Recognition of multiepitope dendrimeric antigens by human immunoglobulin E

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Abstract

In vitro drug allergy tests have limited sensitivity, partly due to a poor understanding of the immunological recognition of in vitro drug–protein conjugates. We have designed and synthesized multivalent mono- and bi-epitope dendrimeric antigen (DeAn) conjugates and studied their chemical and tridimensional structures. We describe differences in the spatial distribution and conformation of these conjugated epitopes for the first time: a partially hidden benzylpenicilloyl and a more exposed amoxicilloyl. Our data suggest that DeAn conjugates provide a useful model for studying IgE recognition in patients who suffer from an allergic reaction to benzylpenicillin and/or amoxicillin. 1D and 2D NMR, MDS and immunochemical studies provide evidence that both antigen composition and tridimensional distribution play key roles in IgE-antigen recognition. Bi-epitope DeAn conjugates could potentially allow the diagnosis of patients allergic to any of these two drugs with a single test and represent the basis for a broadly-applicable in vitro assay.

From the Clinical Editor: The prevalence of allergic drug reactions is rising and there is an imperative need to identify patients at risk. In this interesting and important article, the authors developed a novel method for detecting drug specific IgE antibodies, responsible for allergic reactions, by using multivalent mono- and bi-epitope Dendrimeric Antigen (DeAn) conjugates. The continued success of this research may pave way of eventual development of a simple diagnostic test.

Key words: Immune response; Immunochemistry; In vitro test; Dendrimer; Allergy; Molecular modeling

The study, understanding and precise manipulation of the immune system requires a high degree of knowledge of how the immune system recognizes and responds toward specific targets.1,2 Insight into the exact chemical nature of immune system target moieties, effectors or inhibitors, depends on understanding precisely how chemical structures can drive immune responses involving polyvalent interaction processes.3,4 Allergic drug reactions are one of the most important problems facing the healthcare community. Immunoglobulin E (IgE) mediated-reactions are involved in a high proportion of cases, ranging from simple skin symptoms to anaphylactic shock.5,6 The study of these immunological manifestations is handicapped by a variety of factors including a lack of knowledge of the actual drug derivatives involved in the reaction, changes to the recognized haptenic pattern over time in selected populations, the possibility of cross-reactivity between related chemicals, and an increase in adverse patient responses due to environmental and, probably, genetic factors.1,7-9 In addition, the low sensitivity of in vivo and, especially, in vitro tests is currently a problem for drug allergy diagnosis.7

In order to emulate the in vivo recognition process in vitro, much effort has been invested into the preparation of hapten(drug)–carrier(protein) conjugates that attempt to simulate the antigen responsible for the allergic drug reaction, generally with the objective of developing an in vitro test for the detection of IgE antibodies to a particular drug.10 Human serum albumin (HSA) has been proposed as the carrier protein responsible for antigenic hapten–carrier conjugate formation in vivo, which induces the allergic response. However, its usage in allergy testing has been limited due to the possibility of infections and difficulties in both the chemical handling and characterization of the haptenized protein.

Poly-L-lysine (PLL) has become the most widely used artificial carrier alternative. Due to its accessibility and easy chemical modification with appropriate haptens, PLL has been used both for research purposes and by pharmaceutical companies for the development of in vitro and in vivo drug allergy tests.10,11 On one hand, their multivalency allows a higher density of haptens in the carrier, increasing the possibility of detecting IgE antibodies. However, their inherent polydispersity impedes adequate verification of chemical modification consistency. As a consequence, the chemical characterization of the resulting conjugate is only approximate and subsequent medical interpre- tations can become complex and unreliable.

