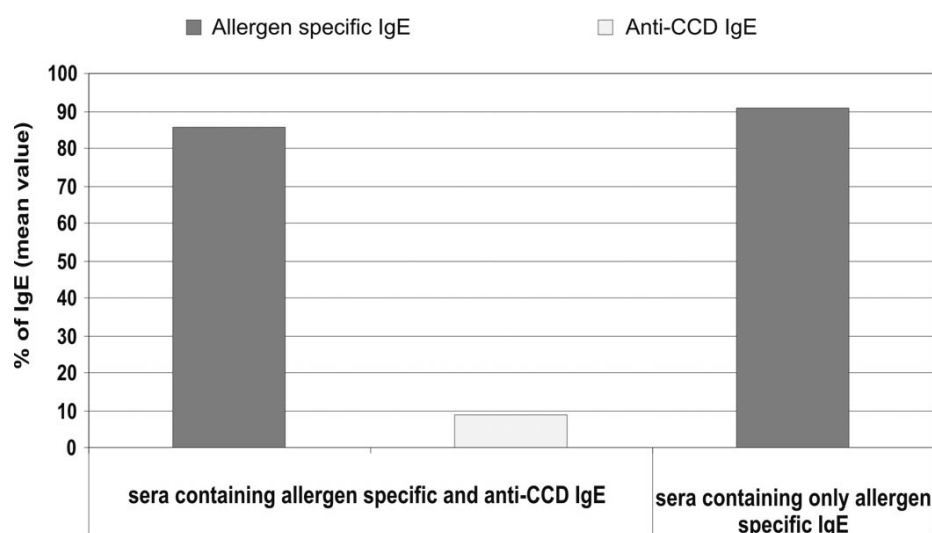


Fig. 46: Final CAP results for IgE content of various sera after incubation with 2mg/ml MUXF-Lysozyme ECH Sepharose cartridges. Sera were applied to the gels in a ratio of 1:1. In this graph the mean value of all tested sera is shown.

As shown in Fig. 46 up to 90 % of allergen specific IgE antibodies retained in the sera after incubation with the MUXF-Lys cartridge. About 91 % of anti-CCD IgE could be removed from patients sera by treatment with 2 mg/ml MUXF-Lys ECH Sepharose.

Results

3.5 Other Agarose beads

3.5.1 Aminoethyl agarose beads

With Aminoethyl agarose beads target molecules are covalently bound by their acid groups from their amino acids.

From this agarose, beads with very low density 4 % (4BCL) and with low density 6 % (6BCL) agarose were tested. 0,8 mg/ml MUXF-BSA were coupled to the beads and 50 μ l of this gel were incubated with 25 μ l, 50 μ l and 100 μ l of an anti-CCD IgE rich serum pool.

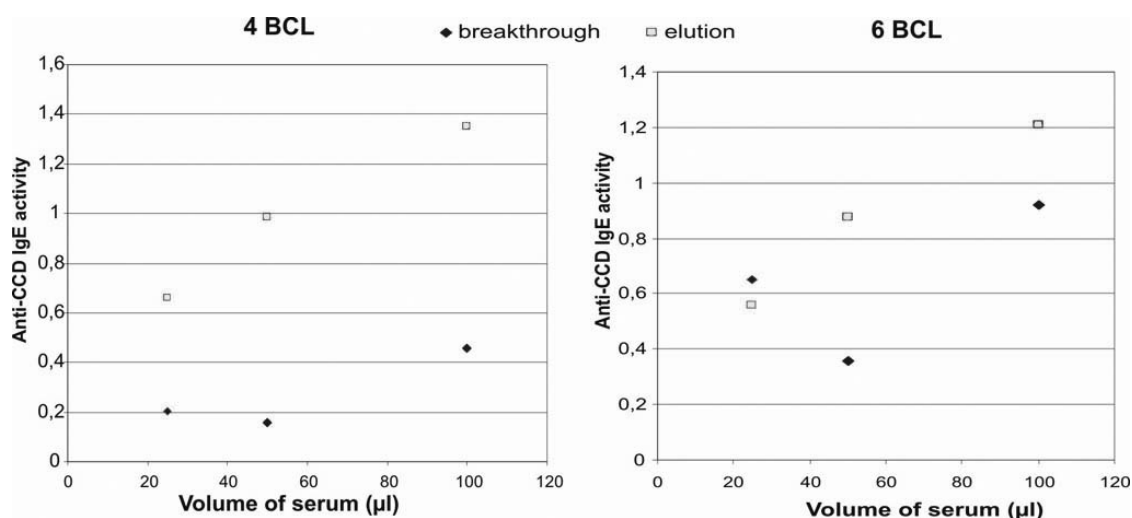


Fig. 47: Testing anti-CCD IgE binding efficiency of 4BCL and 6BCL aminoethyl agarose beads coupled with 0,8mg/ml MUXF-BSA. 50 μ l of each matrix were incubated with 25, 50 and 100 μ l of serum pool. Breakthrough and elution were evaluated via ELISA.

The breakthrough after very low density 4BCL Agarose showed less anti-CCD IgE than the serum after 6BCL Agarose. The readout of the serum pool before column (in a dilution of 1:10) was $OD_{405nm} = 1,9$, so the reduction of anti-CCD IgE lay over 90 % for MUXF-BSA coupled 4BCL Agarose (for a ratio of gel to serum of 1:1), while for MUXF-BSA-6BCL it was 81,1%.

As already observed when working with other matrices the column started leaking antibodies at a ratio gel:serum of 1:2. It is not clear why, appearing in tests with 4BCL and 6BCL gels, the breakthrough after 25 μ l serum on 50 μ l coupled agarose showed a higher anti-CCD IgE content than breakthrough after a 1:1 ratio of gel to serum (presumably an experimental error).

3.5.2 Glyoxal agarose beads

When using Glyoxal agarose beads bio molecules are immobilized through their amino groups. Low density Glyoxal Agarose 4 BCL (= 4 % agarose) and 6BCL (= 6 % agarose) were chosen for coupling with 0,8 mg/ml MUXF-BSA.

