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Research paper

IgE to penicillins with different specificities can be identified by a multiepitope macromolecule

Bihaptenic penicillin structures and IgE specificities

A. Ariza^a, C. Mayorga^a, M.I. Montañez^a, E. Perez-Inestrosa^{b,c}, A. Ruiz-Sánchez^{b,c}, R.M. Rodríguez-Guéant^d, T.D. Fernández^a, J.L. Guéant^d, M.J. Torres^{e,1}, M. Blanca^{d,e,*,1}

^a Research Laboratory for Allergic Diseases, Hospital Regional Universitario de Málaga – IBIMA, Plz. del Hospital Civil s/n, 29009 Malaga, Spain

^b Andalusian Centre for Nanomedicine and Biotechnology – BIONAND, C/Severo Ochoa 35, 29590, Campanillas, Malaga, Spain

^c Department of Organic Chemistry, Faculty of Sciences, University of Malaga, Campus de Teatinos s/n, 29071 Malaga, Spain

^d Laboratory of Cellular and Molecular Pathology in Nutrition, Faculty of Medicine, University of Nancy, 9 avenue de la Forêt de Haye, B.P. 184, 54505 Vandoeuvre-les-Nancy Cedex, France

^e Allergy Service, Carlos Haya Hospital, Plaza. Hospital Civil s/n, 29009 Málaga, Spain

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ABSTRACT

Quantitation of specific IgE by immunoassay is a recommended in vitro test for the diagnosis of immediate hypersensitivity reactions to betalactams (BLs), particularly when skin test results are negative. IgE antibodies that recognize the common nuclear structure of all BLs or the specific side chain structure can be mainly distinguished by immunoassays. The aim of this study was to develop an immunoassay system to detect IgE antibodies with different specificities. Cellulose discs conjugated with benzylpenicillin (BP), amoxicillin (AX) or both drugs, with poly-L-lysine (PLL) as carrier molecule, were used as solid phases in the radioallergosorbent test (RAST). Direct and inhibition radioimmunoassay studies were made to verify the structures recognized by serum IgE antibodies from penicillin-allergic patients. Our results indicated that the addition of both haptens did not decrease the capacity to capture IgE when serum specific to either BP or AX was used, at least in terms of sensitivity. In addition, the inclusion of two haptens improved significantly the levels of IgE detection in patients who recognized both BP and AX. Therefore, the use of a solid phase with a carrier molecule conjugated with two determinants (AX and BP) is helpful to recognize IgE antibodies against either of these determinants and is useful for screening sera with different specificities.

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1. Introduction

Hypersensitivity reactions to betalactams (BLs) are produced by specific immunological mechanisms that are mediated by IgE

in immediate responses (Torres and Blanca, 2010; Antunez et al., 2006a). Several penicillin determinants can be recognized by IgE, the best known of which is benzylpenicilloyl (BPO). BPO is generated from the binding of benzylpenicillin

Abbreviations: BL, betalactam; BPO, benzylpenicilloyl; BP, benzylpenicillin; MDM, minor determinants; AX, amoxicillin; PPL, penicilloyl poly-L-lysine; RAST, radioallergosorbent test; PLL, poly-L-lysine; AXO, amoxicilloyl; 6-APA, 6-aminopenicillanic acid; BA, butylamine; PA, phenylacetic acid; PHPG, para-hydroxyphenyl glycine; Clav, clavulanic acid; AMP, ampicillin.

* Corresponding author at: Allergy Service, pabellón 6, primera planta, Carlos Haya Hospital (Pabellón C), Plaza del Hospital Civil s/n, 29009 Malaga, Spain. Tel.: +34 951290346; fax: +34 951290302.

E-mail addresses: adriana.ariza@fundacionimabis.org (A. Ariza), mayorga.lina@gmail.com (C. Mayorga), maribelmv@uma.es (M.I. Montañez), inestrosa@uma.es (E. Perez-Inestrosa), ajruizs@uma.es (A. Ruiz-Sánchez), rosa-maria.gueant-rodriguez@univ-lorraine.fr (R.M. Rodríguez-Guéant), tahiadfd@gmail.com (T.D. Fernández), jean-louis.gueant@univ-lorraine.fr (J.L. Guéant), mjtorresj@gmail.com (M.J. Torres), mblancago@gmail.com (M. Blanca).