To achieve dense and reproducible hapten–carrier conju- gates, we have previously reported the preparation of synthetic dendrimeric antigens (DeAns) and their ability to be recognized by specific IgEs directed toward haptenic structures covalently attached to their surface.12-17 For these studies, we chose PAMAM dendrimers18 due to the fact that, in contrast to PLL or HSA, these conjugated macromolecules can be chemically characterized by conventional spectroscopic techniques12,15 and have a high degree of structural accuracy between generations. Applying our non-commercial RadioAllergoSorbent test (RAST) and RAST-inhibition experiments to sera from patients allergic to benzylpenicillin, we have shown that IgEs directed to benzylpenicilloyl (BPO) groups recognize penicilloylated dendrimers as DeAns and that they can be used to detect IgE in sera from these patients.12-14

β-Lactams are the most commonly prescribed antibiotics and the ones most frequently involved in allergic drug reactions.19 Recent studies have shown that the pattern of β-lactam allergic responses has moved from the more traditional benzylpenicillin antibiotic to the now more commonly prescribed amoxicillin.20 Patients can be classified according to their specific IgE recognition, being allergic to only one kind of penicillin (e.g., either benzylpeni- cillin or amoxicillin), to several penicillins (e.g., benzylpenicillin and amoxicillin), or having cross-reactivity with other β-lactams which have the same or similar side chain.21-27 Figure 1, A summarizes the classes of patients of interest in this study.

Nowadays, diagnosing allergy to different penicillins requires testing each antibiotic separately, due to an a priori uncertainty regarding patient antibody specificity. In order to improve the detection of β-lactam-specific IgE antibodies and advance toward a method for the universal recognition of β-lactam-directed IgEs, we used the ability of dendrimers to emulate carrier proteins for the preparation of novel DeAns. In this work, two mono-epitope DeAns were made by including either BPO or AXO (the amoxicilloyl epitope corresponding to the antigenic determinant of amoxicillin) at the dendrimer periphery. With the eventual goal of developing a single diagnostic assay, here we present an innovative DeAn preparation that simultaneously includes both BPO and AXO epitopes in the same dendrimer carrier conjugate. We compare IgE recognition between mono- and multiepitope DeAns. Moreover, we relate the ability of IgEs to recognize different DeAns due to their chemical composition and tridimen- sional structure.

Methods

Materials

Poly(amidoamine) dendrimer (ethylenediamine core) with amino surface groups (PAMAM 4th generation) was purchased from Dendritic Nanotechnologies, Inc. (dnt). Benzylpenicillin sodium salt was supplied by CEPA, and amoxicillin was obtained from GlaxoSmithKline. Standard chemicals were obtained from Aldrich or Merck, and used without further purification.

General procedure for the synthesis of antibiotic conjugated dendrimers

An excess of antibiotic (penicillin G sodium salt and/or sodium amoxicillin) was added to a stirred solution of 4th generation Starburst PAMAM dendrimer, in 0.02 M aqueous carbonate buffer, pH 10.8, at 4 °C. Fresh antibiotic (20% of the starting amount) was added to the mixture approximately every 24 h. The mixture was stirred for six days and then purified by gel filtration using Sephadex G-10 as stationary phase and distilled water as eluent. The solvent was then evaporated in vacuum to obtain the corresponding pure product.

Further details of the synthesis, characterization and spectroscopic data for all the DeAns can be found in the Supplementary Data.

NMR experiments

1H NMR, 13C NMR and 1H,1H-NOESY experiments were performed on a BrukerAvance III 400 MHz NMR instrument. Chemical shifts (δ) are given in ppm relative to the residual solvent peak and coupling constants (J) in Hz. All monodimen- sional NMR spectra were recorded using D2O as solvent (with Na2CO3 to ensure a basic pD = 11) at room temperature. 1H NMR spectra were acquired with a spectral window of 14 ppm, an acquisition time of 3 s and a relaxation delay of 1 s. 13C NMR spectra were acquired with a spectral window of 240 ppm, an acquisition time of 0.7 s and a relaxation delay of 2 s. Bidimensional NMR spectra were recorded using deuterated PBS (pD = 7.4) as solvent at room temperature. NOESY 2D-NMR conjugate spectra were collected with 3605 Hz spectral windows in f1 and f2, a 0.5 s mixing time and 9.28 μs 1H 90° pulse width. Experiments were done with a 1 s relaxation delay and a 0.284 s acquisition time. Eight transients were averaged for each 256 × 2048 complex t1 increment.