50 μ l of coupled beads were incubated with 25 μ l, 50 μ l and 100 μ l anti-CCD IgE rich serum pool.

Results

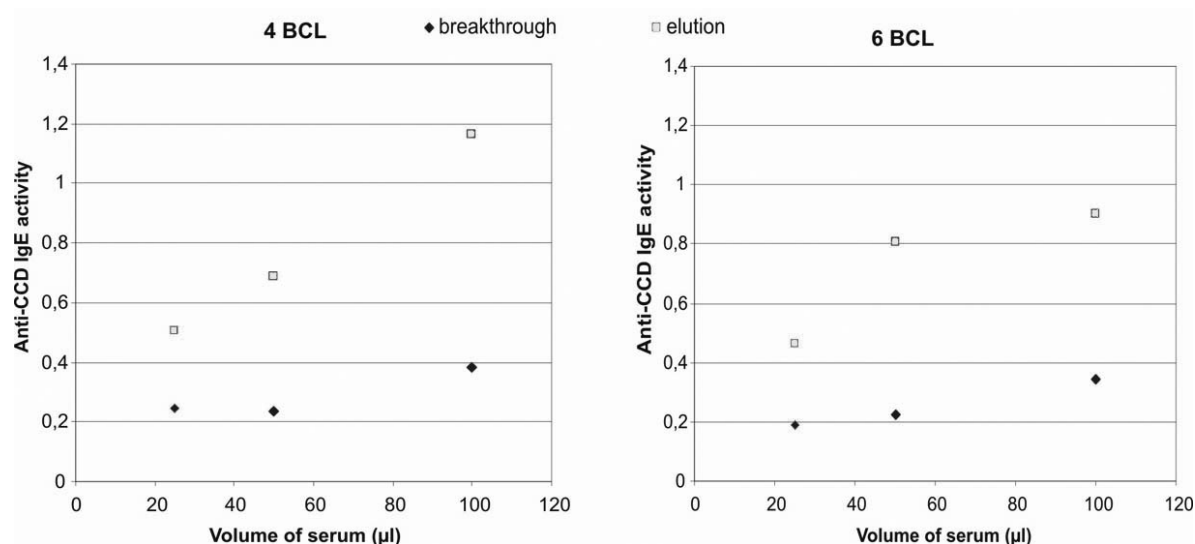


Fig. 48: Testing anti-CCD IgE binding efficiency of 4BCL and 6BCL glyoxal agarose beads coupled with 0,8mg/ml MUXF-BSA. 50 µl of each matrix were incubated with 25, 50 and 100 µl of serum pool. Breakthrough and elution were evaluated via ELISA.

As shown in Fig. 48 the anti-CCD IgE content in the breakthrough after 4BCL and 6BCL glyoxal agarose was almost the same (removal of anti-CCD IgE 87,6% and 88,3%, respectively, 1:1 ratio). Again a ratio gel:serum of 1:1 lead to the best results, although the column was leaking less antibody at a ratio of 1:2 compared to other matrices.

When comparing MUXF-BSA coupled aminoethyl agarose beads and glyoxal agarose beads, glyoxal beads showed a better binding capacity for anti-CCD IgE than aminoethyl agarose. Compared to other affinity matrices tested so far, a quite high amount of anti-CCD IgE could be eluted from all 4 agarose gels.

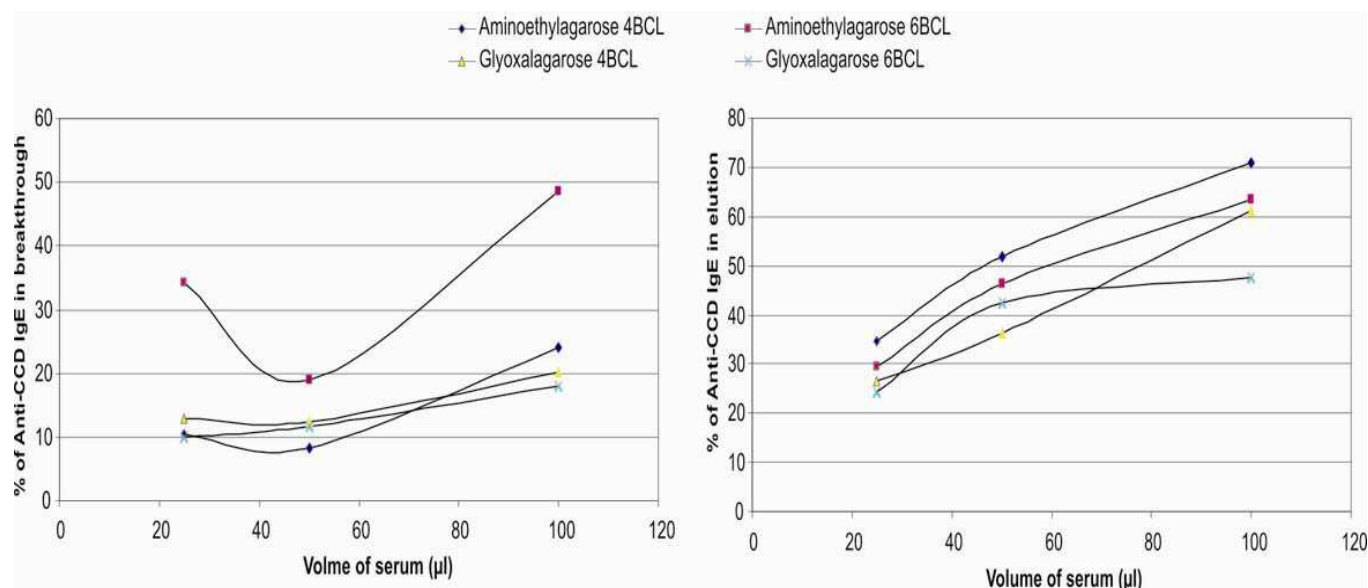


Fig. 49: Final comparison of aminoethyl and glyoxal agarose beads coupled with 0,8mg/ml MUXF-BSA. 50 µl of each gel were incubated with 25, 50 and 100 µl of serum pool. The percentage of anti-CCD IgE in breakthrough and elution was calculated from ELISA results.

