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Role of minor determinants of amoxicillin in the diagnosis of immediate allergic reactions to amoxicillin

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Abstract

Background: Skin testing of subjects with immediate hypersensitivity to amoxicillin is performed using major and minor determinants of benzylpenicillin plus amoxicillin. However, sensitivity is not optimal, and other determinants need to be considered. We assessed the sensitivity of stable, well-characterized minor determinants of amoxicillin in subjects with immediate allergic reactions to amoxicillin to improve skin test sensitivity.

Methods: Amoxicillin, amoxicilloic acid, and diketopiperazine were prepared and characterized by reverse-phase HPLC, tested *in vivo* by skin testing and *in vitro* by basophil activation test and RAST inhibition assay.

Results: Patients with immediate hypersensitivity to amoxicillin were selected: Group A (n = 32), skin test positive just to amoxicillin; Group B (n = 19), skin test positive to benzylpenicillin determinants; Group C (n = 10), skin test negative and amoxicillin drug provocation test positive. In Group A, 27 subjects (81.8%) were skin test positive to amoxicillin, ten (30.3%) to amoxicilloic acid, two (6.1%) to diketopiperacine, and six (18.2%) negative. In Group B, nine (50%) were positive to amoxicillin, eight (42.1%) to amoxicilloic acid, none to diketopiperacine, and nine (50%) negative. In Group C, skin tests were negative. BAT was positive to amoxicillin in 26 patients (50.9%), to amoxicilloic acid in 15 (29.1%), and diketopiperazine in four (7.8%). RAST inhibition studies showed > 50% inhibition in all sera, with the highest concentration of amoxicillin and amoxicilloic acid.

Conclusions: The combination of minor determinants of amoxicillin, amoxicilloic acid, and diketopiperazine seems to be of no greater value than the use of amoxicillin alone. Further efforts are needed to find new structures to improve sensitivity in the diagnosis of immediate hypersensitivity to betalactams.

Immediate allergic reactions to penicillins usually appear within 1 h of drug intake and are the most frequent cause of drug reactions mediated by specific immunological mechanisms (1). These reactions have usually been diagnosed by skin testing using the so-called major and minor determinants of benzylpenicillin (2). The major determinant is formed by the conjugation of benzylpenicillin (BP) to the polylysine reagent, penicilloyl-polylysine (PPL) (3). Minor determinants are formed by BP, benzylpenicilloic and benzilpenilloic. The remaining metabolites identified from benzylpenicillin are not included because of their chemical instability (4).

Since the late 1980s, accumulating evidence has shown the side chain of amoxicillin (AX) to be the relevant part of the structure of the allergenic determinant (5, 6). Accordingly, the addition of AX to the panel of haptens for skin testing has been supported by different studies (1, 2, 6, 7), and it is currently recommended by the European Network for Drug

Abbreviations
BP, Benzylpenicillin; PPL, Penicilloyl-polylysine; ENDA, The European Network for Drug Allergy; AX, Amoxicillin; MDM, Minor determinant mixture; DPT, Drug provocation test; BAT, Basophil activation test; SI, stimulation index; RAST, Radioallergosorbent test; PLL, Polylysine.

Allergy (ENDA) for routine skin testing (8). In spite of this inclusion, skin test sensitivity in subjects with immediate allergic reactions to penicillins is not optimal, ranging from 50% to 70% (1, 2, 6, 7). This raises the question of whether the use of additional minor determinants of AX would improve the sensitivity, as was initially reported with BP (9–11).

The aim of this study, therefore, was to use well-characterized determinants of amoxicillin (8) that include amoxicillin itself, amoxicilloic acid and diketopiperazine. The purity of these determinants was assessed by HPLC, after which they were used *in vivo* by skin testing and *in vitro* by RAST inhibition and the basophil activation test to assess their value in subjects with immediate allergic reactions to amoxicillin.

Patients and methods

Amoxicillin minor determinants

The three amoxicillin minor determinants used in this study were AX, amoxicilloic acid, and diketopiperazine. The chemical structures are shown in Fig. 1. AX was obtained from Glaxo Smithkline Beecham (Madrid, Spain), amoxicilloic acid was prepared following the same approach as for benzylpenicilloic acid (7), and diketopiperazine was prepared according to Llins (12).