¹ Both authors contributed equally to this manuscript.

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(BP) to a carrier molecule that has not yet been identified, although human serum albumin is considered the main candidate (Dewdney, 1977). Other BP determinants have been defined, the so-called minor determinants (MDM), based on the skin response rather than on detailed immunochemical studies (Levine and Redmond, 1969).

Amoxicillin (AX) is the drug mostly involved in IgE-mediated hypersensitivity reactions (Blanca et al., 1988, 1990, 2002). Although there are small changes in the side chain structures, it nevertheless contributes to induce important differences in recognition (Blanca et al., 1991a, 1991b), since some subjects develop allergic reactions to AX but tolerate the administration of BP (Blanca et al., 1990; Silviu-Dan et al., 1993; Fontaine et al., 2007). The use of AX has been recommended in skin and/or in vitro testing together with major and minor BP determinants (Torres et al., 2003).

Cephalosporins are another group of BL from which side chain specific determinants are generated. In vivo and in vitro studies have demonstrated the induction of selective responses to cephalosporins with good tolerance to penicillins and even cephalosporins with different side chain structures (Montañez et al., 2011; Perez-Inestrosa et al., 2005; Romano et al., 2000, 2010; Gueant et al., 2006; Antunez et al., 2006b).

Both BP and AX determinants have to be used when evaluating immediate hypersensitivity reactions to BL because there are different patterns of IgE recognition. In fact, the inclusion of both determinants in diagnostic tests has increased their sensitivity from 68% and 41% for BP and AX, respectively to 74%, with no changes in their specificity (Blanca et al., 2001). The objective of this work was to include BP and AX determinants together on a solid phase in order to detect IgE antibodies with different specificities.

2. Methods

2.1. Patient selection

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 included in the study. Two clinical categories were established: anaphylaxis and urticaria. Anaphylaxis was considered to be the presence of several of the following symptoms: pruritus on palms or soles, later becoming generalized, generalized erythema, urticaria, dyspnoea, difficulty in speaking or swallowing and/or tachycardia, accompanied or not by hypotension and/or loss of consciousness. Urticaria was defined as rapidly evolving and transient pruriginous wheals occurring at different body sites.

Patients were classified into three groups: Group A, included patients with a positive skin test to AX (prick or intradermal test), with a negative skin test to major and minor determinants of BP and good tolerance to BP in a drug provocation test; Group B, included patients with a skin test positive to penicilloyl poly-L-lysine (PPL) and/or MDM and negative to AX; Group C, included patients with a skin test positive to PPL and/or MDM and AX determinants. Nine subjects with confirmed good tolerance to BP and AX were included as negative controls (Group D). The study was conducted according to the principles of the Declaration of Helsinki and approved by the hospital Ethics Committee. Informed consent for the diagnostic procedures was obtained from all the participants in the study.

2.2. Skin test

Skin testing was carried out as previously described (Torres et al., 2001), using 0.02 mL of solution prepared daily. The reagents were PPL (5×10^{-5} M), MDM (2×10^{-2} M) and AX (20 mg/mL) (Diater, Madrid, Spain for all). These were the maximum concentration used for each hapten. In all cases with anaphylaxis we started by prick testing and if negative intradermal test were performed at 1:10 of the maximal concentration recommended (Torres et al., 2003). In the skin prick tests, a wheal larger than 2 mm with a negative response to the control saline was considered positive. In the intradermal tests the wheal area was marked initially and 20 min after testing, and an increase in diameter greater than 3 mm was considered positive (Torres et al., 2001, 2003).

2.3. Specific IgE antibody determination

The radioallergosorbent test (RAST) for quantifying specific IgE was made as previously described (Blanca et al., 1991c). BP and AX at different concentrations (0, 1, 5, 10 and 20 mg/mL) were conjugated to poly-L-lysine (PLL) (Sigma, St Louis, MO, USA) previously bound to cyanogen bromide-activated cellulose discs. 25 discs were obtained from different combinations of hapten concentrations including 1 disc with PLL, 4 with amoxicilloyl (AXO) bound to PLL (AXO-PLL) and 4 with BPO-PLL, and 16 discs with BPO-AXO-PLL at different concentrations of both haptens.