As tests with ECH Sepharose, which were done at the same time, led to very promising results, working with agarose beads was interrupted (also by limited material) so far.

Results

3.6 Glass beads

The Prosep-9 CHO matrix binds molecules over their primary amino groups.

Two different approaches were tested, one with coupling MUXF-BSA to the beads, the other one with coupling MUXF-Lysozyme.

3.6.1 Coupling MUXF-BSA

Different amounts of MUXF-BSA (2, 4, 8, 16 mg/ml) were coupled to 500 μ l glass beads, respectively.

Then the coupled glass beads were tested with an anti-CCD IgE rich serum pool (ratio gel to serum was 1:1).

As a negative control glass beads without MUXF-BSA, but blocked with polyethylene glycol, were also incubated with serum, to find out if the matrix itself is able to bind anti-CCD IgE.

8mg/ml MUXF-BSA glass beads were tested twice: once with a normal centrifugation speed of 1000 x g before incubation with the serum, and the other time the beads were centrifuged with 11000 x g before the serum was added for incubation. This should lead to a minimal dilution of the serum with PBS buffer (which could be still in the glass beads) and also test the stability of the beads.

About 2 μ l of PBS was removed from the beads after a centrifugation speed of 1000 x g, while it was about 5 μ l PBS after a centrifugation with 11000 x g.

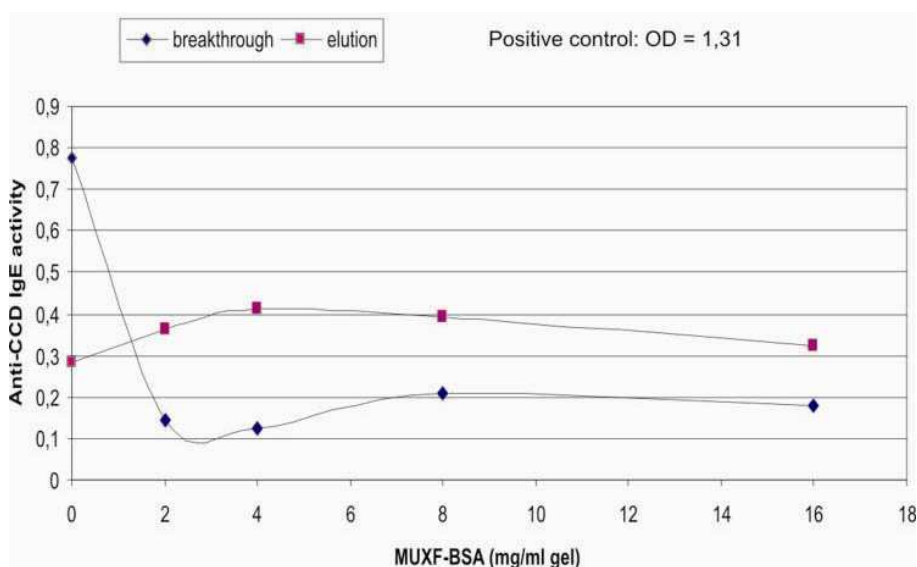


Fig. 50: Various amounts of MUXF-BSA coupled to Prosep-9 CHO glass beads. Binding capacity and efficiency for anti-CCD IgE was determined by evaluation of anti-CCD IgE content in the samples after column by ELISA. The ELISA plate was coated with 2 μ g/ml HRP.

With a centrifugation speed of 1000 x g the lowest amount of anti-CCD IgE after column was achieved with the 4 mg/ml MUXF-BSA gel. The higher ligand concentration may have led to clogging of the matrix and hence led to more IgE in the breakthrough. There also was observed a quite high unspecific binding of anti-CCD IgE to the negative control (about 40 %).

Results

However a centrifugation speed of 11000 x g seemed to affect the binding capacity of the gel positively as can be seen in Fig. 51.

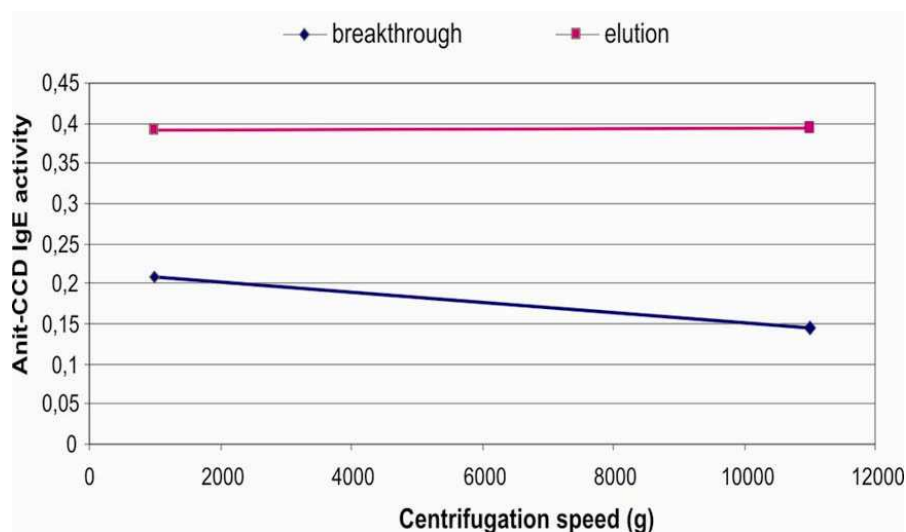


Fig. 51: 8mg/ml MUXF-BSA coupled glass beads were centrifuged with different speed (1000g and 11000g) before incubating with the serum pool. Anti-CCD IgE amount in the samples after column was detected by ELISA.

While anti-CCD IgE in the elution stayed the same for both gels, there were fewer antibodies in the breakthrough of the 8 mg/ml MUXF-BSA glass beads that had been centrifuged with 11000g before applying the serum to the matrix. To test this new matrix in CAP test, 150 µl 4 mg/ml MUXF-BSA glass beads and 150 µl of the negative control were incubated with 150 µl of a mixed serum. The mixed serum consisted of a patient serum (MG) diluted to 1:8 with PBS and further 1:2 with an anti-CCD IgE rich serum pool (so the end dilution of the mixed serum was 1:16). The matrices were centrifuged with 11000 x g before the serum mix was added for incubation. To prevent unspecific binding to the negative control, it was additionally blocked with 0,5 % BSA in TTBS. Breakthrough, elution (100 µl glycine + 50 µl Tris HCl) and the first washout (done with 150 µl TTBS) were sent to the FAZ for CAP analysis.