Figure 1. Schematic description of (A) allergic patients involved in this study: benzylpenicillin-specific, cross-reactive to both benzylpenicillin and amoxicillin, and amoxicillin-specific; and (B) monohaptenic and bihaptenic dendrimeric antigens.

Molecular dynamics simulations

They were performed with the NAMD2 program28 as described in the Supplementary Data. Briefly, MDS utilized AMBER parameters for the description of PAMAM dendrimers,29,30 BPO and AXO. Initial dendrimer configurations were made using the Starmaker program which is part of Silico.31 Simulations were run at physiological pH (7.4) and 300 K. Non-bonded interactions were cutoff at 12 Å and switched from 10 Å. Time steps of 2 fs were taken with implementation of the SHAKE routine. Dendrimers were equilibrated for 2 ns and starting from these configurations, production runs of 16 ns trajectories were performed under an NPT ensemble. Radial density distribution from MDS results within this paper were created with VMD software.32

Selection of patients

Patients with a clinical history of an immediate allergic reaction to penicillin, with a positive skin test and in vitro detection of specific IgE antibodies were diagnosed following European Network of Drug Allergy (ENDA) guidelines.33 Depending on the patients response to drugs, they were further classified into three groups: benzylpenicillin reactors, which includes patients with a positive skin test for benzylpenicilloylpolylysine (PPL) and/or minor determinants mixture (MDM) and negative for amoxicillin; amoxicillin reactors, which includes patients with a positive skin test for amoxicillin, a negative skin test for determinants of benzylpenicillin (PPL or MDM) and tolerance to benzylpenicillin in a drug provocation test; and cross-reactors, which include patients with a positive skin test for both determinants of benzylpenicillin (PPL and/or MDM) and amoxicillin.

The institutional review board “Comisión de Ética y de Investigacióndel Hospital Regional Universitario Carlos Haya” approved the study that was carried out in accordance with the Declaration of Helsinki. Oral and written informed consent for all the diagnostic procedures was obtained from patients.

From patients with a confirmed diagnosis of an immediate reaction to penicillins, we selected six cases that showed high levels (greater than 7%) of IgE antibodies specific to the penicillin involved in the reaction measured by direct RAST. Data from the patients included in the study are given in Table S4 (Supplementary Data).

Radioimmunoassay for IgE determination Radioimmunoassays were performed by RAST as described26 using the following solid phases. Briefly, PLL (Sigma, St. Louis, MO) was conjugated to cyanogen bromide-activated cellulose disks,13 and then benzylpenicillin or amoxicillin was conjugated to the PLL coupled to the cellulose disks, yielding BPO-PLL or AXO-PLL cellulose disks, respectively. RAST to benzylpenicillin and amoxicil- lin was performed in duplicate by incubating 30 µL of patient serum with BPO-PLL or AXO-PLL cellulose disks for 3 h. After three washes, radiolabeled anti-IgE antibody (kindly provided by ALK-A- bello) was added and allowed to incubate overnight before washing to remove non-specifically bound anti-IgE. Radioactivity was measured using a gamma counter (Packard BioScience Company, Frankfurt, Germany) as counts per minute (cpm). Results were calculated as a percentage (% RAST) obtained from the mean of duplicate value for each condition divided by the mean of the maximum cpm and considered positive if they were higher than 2.5% of label uptake, which was the mean ± 2 SD of the negative control group.

RAST inhibition studies

The competition assays were performed as described previously,12 in duplicate using six selected cases that showed

Figure 2. NMR spectroscopy of DeAn conjugates. (A-F) Aromatic region of the 1H NMR spectrum corresponding to the complete series of DeAn conjugates.