RAST was made by incubating sera from patients in the solid phase for 3 h. Radiolabelled anti-IgE antibody (kindly provided by ALK-Abello Laboratory, Madrid, Spain) was then added and samples were incubated overnight. The radioactivity was then measured in a gamma counter (Packard BioScience Company, Frankfurt, Germany) as count per minute (cpm). Results were calculated as a percentage of the maximum of cpm (% RAST) and considered positive if they were higher than 2.5% of label uptake, which was the mean $\pm$ 2 SD of the negative control group.

2.4. Determination of IgE specificity and cross-reactivity

Sera from patients with RAST values higher than 10% were incubated with BP, AX and 6-aminopenicillanic acid (6-APA) coupled to butylamine (BA) (Sigma, St Louis, MO, USA) (BPO-BA, AXO-BA and 6-APA-BA) as well as with the side chain structure of BP (phenylacetic acid (PA)) and AX (para-hydroxyphenyl glycine (PHPG)) at 10-fold decreasing concentrations (100, 10 and 1 mM) for 18 h. Then, solid phase was added and the RAST procedure continued as described above. Results were expressed as percentage inhibition calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{(\% \text{ RAST non-inhibited} - \% \text{ RAST inhibited}) \times 100}{\% \text{ RAST non-inhibited}}$$

2.5. Preparation of monomeric conjugates

Monomeric conjugates of AX, BP and 6-APA with BA were prepared as described elsewhere (Moreno et al., 1995). BPO-BA conjugates were prepared by dissolving 1.03 g of BP in 1 mL

of water. Then, 1.4 mL of BA (14 M) was added and the mixture was shaken for 1 h. After the addition of 20 mL of water, 30 mL of HCl was added slowly and the resulting pellet was washed and filtered with water. The AXO–BA and 6–APA–BA conjugates were prepared by dissolving slowly 1.23 g of AX or 6–APA in a solution of BA (14 M). The solution was stirred for 1 h and lyophilized, and the resulting conjugates were purified by recrystallization.

2.6. Statistical studies

Comparative analysis of RAST values between related samples without a normal distribution was performed using the Mann–Whitney and Kruskal–Wallis tests. All reported p values represent two-tailed tests, with values <0.05 considered statistically significant.

3. Results

Twenty-seven patients who had been diagnosed with immediate hypersensitivity reactions to penicillins were finally

included in this study. The patients were classified into three groups according to skin test and in vitro test positivity (Table 1). Patients with positive skin tests to AX and in vitro detection of specific IgE to AX but who tolerated the administration of BP (data not shown) were included in Group A. The drug involved was AX in 5 cases, AX–clavulanic acid (AX–Clav) in 2 patients and ampicillin (AMP) in 2 patients. Regarding clinical entities, 7 cases developed anaphylaxis and 2 cases had an urticaria reaction. This group was representative of AX side chain specific reactions where the responses induced by AMP were produced by the cross-reactivity between the side chains of AX and AMP. Group B consisted of patients who had positive RAST values only to PPL and positive skin tests to BP (4 patients were also positive to MDM). The drug involved was BP in 7 cases, AMP in 1 case and penicillin V in another case. Regarding clinical entities, 5 patients had anaphylaxis and 4 patients developed urticaria. Finally, patients with positive RAST values to BPO and AXO were included in Group C. Skin tests were positive to BP in all these patients and positive to MDM in 4 cases and/or to AX in 8 cases. The drug involved was AX in 5 cases, AX–Clav in 3 cases and AMP

Table 1

Clinical characteristics and specific IgE of patients from Group A (AX selective reactions), Group B (BP positive patients) and Group C (BP and AX positive patients).