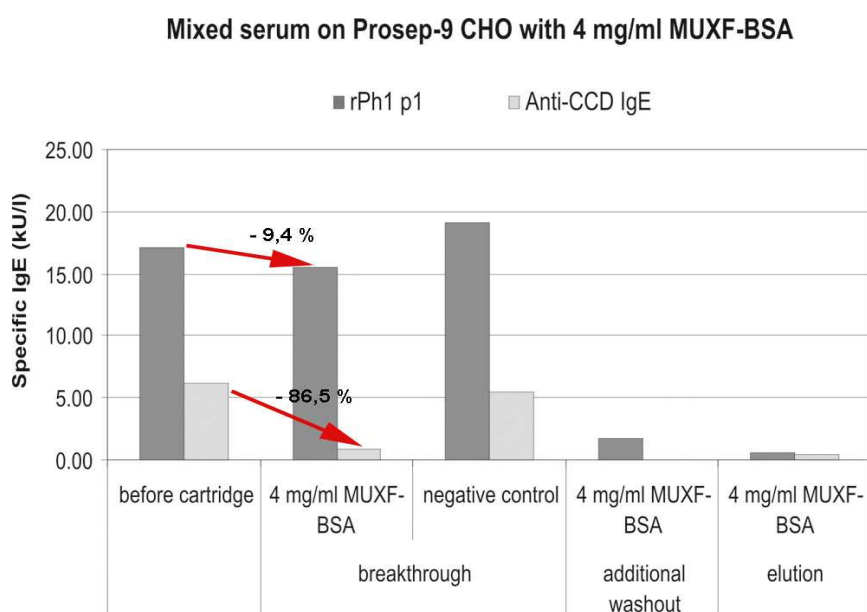


Fig. 52: CAP results for 4mg/ml MUXF-BSA Prosep-9 CHO glass beads. The affinity matrix was incubated with serum in a ratio of 1:1. The mixed serum was checked for anti-CCD and allergen specific IgE before and after cartridge.

Results

As can be seen in Fig. 52 about 86,5 % of anti-CCD IgE could successfully be removed from the serum, while more than 90 % of allergen specific IgE (rPh1 p1) was recovered. The negative control showed almost no binding of antibody, which means that the additional blocking step with 0,5 % BSA in TTBS was necessary to avoid unspecific binding of IgE to the matrix.

Another task was to test the stability of the beads. The binding capacity of 4 mg/ml MUXF-BSA coupled beads and of uncoupled beads after complete drying for 4 days at room temperature was tested. Before applying the sera the dried beads were shortly washed with PBS and centrifuged at 11000g as described in materials and methods. 50 µl beads were incubated with 50 µl of serum pool.

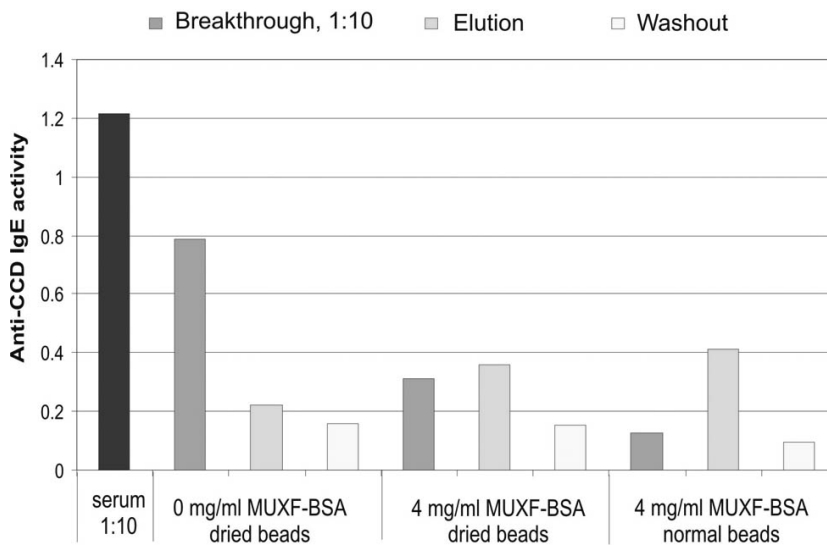


Fig. 53: Checking stability and functionality of 4mg/ml MUXF-BSA coupled glass beads when dried at room temperature. Beads and serum were added in a ratio of 1:1. Non-coupled beads were also tested as a negative control to check unspecific binding of anti-CCD IgE to the matrix. Samples were evaluated with ELISA.

Again about 40 % of anti-CCD IgE bound to the matrix unspecific (the beads have not been blocked with 0,5 % BSA in TTBS). Drying of the coupled glass beads also led to a decrease of the binding capacity. The serum after treatment with the non-dried Prosep cartridge exhibited about 59 % less anti-CCD IgE in the breakthrough than the one from the dried column.

The experiment was repeated and this time the dried beads were left in PBS buffer for 2 hours at room temperature before applying the serum to the column.