The purity of the compounds was analyzed by HPLC using a UV detector at 276 nm. In brief, samples of AX and amoxicilloic acid were dissolved in phosphate tampon pH 7 and phosphate tampon pH 7: methanol (7 : 3) for diketopiperazine to a final concentration of 2 mg/ml. Samples were applied to a HP-1090 HPLC equipped with a Kromasil 100C18 5.0 μm (250 × 3 mm) chromatographic column previously equilibrated with water/acetonitrile (95/5 containing 0.1% of trifluoroacetic acid, TFA). Samples were eluted with a linear gradient from 95/5 to 85/15 in water/acetonitrile (0.1% TFA) in 30 min. Injection volume: 1 μl; Flow: 1 ml/min.

Patients and controls

The study included patients from four Spanish hospitals who had been diagnosed within the previous year with an immediate allergic reaction to AX using the diagnostic procedure described in the ENDA protocol (2). This consisted basically of performing skin tests with PPL, MDM, BP, and AX, and if negative, a drug provocation test (DPT). The patients were classified into three groups: Group A, those with skin tests positive to AX and negative to PPL, MDM, and BP, and good tolerance to BP, and therefore considered to be selective reactors to AX; Group B, those with skin tests positive to at least one of the BP determinants (PPL, MDM, or BP), independently of the AX results and therefore considered to be allergic to the penicillin group; Group C, patients with negative skin tests to all determinants and a positive drug provocation test to AX. Two clinical categories were established: anaphylaxis and urticaria, as reported previously (2).

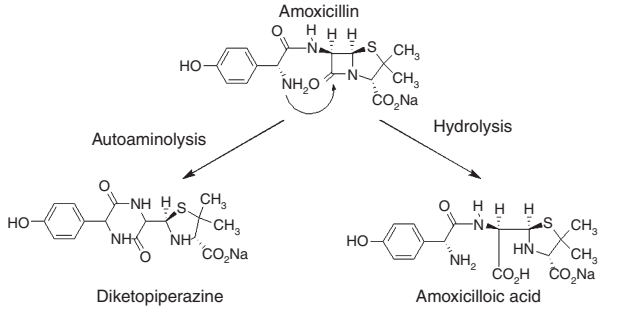

Figure 1 Chemical structure and route of formation of the amoxicillin minor determinants, amoxicilloic acid and diketopiperazine.

Thirty subjects with negative skin tests to PPL, MDM, BP, and AX and with good tolerance to AX were used as controls.

The study was approved by the relevant institutional review boards, and informed consent for the diagnostic procedures was obtained from the patients and controls.

Skin test

Skin testing was carried out as described (2), using 0.03 ml of solution prepared daily. The reagents used were amoxicillin, amoxicilloic acid and diketopiperazine at different concentrations (1, 5, and 10 mg/ml).

In the skin prick tests, a wheal larger than 3 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.

Basophil activation test by flow cytometry (BAT)

The BAT was performed as described, with a few modifications (13). The concentrations used for the different determinants (1.25 and 0.25 mg/ml) were chosen based on dose–response curves and cytotoxicity studies. The cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) by acquiring at least 1000 basophils per sample, and results were considered as positive when the stimulation index (SI), calculated as the ratio between the percentage of degranulated basophils with the different haptens and the negative control, was ≥ 2 to at least one of the dilutions mentioned above.

Radioallergosorbent test inhibition

As a first step, a radioallergosorbent test (RAST) using AX conjugated to polylysine (PLL) (Sigma, St Louis, MO, USA) in the solid phase was carried out as described (14). Results were calculated as a percentage of the maximum, and samples considered positive if higher than 2.5% of label uptake, which was the mean + 2 SD of the negative control group.