Group	Pat.	Age	Sex	Culprit drug	Clinical entity	Skin tests			SlgE		SlgE
						BP	MDM	AX	BP	AX	BP/AX
A	1	42	M	AX	Anaphylaxis	–	–	+	0.7	14.2	13
	2	38	F	AX–Clav	Urticaria	–	–	+	1.2	23	20
	3	37	M	AMP	Anaphylaxis	–	–	+	1.7	29	27
	4	25	F	AX	Anaphylaxis	–	–	+	1.2	13.8	15
	5	22	F	AX	Anaphylaxis	–	–	+	1.3	19	17
	6	18	M	AX–Clav	Anaphylaxis	–	–	+	1.9	17	15.4
	7	23	F	AX	Urticaria	–	–	+	1.5	12	10.5
	8	50	F	AX	Anaphylaxis	–	–	+	1.2	39	37
	9	37	M	AMP	Anaphylaxis	–	–	+	0.9	12	10.5
	10	35	F	BP	Anaphylaxis	+	–	–	15.6	1.9	16.5
B	11	34	M	BP	Urticaria	+	–	–	13.9	1.7	14
	12	39	M	AMP	Urticaria	+	–	–	12.4	0.7	13.1
	13	55	M	BP	Anaphylaxis	+	+	–	16.5	1.3	15.4
	14	49	F	PV	Anaphylaxis	+	+	–	25	1.8	23.2
	15	59	F	BP	Anaphylaxis	+	+	–	13.8	1.4	11.5
	16	60	M	BP	Urticaria	+	–	–	12	1.3	9.7
	17	47	F	BP	Anaphylaxis	+	+	–	9.6	0.7	11.5
	18	71	F	BP	Urticaria	+	–	–	8.9	0.9	7.2
	19	53	F	AX–Clav	Anaphylaxis	+	–	+	34.7	40.2	38.41
	20	27	M	AX	Anaphylaxis	+	–	+	7.10	12.0	9.28
C	21	23	F	AX–Clav	Anaphylaxis	+	+	+	25.3	27.8	31.5
	22	35	M	AX	Anaphylaxis	+	–	+	14.5	5.8	15.9
	23	42	F	AX	Urticaria	+	+	+	12	18	23
	24	28	M	AX	Anaphylaxis	+	+	+	29	23	27
	25	63	F	AMP	Anaphylaxis	+	–	–	9	13.9	18.7
	26	58	F	AX–Clav	Urticaria	+	–	+	41	40	39.2
	27	46	F	AX	Urticaria	+	+	+	18.3	25.4	33.1
	28	18	F			–	–	–	0.54	.67	.81
	29	23	F			–	–	–	1.01	1.02	0.95
	30	51	M			–	–	–	0.35	0.65	0.85
D	31	36	M			–	–	–	0.82	0.80	0.96
	32	39	M			–	–	–	0.74	0.58	1.10
	33	44	M			–	–	–	2.50	3.40	4.60
	34	23	M			–	–	–	0.98	0.76	0.53
	35	25	F			–	–	–	0.63	1.45	2.10
	36	35	F			–	–	–	0.67	1.10	1.46

In all skin tests with negative results, the concentration of hapten used was BP 5×10^{-5} M, MDM 2×10^{-2} M, AX 20 mg/mL. All those positive results with (*) were obtained by prick tests and those with (**) were obtained with a dilution 1/10 from the maximum recommended concentration.

AX: amoxicillin; BP: benzylpenicillin; AMP: ampicillin; Clav: clavulanic acid; PV: penicillin V; BPO: benzylpenicilloyl-PLL; MDM: minor determinant mixture; slgE: specific IgE; BPO/AX: discs combined with BPO and AX.

in 1 case. Clinically, 3 patients developed anaphylaxis and 3 patients had urticaria.

We evaluated the IgE binding capacity of the different bihaptenic solid phases by RAST. When we analyzed serum from a Group A patient (patient 8 in Table 1), we found that the increase in RAST values was mainly related to the AX concentration and no important changes occurred with increasing concentrations of BP (Fig. 1A). In fact, the IgE level of detection decreased when discs with increasing concentrations of BP were used. However, results obtained using serum positive to just BP (patient 16 in Table 1) showed an increase in RAST values with increasing BP concentrations, with no differences related to AX concentrations (Fig. 1B). These data indicated that the inclusion of two haptens did not modify the label uptake capacity either for sera specific to BP or for sera specific to AX. The same experiments were made using sera from patients with specific IgE antibodies to AX and BP, with either intermediate (patient 23 in Table 1) or high values of label uptake (patient 26 in Table 1). Although at lower concentrations of AX increasing BP concentrations influenced IgE recognition, the highest IgE level was detected at the maximum AX concentration (20 mg/mL), even in the absence of BP (Fig. 1C). These results were more evident in sera with high values of label uptake where the IgE binding was high independently of the BP concentration on the disc, even at an AX concentration of 1 mg/mL (Fig. 1D). We detected no binding with control discs with PLL. All these results indicated that

20 mg/mL was the optimal concentration for binding AX and BP on the solid phases and this was therefore the concentration used in the subsequent studies.