Results

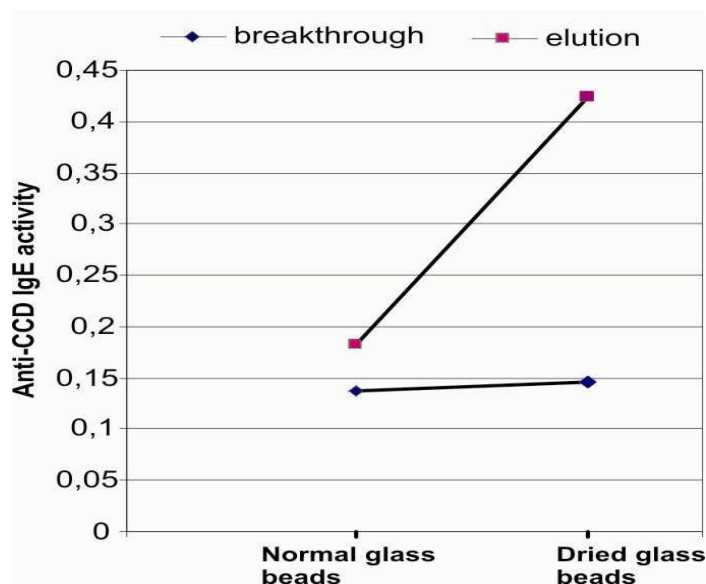


Fig. 54: New comparison of normal and dried 4mg/ml MUXF-BSA glass beads. This time the dried beads were left in PBS buffer for 2 hours before applying the serum to the column. Gel and serum were tested in a ratio of 1:1. Anti-CCD IgE levels were determined by ELISA.

Although the Prosep beads should not be affected by swelling or shrinking in buffer, this time a clear improvement of anti-CCD IgE binding capacity for dried glass beads could be observed compared to the first test. The breakthroughs of normally treated and dried beads showed no difference in ELISA testing. The results even suggested that the antibody could be eluted with more efficiency from the dried beads.

3.6.2 Coupling MUXF-Lysozyme

2 mg/ml and 6 mg/ml MUXF-Lysozyme (MUXF-Lys) were coupled to 500 μ l Prosep-9 CHO glass beads. This time the glass beads were blocked with 0,5 % BSA in TTBS before using the matrix for affinity chromatography. 50 μ l coupled beads were incubated with 50 μ l of a serum pool rich in anti-CCD IgE. Breakthrough and elution were collected and evaluated by ELISA.

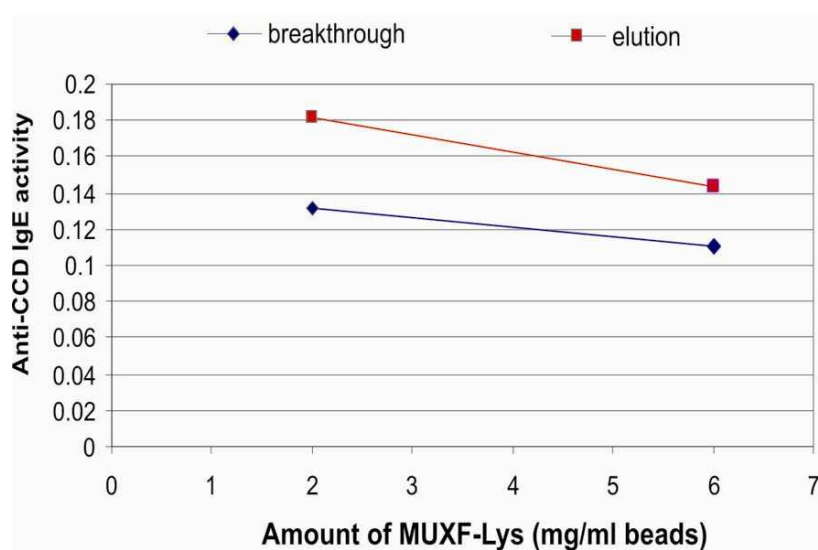


Fig. 55: Testing binding capacity of 2mg/ml and 6mg/ml MUXF-Lys coupled glass beads. 50 μ l of each matrix were incubated with 50 μ l of serum pool. Breakthroughs and elutions were evaluated via ELISA. The readout for the serum (1:10) before column was $OD_{405nm} = 1,22$.

Results

The ELISA test revealed almost no difference in the anti-CCD IgE content in the serum after column for the 2 mg/ml and 6 mg/ml MUXF-Lys glass beads. On average about 90% of anti-CCD IgE was removed from the sera by both matrices. Although the 6 mg/ml coupled beads showed an insignificantly better binding capacity, the 2 mg/ml MUXF-Lys glass beads were chosen for further testing, as for these coupling less material (MUXF-Lys) had to be used and could be compared with other matrix in similar ligand concentration.

The next step was to compare MUXF-BSA coupled glass beads with MUXF-Lys coupled beads. Therefore 50 μ l of 2 mg/ml MUXF-BSA- and 2 mg/ml MUXF-Lys- Prosep-9 CHO were each incubated with an anti-CCD serum pool, in the ratios 1:1 and 1:2, and the serum after column was evaluated via ELISA.

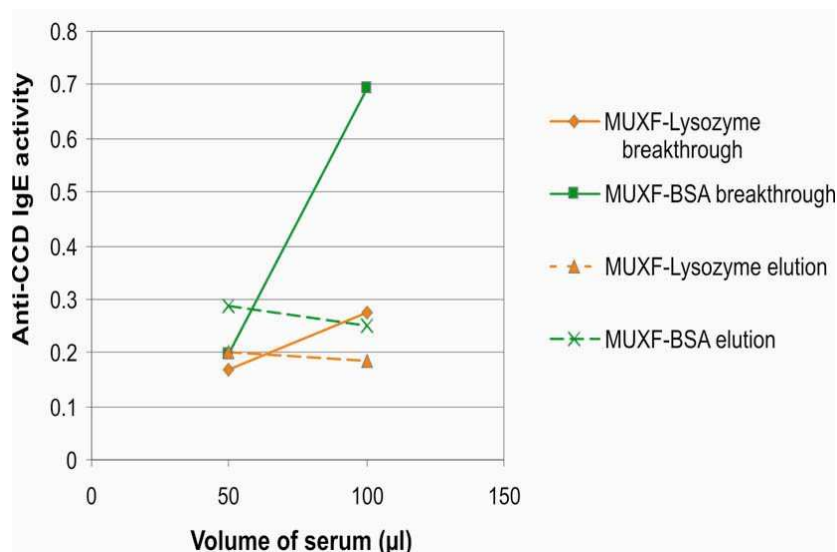


Fig. 56: Comparison of 2mg/ml MUXF-BSA and 2mg/ml MUXF-Lys coupled Prosep glass beads. The affinity matrices were incubated with a serum pool in the ratios 1:1 and 1:2. Anti-CCD IgE levels in the samples before and after column were determined by ELISA.