In those positive sera with RAST values to AX-PLL higher than 7%, cross-inhibition studies were carried out using AX-PLL in the solid phase, as reported (15), by incubating sera from patients with amoxicillin, amoxicilloic acid, and diketopiperazine at tenfold concentrations (100, 10, 1, 0.1, and 0.01 mM). The results were expressed as percentage inhibition with respect to the noninhibited serum. Compari-

Table 1 Clinical characteristics of the patients evaluated

Patient	Age	Reaction	Drug	Interval months
1A	40	Anaphylaxis	Amoxicillin-clavulanic	10
2A	49	Anaphylaxis	Amoxicillin-clavulanic	5
3A	64	Urticaria	Amoxicillin	9
4A	20	Urticaria	Amoxicillin	8
5A	38	Urticaria	Amoxicillin	2
6A	52	Anaphylaxis	Amoxicillin-clavulanic	14
7A	33	Urticaria	Amoxicillin	6
8A	39	Anaphylaxis	Amoxicillin-clavulanic	1
9A	38	Urticaria	Amoxicillin	36
10A	43	Anaphylaxis	Amoxicillin-clavulanic	36
11A	31	Anaphylaxis	Amoxicillin	36
12A	58	Anaphylaxis	Amoxicillin	8
13A	27	Anaphylaxis	Amoxicillin	3
14A	60	Anaphylaxis	Amoxicillin/ Amoxicillin-clavulanic	3
15A	43	Anaphylaxis	Amoxicillin-clavulanic/ Amoxicillin	2
16A	50	Anaphylaxis	Amoxicillin	24
17A	50	Anaphylaxis	Amoxicillin	3
18A	43	Anaphylaxis	Amoxicillin-clavulanic	5
19A	48	Anaphylaxis	Amoxicillin-clavulanic	70
20A	58	Anaphylaxis	Amoxicillin	6
21A	48	Anaphylaxis	Amoxicillin	36
22A	34	Anaphylaxis	Amoxicillin-clavulanic	5
23A	47	Anaphylaxis	Amoxicillin	84
24A	35	Anaphylaxis	Amoxicillin	4
25A	47	Anaphylaxis	Amoxicillin	12
26A	53	Anaphylaxis	Amoxicillin	2
27A	49	Anaphylaxis	Amoxicillin	5
28A	44	Anaphylaxis	Amoxicillin-clavulanic	6
29A	36	Anaphylaxis	Amoxicillin	5
30A	51	Anaphylaxis	Amoxicillin-clavulanic	24
31A	52	Anaphylaxis	Amoxicillin	8
32A	28	Anaphylaxis	Amoxicillin	24
33A	37	Anaphylaxis	Amoxicillin-clavulanic	4
34B	14	Urticaria	Amoxicillin	132
35B	25	Urticaria	Amoxicillin-clavulanic	60
36B	25	Urticaria	Amoxicillin	8
37B	65	Urticaria	Amoxicillin-clavulanic	12
38B	34	Urticaria	Amoxicillin	60
39B	74	Urticaria	Amoxicillin	11
40B	76	Anaphylaxis	Amoxicillin-clavulanic	18
41B	43	Anaphylaxis	Amoxicillin	2
42B	64	Anaphylaxis	Amoxicillin-clavulanic	30
43B	67	Anaphylaxis	Amoxicillin	16
44B	56	Anaphylaxis	Amoxicillin	204
45B	53	Anaphylaxis	Amoxicillin-clavulanic	36
46B	67	Urticaria	Amoxicillin	24
47B	47	Anaphylaxis	Amoxicillin-clavulanic	5
48B	68	Urticaria	Amoxicillin	120
49B	48	Anaphylaxis	Amoxicillin-clavulanic	24
50B	58	Anaphylaxis	Amoxicillin-clavulanic	5
51B	66	Urticaria	Amoxicillin	51
52C	15	Anaphylaxis	Amoxicillin	12
53C	31	Anaphylaxis	Amoxicillin	7
54C	57	Anaphylaxis	Amoxicillin-clavulanic	24
55C	44	Anaphylaxis	Amoxicillin	5
56C	21	Anaphylaxis	Amoxicillin-clavulanic	62
57C	61	Urticaria	Amoxicillin	18
58C	59	Urticaria	Amoxicillin-clavulanic	9
59C	23	Anaphylaxis	Amoxicillin	3
60C	38	Urticaria	Amoxicillin	27
61C	42	Anaphylaxis	Amoxicillin	10

son of the inhibition capacity of the reagents was made at 50% inhibition.