Comparisons between RAST values using different solid phases with sera from Groups A, B, C and D are shown in Fig. 2. In Group A, there was a significant increase in RAST values obtained with BPO-AXO-PLL discs compared to BPO-PLL discs ($p = 0.017$), with no changes in BPO-AXO-PLL or AXO-PLL (Fig. 2A). In Group B, there was a significant increase in RAST values with BPO-AXO-PLL discs compared to AXO-PLL discs ($p = 0.008$) with a significant decrease in BPO-AXO-PLL and BPO-PLL discs ($p = 0.004$) (Fig. 2B). In Group C, there was a significant increase in the level of detection of IgE with BPO-AXO-PLL discs compared to BPO-PLL and AXO-PLL discs ($p = 0.028$ and $p = 0.015$, respectively) (Fig. 2C). No significant differences in RAST values were observed with the different discs in Group D.

We studied the pattern of recognition obtained with different solid phases by RAST inhibition assays. In Fig. 3A–B, the inhibition curves from a representative case of a patient with sera positive to just AX are shown (patient 1 in Table 1). The results obtained using AXO-PLL discs showed that the monomeric conjugate AXO-BA was the best inhibitor at the three concentrations tested, followed by PHPG; BPO-BA did not achieve 50% inhibition even at the maximum concentration and 6-APA-BA and PA did not produce any inhibition (Fig. 3A). Similar results were obtained when the experiments were

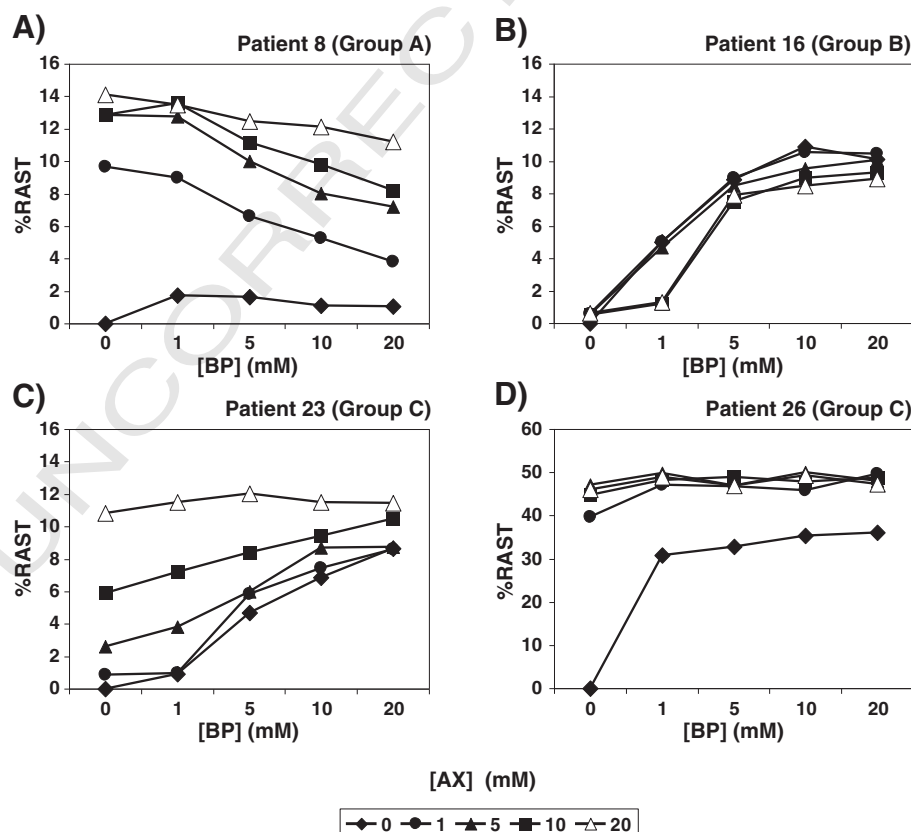


Fig. 1. Concentration effect curves using the 25 solid phases produced were used in RAST with different sera: (A) Group A positive to AX; (B) Group B positive to BP; (C) Group C positive to both BP and AX with a medium IgE level; (D) Group C positive to both BP and AX with a high IgE level.