When using a ratio gel:serum of 1:1 the anti-CCD IgE content in the breakthroughs was almost the same for MUXF-BSA and MUXF-Lys beads and over 80 % of anti-CCD IgE could be removed in both cases. But when going on to a ratio of 1:2 the MUXF-Lys Prosep-9 CHO showed a much better binding capacity. The MUXF-BSA coupled glass beads were leaking more than 62 % of anti-CCD IgE at a ratio of 1:2, while the MUXF-Lys column still was able to remove more than 75 % of anti-CCD IgE from the serum under the same conditions.

As BSA has an average size of about 66 kDa it may have blocked the pores for more IgE and hence led to displacement of IgE from the matrix. On the contrary Lysozyme has a size of about 14 kDa which allows more IgE to enter the matrix and bind to the MUXF glycopeptide than BSA does.

Due to comparison and similar tests with ECH Sepharose, further tests with Prosep-9 CHO glass beads were stopped as coupled sepharose led to slightly better results.

Results

3.7 Summary of results

To design a proper affinity column for depletion of clinically irrelevant anti-CCD IgE antibodies from patients' sera, several matrixes coupled with MUXF glycopeptide (-conjugates) and evaluated in ELISA (see Tab. 8) and CAP test (Tab. 9).

Tab. 8: Summary of results obtained with various combinations of different solid supports coupled with MUXF glycopeptide, MUXF-BSA or MUXF-Lysozyme conjugate. All gels were tested with serum in a ratio of 1:1. The percentage of absorbed anti-CCD IgE was measured in ELISA tests. The concentration of ligand leading to this result is indicated in brackets next to the % values.

Matrix	Ligand	Ligand concentration tested (mg/ml matrix)	Absorption of anti-CCD IgE (%) measured in ELISA
Poly-L-lysine agarose (Sigma-Aldrich)	MUXF-BSA	1 - 40	84.6 (40)
CIM EDA disk (BIA Separations)	MUXF-BSA	2	90.2 (2)
CIM EPOXY disk (BIA Separations)	MUXF-BSA	3	89.4 (3)
Eupergit C (Sigma-Aldrich)	MUXF-BSA	0.8 - 16	75.1 (4)
Eupergit CM (Sigma-Aldrich)	MUXF-BSA	0.8	85.7 (0.8)
Eupergit C250L (Sigma-Aldrich)	MUXF-BSA	0.8	90.4 (0.8)
Glyoxal Agarose 4 BCL (Hispanagar)	MUXF-BSA	0.8	87.6 (0.8)
Glyoxal Agarose 6 BCL (Hispanagar)	MUXF-BSA	0.8	88.3 (0.8)
Aminoethyl Agarose 4 BL (Hispanagar)	MUXF-BSA	0.8	89.5 (0.8)
Aminoethyl Agarose 6 BL (Hispanagar)	MUXF-BSA	0.8	81.1 (0.8)
PROSEP-9 CHO (Millipore)	MUXF-BSA	2 - 16	82.1 (2)
PROSEP-9 CHO	MUXF-Lyso	2 - 6	90.9 (6)
ECH-Sepharose (GE Healthcare)	MUXF-BSA	0.1 - 25.6	95.8 (3)
ECH-Sepharose	Bromelain glycopeptide (MUXF)	0.005 - 1.28	59.6 (1.25)
ECH-Sepharose	MUXF-Lyso	2 - 6	90.6 (3)

When comparing all tested matrices, ECH Sepharose and Prosep-9 CHO glass beads coupled with MUXF-BSA or MUXF-Lysozyme appeared to most efficiently remove CCD directed IgE from the applied sera in ELISA tests as well as in CAP tests.

Results

For ELISA more than 95 % of anti-CCD IgE could be depleted from the serum pool by the ECH-Sepharose MUXF-BSA (3 mg/ml) affinity matrix, followed by the MUXF-Lysozyme coupled ECH Sepharose where more than 90 % of CCD directed IgE could be removed.

Tab. 9: CAP results for ECH Sepharose and glass beads coupled with either MUXF-BSA or MUXF-Lysozyme.

CAP results	ECH Sepharose with 3mg/ml MUXF-BSA	ECH Sepharose with 2mg/ml MUXF-Lys	Prosep glass beads with 4mg/ml MUXF-BSA
Anti-CCD IgE removed (%)	96,7	91,1	86,5
Allergen specific IgE removed (%)	7,6	10	9,4

With MUXF-BSA coupled ECH Sepharose, IgE values in CAP test could be reduced beneath the positive cut-off value of 0,34 kU/l. With MUXF-Lysozyme the cut-off could be reached in the last CAP tests with different sera (mean value for all tested sera was 0,3495 kU/l). Both results were achieved when sera were incubated with matrices in a ratio of 1:1.

In the last tests with MUXF-Lys coupled ECH Sepharose a ratio of 2:3 also showed successful removal of anti-CCD IgE. Further trials with MUXF-Lysozyme glass beads and ECH Sepharose, where matrix and serum were incubated in a ratio of 1:2, exhibited a removal of CCD directed IgE from the serum of over 80 % in ELISA tests.

4 Discussion

In vitro allergy diagnosis methods like RAST or CAP tests frequently display false positive allergy detections caused by cross-reactive IgE antibodies against N-glycan structures of allergens. To avoid erroneous diagnosis and enhance accuracy of these tests we developed an affinity matrix, which depletes anti-CCD IgE from patients' sera before subjecting them to determination of allergen-specific IgE. For the practical realization of this goal two main steps had to be taken.

First, it was necessary to find an appropriate solid support which could successfully be coupled with a CCD structure (in our case MUXF-glycopeptide either alone or - rather - conjugated to a carrier protein) and efficiently remove anti-CCD IgE from patients' sera. Also the amount of coupled glycan structure for satisfactory IgE removal had to be quantified. Many different matrices were coupled with various amounts of glycopeptide and elimination of anti-CCD IgE was determined with ELISA tests (Tab. 8).