(F) Schematic representation of a DeAn BPO residue and NOE effects. (G) Schematic representation of a DeAn AXO residue. (H-J) 2D-NOESY spectrum of DeAn conjugates. (H) Spectrum of G4-[BPO/AXO]1/1 after setting the threshold to an intensity at which crosspeaks between each hapten (BPO and AXO) and dendrimer chains become visible. Arrows indicate crosspeaks between protons corresponding to the hapten units (AXO—red arrows/BPO—green arrows) and dendrimer branches. (I) Spectrum of G4-[BPO]. Arrow indicates crosspeaks between protons corresponding to the benzyl side chain of BPO units and the dendrimer skeleton. (J) Spectrum of G4-[AXO]. No NOE effects were found between AXO units and the dendrimer skeleton.

high levels (%RAST greater than 7) of IgE antibodies specific to the penicillin involved in the reaction measured by direct RAST, employing BPO-PLL or AXO-PLL conjugates coupled to the solid phase. In the fluid phase the sera from selected patients were incubated with each DeAn conjugate at two concentrations, 1 and

0.1 mM. Assays were then followed as described above in the radioimmunoassay section. Results are expressed as inhibition percentages from the non-inhibited serum calculated according to the following formula:

%Inhibition ¼ %RAST non-inhibited−%RAST inhibited x 100

%RAST non-inhibited

Specific IgE recognition was considered when % of inhibition was greater than 50%.

Results

DeAn synthesis

In our efforts to exploit dendrimer properties for interaction with the immune system, a fourth-generation PAMAM dendri- mer, with 64 surface amino groups, was chosen as the carrier due to both its high multivalency and our previous findings that this generation is optimal for IgE detection.13,14 Thus, we prepared and characterized a series of G4-PAMAM dendrimers covalently modified in the periphery with BPO and AXO, the chemical structures responsible for the allergic response to the β-lactam antibiotics, benzylpenicillin and amoxicillin, respectively. These molecules were derived from the nucleophilic attack of free amine groups at the PAMAM dendrimer periphery (which act as emulators of free amine groups of, e.g., lysines, in natural sera proteins) to the exceptionally electrophilic β-lactam carbonyl

Table 1

Radius of gyration Rg, aspect ratios (Iz/Ix and Iz/Iy), asphericities (δ), radius of the solvent accessible surface area (RSASA) and partial solvent accessible area (PSASA) occupied by penicillin units of G4-[BPO], G4-[AXO] and G4-[BPO/AXO]1/1.

DeAn	Rg (Å)	Iz/Ix	Iz/Iy	δ	RSASA (Å)	PSASA (%)	
G4-[BPO]	21.90 ± 0.24	1.25 ± 0.05	1.17 ± 0.05	0.005 ± 0.002	32.88	83	
G4-[AXO]	22.03 ± 0.19	1.61 ± 0.08	1.07 ± 0.02	0.019 ± 0.004	32.44	85	
G4-[BPO/AXO]1/1 46[BPO]36[AXO]	22.18 ± 0.21	1.50 ± 0.05	1.13 ± 0.03	0.013 ± 0.002	32.63		

group. As a result, the β-lactam ring becomes opened with the resulting molecule covalently bound to the initial amine group through a new amide bond. DeAn chemical structures can be found in Figure 1, B and in the Supplementary Data.

A series of DeAns were prepared as two mono-epitope conjugates, “”G4-[BPO] and G4-[AXO], and three bi-epitope conjugates, G4-[BPO/AXO]3/2, G4-[BPO/AXO]1/1 and G4-[BPO/AXO]2/3.

The bi-epitope conjugates were synthesized by reacting the dendrimer with 3/2, 1/1 and 2/3 molar ratio mixtures of benzylpenicillin and amoxicillin, respectively.