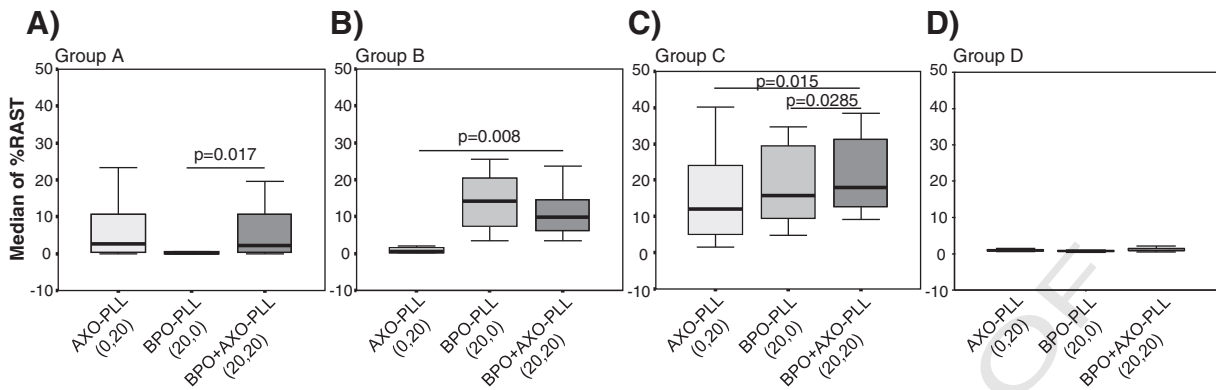


Fig. 2. Box plots represent the median percentage of RAST using sera from Groups A, B, C and D with different solid phases: BPO–PLL (20,0), AXO–PLL (0,20) and BPO–AXO–PLL (20,20). Differences with a non-parametric test for related samples were considered to be significant if $p < 0.05$.

made using BPO–AXO–PLL discs (Fig. 3B). In Fig. 3C–D, the inhibition curves from a representative case of a patient with sera positive to BP are shown (patient 14 in Table 1). Results obtained in the inhibition assay using BPO–PLL and BPO–AXO–PLL discs showed that BPO–BA was the best inhibitor, with 87% inhibition, followed by AXO–BA with 55% inhibition, both with the lower concentration and showing high cross-reactivity when BPO–PLL discs were used (Fig. 3C). When the same experiments were made using bihaptenic solid phases, a similar

pattern of inhibition was observed, with a higher inhibition obtained with BPO–BA and AXO–BA followed by 6-APA–BA, PA and PHPG (Fig. 3D).

RAST inhibition studies were also performed using sera with specific IgE antibodies to both AX and BP (patient 24 in Table 1). When AXO–PLL discs (0,20) were used, the best inhibitor was AXO–BA followed by BPO–BA, PA, PHPG and finally 6-APA–BA (Fig. 4A). However, when BPO–PLL discs (20,0) were used the best inhibitor was BPO–BA followed by