Second the amount of gel slurry and serum, with which a satisfying elimination of anti-CCD IgE could be assured had to be optimized. In the beginning of this work, a minimal ratio of gel to serum of 1:1 seemed to be necessary for anti-CCD IgE removal. With ongoing trials we observed enhanced binding capacity of the coupled matrices when increasing the centrifugation speed before incubation with the serum from 1000 x g to 11000 x g. Also the problem of apparently unspecific binding of allergen specific antibodies to the columns could be solved by this step. Further BSA, which was conjugated to MUXF, emerged as being too large. With a size of about 66 kDa it probably filled a large volume of the pores in the matrix leaving only little space for anti-CCD IgE. Hence the same volume of gel (slurry) as serum of 1:1 had to be used. This improved when matrices with new lysozyme as the carrier protein for the MUXF-glycopeptide were prepared.

Of all developed and tested affinity matrices ECH Sepharose and Prosep-9 CHO glass beads coupled with MUXF-BSA or MUXF-Lysozyme appeared to most efficiently remove CCD directed IgE from the applied sera in ELISA tests as well as in CAP tests. However when comparing binding capacities of MUXF-BSA and MUXF-Lys matrices using a 1:1 ratio in CAP test, MUXF-BSA columns showed better results for removing anti-CCD IgE from the serum. Concerning this issue some more trials with MUXF-Lysozyme matrices should be done and maybe the columns' binding capacity could be improved by further optimizing the working procedure or coupling process. Anyway anti-CCD IgE antibodies were successfully removed from patients' sera and hence false positive results in CAP tests could be prevented by treating sera with the 3 mg/ml MUXF-BSA coupled ECH Sepharose before starting the *in vitro* test.

Although our proposed method solves the problem of false positive *in vitro* allergy tests, two alternative strategies for reaching these goal shall no be concealed. The first is addition of a CCD-inhibitor the serum before analysis as has been done for merely scientific purposes with insect venom reactive sera [31].

Another possibility to reach the same goal would be working with recombinant allergens. As anti-CCD IgE recognizes solely glycosylated structures on allergens, it would only bind to natural allergens. A method to avoid or control glycosylation of allergens is producing these recombinant molecules in various expression systems like *E.coli* or yeast. Until now an increasing number of inhalant allergens and food allergens has been produced that way and is replacing naturally derived allergens in *in vitro* tests. Expression of recombinant molecules in *E.coli* leads to non-glycosylated proteins [32-33]. However this can lead to non- or incorrect folding of the produced molecules and hence to allergen specific IgE antibodies not detecting the allergen or recombinant proteins creating new conformational epitopes. If proper protein folding still can be achieved in *E.coli*, it is an excellent expression system for recombinant allergens. Other organisms that are used for this purpose are yeast (*pichia pastoris*), insect cells or tobacco plants. These eukaryotic expression systems facilitate the correct folding of proteins in most cases. Several recombinant allergens have

Discussion

already been produced in yeast, which is known for hyperglycosylation of proteins [34]. A big advantage of this organism is that it does not produce proteins with fucose and xylose N-glycans and hence would be a good alternative to *E.coli* for producing recombinant allergens. On the other hand glycoproteins from yeast sometimes are recognized by IgE from patients displaying yeast- or mould allergies [8]. The same is true for expression of non-plant glycoproteins in tobacco plants. Although they would be the system of choice when recombinant pollen and plant food allergens have to be produced, expression of non-plant glycoproteins would be a problem as tobacco would form IgE-binding glycans on these proteins [8]. In conclusion, concerning recombinant allergens, it seems that for each allergen the appropriate expression system still has to be determined to assure correct folding and presentation of the allergen to antibodies.

The system designed in our work would, in any case, be a momentary possibility to successfully prevent false positive allergy diagnosis which is due to anti-CCD IgE antibodies.

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Appendix

6 Appendix

Sera list for final testing of 2 mg/ml MUXF-Lys ECH Sepharose via CAP test:

- Sera containing anti-CCD and allergy specific IgE:

Tab. 10: List of patients sera, with allergen specific and anti-CCD IgE levels. YJ = yellow jacket (wasp)

Serum No.	Allergy type	Allergen-specific IgE (kU/l)	Allergen-specific IgE (kU/l)	CCD-specific IgE (kU/l)
P3565/170	wasp	wasp 3.0	YJ, 12.5	3
P3612/151	insect allergy, honeybee-wasp-double positive	wasp 2.1 bee 2.2	YJ, 6.37	2.27
P3273/173	Insect allergy, honeybee-wasp-double positive	wasp 2.1 bee 2.3	YJ, 5.27	2.05
P3244/169	wasp	wasp 2.9	YJ, 7.34	2.22
P3599/159	wasp	wasp 3.3	YJ, 6.38	2.26
P5633	Pos CAP, neg skin test	Ragweed, 15.4; birch 11.2; wheat flour 11.2, CCD 8.4	grass pollen, 7.41	5.63
D01			grass pollen, 2.64	1.9
D22	Grass	Birch 0.4; grass >100	rPh1 p1+5, 46.90	14.7
D22	Grass	Birch 0.4; grass >100	nd	1.37
D10	Grass	Bet v 1, 0; Phl p1+5, 87.5; Feld 1, 32.1; Phl p7, 22.4	rPh1 p1+5, 90.10	1.64
P0090/6	birch pollen allergy	birch 4.5 honeybee and wasp neg	Bet v1, 37.8	6.05
85	Monovalent grass pollen allergy	grass 4.6	rPh1 p1+5, 47.4	0.6
91	grass pollen allergy	grass 4.6	rPhl p1+5, 15.0	0.5
P2276/18	grass pollen allergy, positive RAST/negative skin test to latex and birch	grass 4.5 birch 2.6 latex 3.0	rPhl p1+5, 102	4.22
P2492/129	grass, weakly-questionable positive skin tests to latex, birch, mugwort, ragweed and other pollens	latex 3.3 grass 4.3 ash 2.5 platanus 2.6	rPhl p1+5, 69.8	2.25
90	grass + birch	grass 4.9 birch neg	rPhl p1+5, 100	4.2
P0478/3	polyvalent pollen allergy	birch 4.6 grass 4.9 mugwort 2.2 oilseed rape 3.7 ash 4.6	Bet v1, 29.1	3.43
P0496/9	polyvalent pollen allergy	grass 4.9 mugwort 3.5 oilseed rape 3.4 ash 2.3	rPhl p1+5, 187	25.2
68	polyvalent pollen allergy	birch 2.5 grass 4.5 plantain 2.9 oilseed rape 3.0	rPhl p1+5, 186	13
P0701/75	polyvalent pollen allergy	birch 4.8 grass 2.7 mugwort 2.4	Bet v1, 90	2.25
25	polyvalent pollen allergy	grass 4.9 mugwort 2.6 ragweed 3.1	rPhl p1+5, 193	4.73
P0486/10	polyvalent pollen allergy	birch 3.7 grass 4.6 mugwort 2.8 oilseed rape 2.2 Bet v 2 neg	rPhl p1+5, 236	4.48
29	Polyvalent pollen allergy (n>4)	birch 3.3 grass 4.9 ash 3.0	rPhl p1+5, 149	2.99
P2509/49	Polyvalent pollen allergy (n>4)	latex 4.4 birch 4.2 grass 4.0 mugwort 4.3 ragweed 2.4	Bet v1, 111	8.22