1D nuclear magnetic resonance (NMR)

Structural determination of the DeAn series was performed by 1D (1H and carbon decoupled 13C) NMR analyses. The 1H NMR spectra of the two mono-epitope DeAns, G4-[BPO] and G4-[AXO], show a shift in the resonance of dendrimer methylene protons bonded to terminal amino groups from 2.70 to 3.22 ppm, indicating amide bond formation (Figures S1 and S2). The shift and separation of β-lactam proton (H5 and H6) peaks indicate the opening of this ring in BPO and AXO.15 Signals corresponding to the different penicilloyl units (BPO and AXO) only differ in the protons associated with the corresponding acyl side chains: the aromatic region displays a multiplet for the benzyl moiety of BPO and an AA'BB' system for the para-disubstitutedhydroxybenzylamino moiety of AXO (Figure 2, A and B). Analysis of the integrated areas of these separate signals permitted the number of BPO and AXO groups attached to bi-epitope DeAn conjugates to be quantified (Figure 2, C, D and E; also Figures S3, S4 and S5). We found that the use of strictly equimolar amounts of benzylpenicillin and amoxicillin during the reaction yielded a mixed conjugate containing a 1:1 ratio of BPO/AXO; otherwise, the use of different ratios (3:2 and 2:3) resulted in controlled ratios of BPO/AXO. These data show that the ratio of β-lactam-dendrimer coupling correlates closely with the proportion of β-lactams employed in the reaction. Thus, the composition of DeAn conjugates can be controlled by altering the stoichiometry of the reactant mixture.

13C NMR was very homogeneous for all DeAns with the

appearance of new signals between 39.0 and 40.0 ppm corroborating the amide linkage between the dendrimer and the hapten. This is consistent with the disappearance of peaks between 42.5 and 40.9 ppm corresponding to the bond between ethylene residues and the primary amino groups (see 13C NMR spectra in the Supplementary Data).

2D nuclear magnetic resonance

The conformation of DeAn conjugates in aqueous PBS media was studied by 2D NMR (1H,1H-NOESY). NOESY interactions between different protons provide information regarding their spatial locations relative to different parts of the dendrimer branch.34 This method allows us to infer the dominant orientation of peripheral BPO and AXO units in the dendrimer domain in aqueous solutions at pH 7.4 (Figure 2, F and G).

The G4-[AXO] spectrum (Figure 2, J) shows a pattern of

cross peaks (H3 ↔ CH3(β) plus H3 ↔ H6 and a small H5 ↔ H6) similar to G4-[BPO] (Figure 2, I),15 indicating that the stereochemistry of the AXO units derives from the original

amoxicillin, with configuration (5R,6R), and with a preferred anti conformation between H5 and H6.

A NOE interaction between the two thiazolidine ring methyl

groups (α andβ) was found in both mono-epitope conjugates. This contrasts with previous studies that did not find this type of interaction in BPO units conjugated to simple monoamines or second generation dendrimers.15This may indicate shorter dis- tances between the methyl groups of different penicilloyl (BPO and AXO) units, suggesting that G4-[BPO] and G4-[AXO] present a densely packed surface, which is in agreement with molecular simulations of the structure of unfunctionalized higher generation (G ≥ 4) PAMAM dendrimers.35,36

It is important to bear in mind that the dense-packing of these

fourth generation DeAn conjugates reflects the arrangement of BPO and AXO units when they are dissolved in PBS. Unlike G4-[BPO],15 the G4-[AXO] spectrum exhibits no significant NOE effect between the methylene protons of dendrimer chains and penicilloyl moieties (Figure 2, I and J). In fact, in order to observe aromatic AXO and dendrimer chain methylene (t, u, s) crosspeaks, the threshold has to be set to such a high intensity that aromatic protons also show crosspeaks with thiazolidine ring methyl groups (α, β) (Figure S2 in the Supplementary Data). This reveals that the p-hydroxybenzylamino AXO side chain can be placed either outwards, together with thiazolidine moieties, or inwards with the dendrimer skeleton. Higher intensities eventually show background noise and the resulting crosspeaks can be attributed to interactions between the entire AXO residue and the complete dendrimer branch (Figure S2). This does not provide any additional information, other than indicating a more outward disposition of AXO, in comparison to BPO units,15 in the mono-epitope conjugates.