Results

The HPLC analysis showed that the AX contained 91% of AX, 5.4% of a nonidentified product, and traces of amoxicilloic acid. Amoxicilloic acid contained 88% of this product and 12% traces of a nonidentified product. Diketopiperazine contained 92% of this product and 8% of a nonidentified product. These three determinants were tested at different concentrations (10, 5, and 1 mg/ml) in the control group by prick and intradermal testing, with negative results.

The study included 61 patients (33 women) with immediate allergic reactions to AX (Table 1). Their mean age was 45.7 years (range: 14–76), and the mean time interval between the reaction and the study was 24.6 months (range: 1–204). Regarding the clinical symptoms, 44 had developed anaphylaxis, and 17 had developed urticaria. Six patients had had two episodes and the remaining patients had just one. The drugs involved in the reaction were AX ($n = 39$) and AX-clavulanic acid ($n = 24$).

The skin test results using classical determinants are given on the left of Table 2. Thirty-three subjects (Group A) were just positive to AX, 14 by prick, and 19 by intradermal tests. Nineteen subjects (Group B) were positive to PPL, MDM, or BP itself, independently of the AX results. In this group, eight were positive to PPL (two by prick), 14 to MDM (three by prick), seven to BP, and nine to AX (one by prick). The combination was PPL and MDM in 6; MDM in 4; MDM and BP in 3; BP in 3; PPL in 1; and PPL, MDM, and BP in 1. Ten patients (Group C) were skin test negative to all determinants and developed immediate symptoms after DPT with AX (data not shown).

Results of skin testing using AX determinants are given in the three columns assigned to new determinants in Table 2 (right). In Group A, 27 subjects (81.8%) were positive to AX (12 by prick at 10 mg/ml, seven intradermal at 10 mg/ml, and eight intradermal at 5 mg/ml), ten (30.3%) were positive to amoxicilloic acid (seven intradermal at 5 mg/ml and three intradermal at 1 mg/ml), three (9.1%) were positive to diketopiperacine (three intradermal at 10 mg/ml), and six (18.2%) were negative to all the determinants. All those who were positive to amoxicilloic acid and diketopiperacine were also positive to AX. In Group B, nine subjects (50%) were

Table 2 Skin test results with the classical and the new determinants in Group A and Group B

	Skin test						
	Classical determinants				New determinants		
PAT	PPL	MDM	BP	AX	AX	AXCILLOIC	DIKETO
1A	(–)	(–)	(–)	ID (+)	ID (+) (5 mg)	ID (+) (5 mg)	(–)
2A	(–)	(–)	(–)	P (+)	ID (+) (5 mg)	(–)	(–)
3A	(–)	(–)	(–)	ID (+)	ID (+) (5 mg)	ID (+) (5 mg)	(–)
4A	(–)	(–)	(–)	ID (+)	ID (+) (5 mg)	(–)	(–)
5A	(–)	(–)	(–)	ID (+)	(–)	(–)	(–)
6A	(–)	(–)	(–)	P (+)	(–)	(–)	(–)
7A	(–)	(–)	(–)	ID (+)	ID (+) (5 mg)	(–)	(–)
8A	(–)	(–)	(–)	ID (+)	ID (+) (5 mg)	(–)	(–)
9A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
10A	(–)	(–)	(–)	P (+)	ID (+) (5 mg)	(–)	(–)
11A	(–)	(–)	(–)	P (+)	(–)	(–)	(–)
12A	(–)	(–)	(–)	ID (+)	(–)	(–)	(–)
13A	(–)	(–)	(–)	ID (+)	ID (+) (5 mg)	(–)	(–)
14A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	(–)
15A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
16A	(–)	(–)	(–)	ID (+)	(–)	(–)	(–)
17A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
18A	(–)	(–)	(–)	ID (+)	P (+) (10 mg)	(–)	(–)
19A	(–)	(–)	(–)	ID (+)	P (+) (10 mg)	ID (+) (1 mg)	ID (+) (10 mg)
20A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	(–)
21A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	(–)
22A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	(–)	(–)
23A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
24A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
25A	(–)	(–)	(–)	ID (+)	(–)	(–)	(–)
26A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
27A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	ID (+) (10 mg)
28A	(–)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
29A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	(–)	(–)
30A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	(–)	(–)
31A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	(–)
32A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	(–)
33A	(–)	(–)	(–)	P (+)	P (+) (10 mg)	ID (+) (1 mg)	ID (+) (10 mg)
34B	ID (+)	ID (+)	(–)	(–)	(–)	(–)	(–)
35B	(–)	(–)	ID (+)	ID (+)	(–)	(–)	(–)
36B	P (+)	P (+)	(–)	(–)	ID (+) (5 mg)	ID (+) (10 mg)	(–)
37B	(–)	ID (+)	ID (+)	ID (+)	ID (+) (5 mg)	ID (+) (5 mg)	(–)
38B	ID (+)	ID (+)	(–)	ID (+)	ID (+) (10 mg)	ID (+) (10 mg)	(–)
39B	P (+)	P (+)	(–)	(–)	(–)	(–)	(–)
40B	(–)	ID (+)	ID (+)	(–)	(–)	(–)	(–)
41B	(–)	(–)	ID (+)	ID (+)	ID (+) (10 mg)	ID (+) (10 mg)	(–)
42B	ID (+)	(–)	(–)	ID (+)	ID (+) (10 mg)	(–)	(–)
43B	ID (+)	ID (+)	ID (+)	ID (+)	ID (+) (5 mg)	ID (+) (10 mg)	(–)
44B	(–)	ID (+)	(–)	ID (+)	ID (+) (5 mg)	ID (+) (10 mg)	(–)
45B	(–)	ID (+)	ID (+)	ID (+)	P (+) (10 mg)	ID(+) (10 mg)	(–)
46B	(–)	(–)	ID (+)	(–)	(–)	(–)	(–)
47B	(–)	ID (+)	(–)	(–)	(–)	(–)	(–)
48B	ID (+)	ID (+)	(–)	(–)	(–)	(–)	(–)
49B	(–)	P (+)	(–)	P (+)	P (+) (10 mg)	ID (+) (5 mg)	(–)
50B	(–)	ID (+)	(–)	(–)	(–)	(–)	(–)
51B	ID (+)	ID (+)	(–)	(–)	(–)	(–)	(–)