Appendix

20	Polyvalent pollen allergy (n>4)	CCD-positive according to immunoblot	rPhl p1+5, 107	3.61
30	Polyvalent pollen allergy (n>4)	birch 3.4 grass 4.9 mugwort 2.4	rPhl p1+5, 58.9	0.64
33	Polyvalent pollen allergy (n>4)	birch 4.9 grass 3.6	Bet v1, 65.4	0.71
P2738/ 21	birch, grass, mugwort, negative skin test to latex	birch 3.3 grass 4.8 mugwort 3.8 latex 3.0	rPhl p1+5, 196	5.73
P3693/ 122	birch, grass, negative skin test to latex, ragweed, mugwort	birch 4.8 grass 4.8 mugwort 3.3 ragweed 4.2 latex 3.7	Bet v1, 166	3.45
P0750/ 79	birch grass ash	birch 2.9 grass 4.9	rPhl p1+5, 250	5.92
P4096/ 125	birch, grass, mugwort, negative skin test to latex	grass 2.9 mugwort 3.1 latex 3.0 birch 2.9 ficus 3.0	Bet v1, 28.1	3.66
P1929/ 115	allergy to birch, grass, mugwort, ash, house dust mite, cat, horse, positive RAST/negative skin test to latex	latex 2.3	Bet v1, 27.6	4.55
P3902/ 124	Allergy to birch, mugwort, grass pollen, positive RAST/negative skin test to latex		Bet v1, 90.2	2.45
P2238/ 118	Allergy to birch, mugwort, grass pollen, positive RAST/negative skin test to latex		Bet v1, 17.6	3.41
P2259/ 114	polyvalent pollen allergy + questionable positive latex	latex 2.2 birch 3.7 grass 4.4 ash 3.1	rPhl p1+5, 91.6	2.78
P0032/ 46	Polyvalent pollen allergy (n>4)	birch 2.9 grass 2.7	Bet v1, 49.9	2.42
P3887/ 126	polyvalent pollen allergy/negative skin test to latex	birch 4.0 grass 4.0 ash 3.0 latex 2.6	Bet v1, 73.9	3.27
P2264/ 133	grass + birch, negative skin test to mugwort, ficus etc.	birch 4.1 grass 4.1 mugwort 3.2 ficus 2.4	Bet v1, 83.5	3.27
P3334/ 119	grass + birch, questionable ragweed, mugwort, latex	latex 3.2 birch 3.2 grass 2.5 ragweed 2.4	Bet v1, 42.5	5.05
P2459/ 121	grass pollen, weakly peanut latex	latex 3.0 grass 3.6 peanut 2.5	Bet v1, 44.8	5.47

Appendix

- Sera containing only allergen specific IgE:

Tab. 11: List of patients sera with solely allergen specific IgE.

Serum No.	Alltergy type	Allergen-specific IgE (kU/L)	Total IgE (kU/L)	Allergen-specific IgE (kU/L)
D20	Grass	Birch 0; grass 14	67	rPh1 p1+5, 20.6
D03	Grass, birch	Bet v 1, 7; Ph1 p1+5, 5.6; alternaria, 4.2	78	rPh1 p1+5, 5.35
D09	Grass	grass, 25.6	44	rPh1 p1+5, 44.00
D22	Grass	Birch 0.4; grass >100	740	rPh1 p1+5, 20.20
D04	Grass, birch	Bet v 1, 0; Phl p1+5, 5.6	53	rPh1 p1+5, 5.78
D12	Grass, birch	Bet v 1, 7; ph1 p1+5,1.96	368	rPh1 p1+5, 1.98
D06	Birch	Birch >100	444	Bet v 1, 23.9
D21	House dust mite	HDM, 11.2	55	HDM, 8.15
D16	House dust mie, horse and cat dander	Der p1, 5,6; horse, 8.4	211	Der p1, 4.27
D07	House dust mite	HDM, 28.9	143	HDM, 20.0
D19	Birch	Bet v1, 67.5	257	Bet v1, 64.0
D18	Birch	Bet v1, 32.1	82	Bet v1, 37.6
D17	Brich, grass	Bet v1, >100; Phl p1+5, 1.96	539	Bet v1, 26.7
D15	Birch, grass, house dust mite	Bet v1, 1.12; Phl p1+5, 14; Der p1, 1.68	510	Phl p1+5, 11.6; Der p1, 1.52
D14	Birch, grass, house dust mite	Bet v1, 19.1; Ph1 p1+5, 7; Der p1, 2.52	240	Bet v1, 14.1; Der p1, 2.21
D13	Grass	Phl p1+5, 57.5	264	Phl p1+5, 39.8
D11	Grass	Phl p1+5, 4.2	49	Phl p1+5, 9.54
D08	Birch	Birch 11.2	125	Bet v1, 9.44
D05	Birch	Birch >100	177	Bet v1, 8.62
D02	Grass, birch	Bet v 1, 32.1; Phl p1+5, 5.6	860	Bet v1, 28.4

Curriculum Vitae

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