2D-NOESY analysis of G4-[BPO/AXO]1/1 was useful in

allowing the conformation of both haptens in the bi-epitope dendrimer structure to be compared at the same contour threshold (Figures 2, H and S4). The resulting spectra are consistent with the fact that the NOE effect between the more peripheral dendrimer branch methylene protons and the benzyl side chain of BPO units was very intense compared to their weak interaction with the p-hydroxybenzylamino moiety of AXO units. These data suggest that AXO units are exposed to the exterior aqueous media, resulting in their carboxylate,

Figure 3. Density distribution of dendrimers (—) and their BPO (−− −) and AXO ( ) units with respect to the center of mass of DeAn conjugates.

amino and phenol groups facing outwards from the dendrimer skeleton toward the outer surface. This points toward to the almost complete exterior availability of AXO haptens for

recognition by IgE, while BPO haptens may become partially hidden, with benzyl moieties surrounded by dendrimer branches.

Molecular dynamic simulation (MDS)

The study of G4-[BPO], G4-[AXO] and G4-[BPO/AXO]1/1 by MDS can give us some data about size and shape of these DeAn conjugates. The molecular properties obtained are shown in Table 1.

The three DeAn conjugates are similar in size as quantified by the radius of gyration (Rg) and variations of the solvent accessible surface area (SASA). SASA variations were used to obtain an effective outer radius RSASA, which could be considered the effective macromolecular size (Figure S10). Aspect ratios and asphericity, which characterize the shape of dendrimers, increase slowly from the BPO- to AXO-DeAn epitope conjugates, resulting in an increase in the degree of anisotropy, with G4-[AXO] showing the highest deviation and most ellipsoid shape (Figure S11). The radial density distribution profiles of DeAn conjugates (Figure S12) exhibited maximums at small r values that decay monotonically at longer distances with a high plateau at the periphery, suggesting a dense dendrimer shell with a uniform and compact distribution on the outside.

Radial density profiles for BPO and AXO units are displayed in Figure 3, showing that BPO units are distributed throughout the macromolecule, while AXO units tend to be oriented toward the exterior, suggesting a higher degree of backfolding for BPO than for AXO units. In any case, the high values for the Partial Solvent Accessible Area (PSASA) occupied by these penicillin moieties (Table 1) imply that they are accessible on the surface of the three DeAn conjugates.

RAST-inhibition

The ability of β-lactam-specific IgEs to recognize DeAn conjugates was studied by competitive RAST inhibition immunoassays using sera from patients allergic to selected penicillins (Figure 4). At all DeAn concentrations, sera from patient 1 (allergic to benzylpenicillin) was strongly inhibited by G4-[BPO] and the three bi-epitope dendrimers, but was not inhibited by G4-[AXO] (Figure 4, A). We observed a concentration dependent inhibition of amoxicillin-specific sera (patients 2, 3 and 4, Figure 4, B, C and D) by both G4-[AXO] and the three bi-epitope dendrimers. No inhibition was observed with G4-[BPO]. Sera with cross-reactivity to both penicillins (patients 5 and 6, Figure 4, E, F, G and H) recognized all five DeAn conjugates. Inhibition was similar toward the G4-[AXO] conjugate and the three bi-epitope AXO-containing DeAn conjugates. However, inhibition of the AXO-containing conju- gates was at least 10% higher than for G4-[BPO] across the range of concentrations tested. No differences in IgE recognition level were found between the three bi-epitope dendrimers against any of the sera.

Discussion

The multicomponent nature of the conjugates presented here, the degree of confidence of their composition and spatial structure, and the ease, reliability and versatility of their synthesis, make the present methodology ideally suited for achieving well-defined bi-epitope conjugates in the form of DeAns.