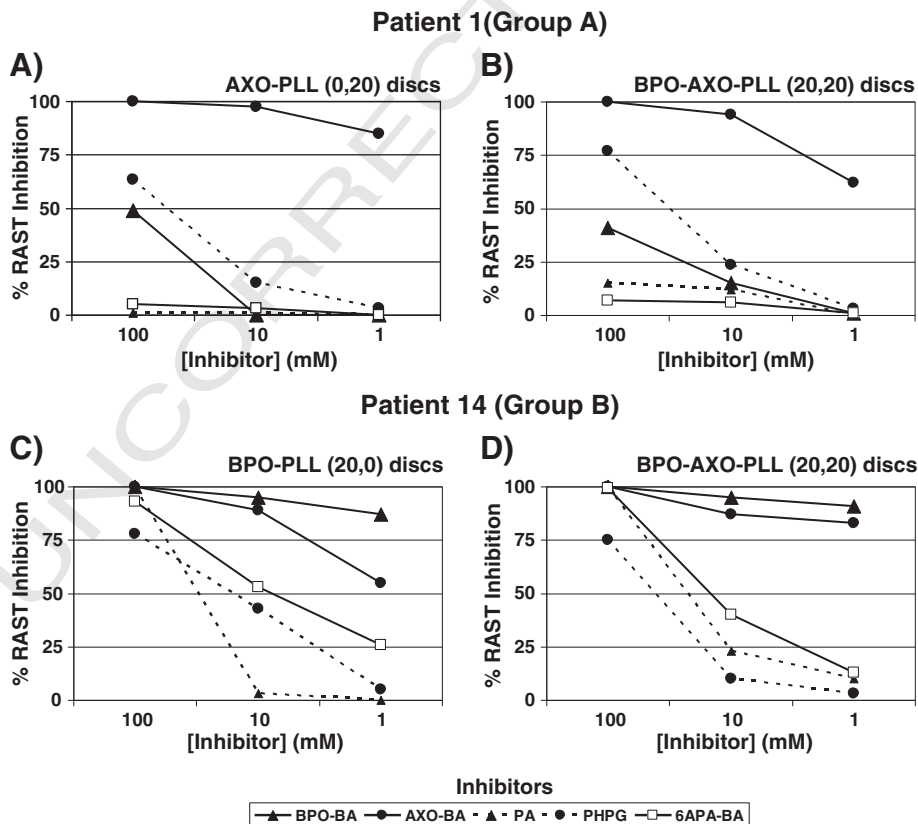


Fig. 3. RAST inhibition study results from a representative serum specific for AX used as a solid phase (A) AXO–PLL (0,20) and (B) BPO–AXO–PLL (20,20) and from a representative serum specific for BP used as a solid phase (C) BPO–PLL (20,0) and (D) BPO–AXO–PLL (20,20). The inhibitors used were the monomeric conjugates BPO–BA, AXO–BA, 6-APA–BA and the side chains of AX (PHPG) and PG (PA) at three different concentrations (100, 10 and 1 mM).

Patient 24 (Group C)

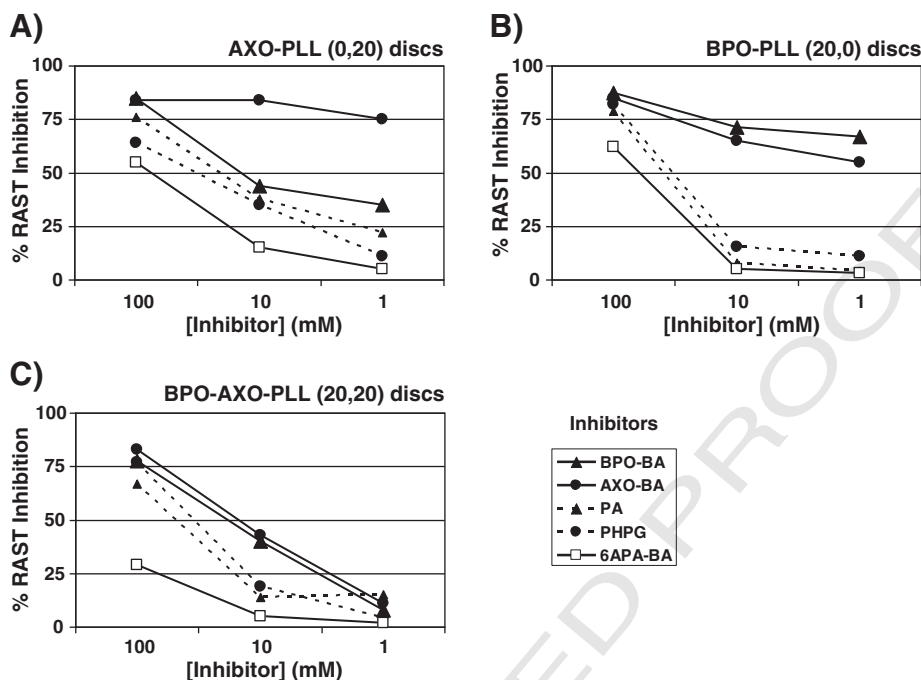


Fig. 4. RAST inhibition study results from a representative serum specific for both BP and AX used as solid phases (A) AXO-PLL (0,20), (B) BPO-PLL (20,0) and (C) BPO-AXO-PLL (20,20). The inhibitors used were the monomeric conjugates BPO-BA, AXO-BA, 6-APA-BA and the side chains of AX (PHPG) and PG (PA) at three different concentrations (100, 10 and 1 mM).