Although the synthesis of some bifunctional dendrimer structures has been reported recently,37-41 to our knowledge the bidirectional biomolecular recognition behavior of dendrimers possessing two different epitopes in the context of immune system interactions has never been studied. 1H and 13C NMR were useful for confirming the functionalization of dendrimers with the related haptenic structures BPO and AXO (the two major determinants of the allergic responses to these drugs). The homogeneity of these spectra points toward an ordered structure of the isolated DeAn conjugates. Moreover, 1H NMR analysis provides information about the ratio of the different β-lactams attached to bi-epitope DeAn conjugates. It is noteworthy that even bi-epitope conjugates exhibit homogenous and harmonized spectra. We also showed how bifunctionality can be controlled through the intrinsic chemical reactivity of both antibiotics, which is related

to the electrophilic properties of β-lactam carbonyls.

The correlation between chemical structures and the way DeAn conjugates are recognized by IgEs from patients allergic to penicillins, permit us to gain insights into how antigens interact with the immune system. From the analysis of data presented in

Figure 4. RAST inhibition assays of different serum samples from patients allergic to penicillins. Every serum contained IgE antibodies directed either specifically to benzylpenicillin (patient 1), specifically to amoxicillin (patients 2, 3 and 4), or to both penicillins (patients 5 and 6). Disks covalently bound to penicilloyl-PLL conjugates were employed as a solid phase, using the penicillin specific for each patient. (A, E, G) PLL-BPO solid phase. (B-D, F, H) PLL-AXO solid phase. The different penicilloylated DeAn conjugates (G4-[BPO], G4-[AXO], G4-[BPO/AXO]3/2, G4-[BPO/AXO]1/1 and G4-[BPO/AXO]2/3) were used as fluid phase inhibitors at two concentrations (1 and 0.1 mM). Specific IgE recognition is considered with inhibition N 50%.

Figure 4, we can conclude that, as a general trend, IgEs recognize a DeAn conjugate if it contains the epitope to which the IgEs are directed, independently of the number of hapten units present in the DeAn. The specificity of the recognition process is confirmed since IgEs from patient 1 (Figure 4, A), allergic only to benzylpenicillin, recognized G4-[BPO], G4-[BPO/AXO]3/2, G4-[BPO/AXO]1/1 and G4-[BPO/AXO]2/3, but did not show any detectable recognition of the conjugate lacking BPO. Converse- ly, IgEs from patients only allergic to amoxicillin (Figure 4, B, C and D) recognized G4-[AXO], G4-[BPO/AXO]3/2, G4-[BPO/AXO]1/1 and G4-[BPO/AXO]2/3, but did not significantly recognize the conjugate lacking AXO units. These data from patients selectively allergic to only one of these β-lactams indicate that molecular recognition takes place mainly via the side chain structure, although it is unclear whether or not the β-lactam nuclear structure is also

involved in some way. Importantly, the presence of the unrecognized epitope in the same bi-epitope DeAn, did not interfere with epitope recognition by IgEs specific to BPO or AXO (Figure 4, A, B, C and D). Also highly significant is the fact that IgEs from patients allergic to both penicillins specifically recognized all the DeAn conjugates studied here. To fully understand the recognition data of the cross-responders it is necessary to take into account more parameters, as discussed below.

From NMR and MDS data, we propose that the BPO epitope side chains in DeAn conjugates are surrounded by dendrimer chains, while the side chains of AXO moieties are more exposed outside the dendrimer skeleton (Figure 3). This more enclosed arrangement for BPO units was higher in mono-epitope than in bi-epitope conjugates, and might imply a reduction in IgE accessibility and lowered molecular recognition. This is the first time in which differences in the spatial distribution and conformation of two hapten moieties have been described in a hapten–carrier conjugate. This fact can explain the trend in Figure 4, E-G for cross-reactive patients, whereby mono-epitope G4-[BPO] consistently showed lower levels of RAST inhibition than the other DeAn conjugates. At this point, extrapolating from this observation to natural conjugates with human carrier proteins would be speculative, but the chemical nature of the two side chains of these antibiotics supports the observations made here.

Two hypotheses may explain the nature of IgEs from patients cross-reactive to drugs as described in Figure 5: model I implies the coexistence of two selective IgE populations, while model II proposes the presence of a single IgE that recognizes both antibiotics. The possibility of coexisting β-lactam-specific IgE antibodies (Figure 5, A and B, model I) has been suggested but not yet clearly demonstrated.42 On the contrary, the cross-

Figure 5. Schematic representation of RAST inhibition assays for two hypotheses explaining cross-reactivity to benzylpenicillin and amoxicillin. Model I involves the presence of two coexisting antibodies specific for each antibiotic: (A) The solid phase is functionalized with PLL-BPO. IgE recognizes two DeAns, but does not recognize G4-[AXO]. (B) The solid phase is functionalized with PLL-AXO. IgE recognizes two DeAns, but does not recognize G4-[BPO]. Model II involves the existence of a single IgE that can recognize both antibiotics: (C) the solid phase is functionalized with PLL-BPO. IgE recognizes all DeAns. (D) The solid phase is functionalized with PLL-AXO. IgE recognizes all DeAns. A more detailed explanation can be found in Figure S13 in the Supplementary Data.

allergic patients selected for this study seem to possess a single IgE class with specificity for both β-lactams, as represented in Figure 5, C and D (model II), since positive inhibition was obtained with all evaluated DeAn conjugates and solid phases activated with both BPO and AXO haptens. Although it is important to stress that these results are derived from individual patients, these are nevertheless in agreement with our previous detailed cross-inhibition studies that did not detect co-existing IgE antibodies in the cases of

benzylpenicillin and amoxicillin.42 These data from cross-reactive allergic patients point to a molecular recognition process in which at least the common nuclear structure, the opened β-lactam residue, is involved. Whether the side chain structures participate in the molecular recognition in cross-reactive patients is still unknown, but our experimental data show less recognition of BPO versus AXO units in those G4-[BPO/AXO] conjugates, where the benzyl side chains might be less accessible to the antibody. This would seem to suggest that the whole structure participates in the recognition process.

In conclusion, the results described here demonstrate the effectiveness of chemical methodologies for obtaining synthetic DeAn conjugates and their value as tools for understanding interactions between immunoglobulins and haptens. Our results suggest that the way in which haptens are arranged and exposed on the antigen surface, as a result of interactions between hapten, carrier and media, can drive IgE recognition and, as a result, the allergic response. The crucial hapten arrangement of antigens is mainly determined by their own chemical structure. A major challenge for current diagnostic practices is to improve in vitro diagnosis in comparison to in vivo diagnosis. Combinations of various haptenic structures on the same carrier could provide an efficient way of evaluating cross-reactivity. This approach could provide information about the structural and chemical require- ments of antigen recognition and help develop new more

efficient approaches for the detection of drug allergy in vitro. Moreover, the bi-epitope DeAn conjugates described in this paper could represent the basis of a novel method for screening a wider proportion of allergic patients with a single test. The use of dendrimers for preparing the hapten–carrier conjugates has allowed achieving a well characterization and control of the structures that is impossible to get with the use of polydisperse polymers, PLL. In fact, our study of DeAn conjugates offers the first evidence suggesting that control of the tri-dimensional distribution of an antigen is an important aspect of the IgE-antigen recognition process. All these make us to consider the bi-epitope DeAns as potential candidates for improved β-lactam allergy diagnosis when including it in solid phases. This will enhance the IgE detection in patients who recognized both BP and AX and therefore useful for screening sera with different specificities. DeAn conjugates may serve as tools for the design of hapten–carrier conjugates that can act as effectors or inhibitors of the immune system and modulate its responses.

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Appendix A. Supplementary Data

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