AXO-BA, with a low contribution of the other inhibitors (Fig. 4B). When BPO-AXO-PLL discs (20,20) were used, BPO-BA- and AXO-BA were the best inhibitors, indicating high cross-reactivity between the two structures, followed by their side chains, with no inhibition with 6-APA-BA (Fig. 4C).

4. Discussion

In vitro tests can be used for the diagnosis of immediate hypersensitivity reactions to BL, particularly when skin test results are negative, in order to avoid a drug provocation test, as well as in those cases where skin testing cannot be performed or in patients with a high risk of experiencing an anaphylactic reaction (Torres and Blanca, 2010; Torres et al., 2003). The in vitro tests recommended are the quantitation of specific IgE antibodies by immunoassays (Blanca et al., 2001; Torres et al., 2002) and, more recently, the basophil activation test (Torres et al., 2004; De Weck et al., 2009).

Data accumulated so far indicate that there are different patterns of BL recognition by IgE antibodies and, for skin testing, it is accepted that in addition to major and minor BP determinants, side chain AX determinants are also needed to achieve a diagnosis (Blanca et al., 2002; Torres et al., 2003). Two main patterns of recognition can be distinguished using immunoassays; a pattern in which the side chain structure mainly contributes to the antigenic determinant and another in which mainly the common structure of the BL is recognized (Moreno et al., 1995). Skin testing and in vitro studies reflect the clinical response in patients allergic to all the penicillins

and in other patients allergic to AX with good tolerance to the closely related BP (Blanca et al., 1988, 1990).

For the evaluation of the different patterns of IgE recognition, a detailed characterization of penicillin conjugates is necessary and important research has been carried out to identify the determinants generated by different BLs (Rodríguez-Bada et al., 2006) and the haptenization of human proteins (Meng et al., 2011; Ariza et al., 2012). In this work we aimed to develop an in vitro model capable of binding different penicillin determinants (BP and AX) using PLL as the carrier molecule (Blanca et al., 1992; Garcia et al., 1997).

Our hypothesis was that the inclusion of different penicillin molecules on the same solid phase would enable detection of antibodies with different specificities or even antibodies with possible coexisting sensitivities. Our results indicate that the addition of both haptens does not decrease the capacity to capture IgE antibodies when serum specific to either BP or AX is used, at least in terms of the sensitivity of any of the individual sera, while it increases the capacity to detect specific IgE antibodies, independently of the pattern of response of the patient. We were, in fact, able to detect all the allergic patients using a bihaptenic solid phase.

We confirmed the existence of different patterns of IgE recognition with an important role of the AX side chain structure in selective allergic patients to AX and recognition of BP with cross-reactivity between AX and 6-APA due to the importance of the common region of the molecule in the conformation of the antigenic determinant. These aspects have been previously demonstrated with serum IgE antibodies from selective allergic patients to BP or AX by several

approaches (Moreno et al., 1995). When serum samples from patients who recognized BP and AX were analyzed by immunoassay, we found different patterns of recognition depending on the hapten bound on the solid phase, which suggests the presence of IgE antibodies with different specificities and with a variable degree of cross-reactivity. However, IgE antibodies with both BP and AX specificity were detected using bihaptenic solid phases. Furthermore, the inclusion of two haptens on a solid phase significantly improved the RAST values obtained with sera from patients who recognized both BP and AX; therefore bihaptenic conjugates are more appropriate in the detection of IgE antibodies with different specificities.

5. Conclusions

In summary, we demonstrated in this work that two relevant haptens can be introduced in the same carrier molecule with the possibility of capturing IgE antibodies. In this way, the analysis of a large series of sera specific to either one or both haptens (BP or AX) enabled detection of a higher number of IgE antibodies than when only one of the specificities was considered. Although we used two common specificities, BP and AX, it is reasonable to suppose that other penicillin determinants or cephalosporins could also be used to analyze serum with unknown specificities. Further experiments are in progress to improve this model and seek the possibility of capturing IgE antibodies to BL antibiotics including cephalosporins.

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