PPL, Penicilloyl-polylysine; MDM, Minor determinant mixture; BP, Benzylpenicillin; AX, Amoxicillin; AXCILLOIC, Amoxicilloic; DIKETO, Diketopiperazine; ID, Intradermal test; P, Prick test.

positive to AX (two by prick test at 10 mg/ml, three intradermal at 10 mg/ml, and four intradermal at 5 mg/ml), eight (44.4%) to amoxicilloic acid (six intradermal at 10 mg/ml and two intradermal at 5 mg/ml), none to diketopiperacine, and nine (50%) were negative to all the determinants. All those who were positive to amoxicilloic acid were also positive to AX. All those who were negative to the classical AX determinants (Group C) were also negative to the new minor AX determinants (data not shown). Patients from Groups A and B underwent further *in vitro* analysis. BAT was positive to AX in 26 patients (50.9%), to amoxicilloic acid in 15 (29.41%), and to diketopiperazine in four (7.8%). All cases that were positive to amoxicilloic acid or diketopiperazine were also positive to AX. There were no differences in BAT results between those patients with positive and negative skin tests with the new determinants in Groups A and B (data not shown). Figure 2 shows prototypes of the positive results, three from Group A and one from Group B.

RAST inhibition studies were performed in ten cases that had a sufficient RAST binding value (> 7%). Of these, seven had positive skin tests (1A, 2A, 18A, 21A, 37B, 41B, 43B) to the new determinants, and three were skin test negative (5A, 11A, and 39B). The remaining sera were not inhibited, because the RAST value was not high enough (< 7%) to do an accurate inhibition. RAST inhibition results were similar in the ten sera, independently of the skin test results. Moreover, there were no differences in the RAST inhibition results depending on the time interval between the reaction and the study. Figure 3 shows three cases from Group A and one from Group B. A parallel inhibition was obtained with AX and amoxicilloic acid in all cases in Group A, although inhibition was higher with AX, suggesting that this was the determinant mainly recognized by the IgE antibody. No significant inhibition was obtained with diketopiperazine. Concerning Group B, a very similar inhibition was obtained with AX and amoxicilloic acid and some inhibition with diketopiperazine, indicating that a common BL structure was recognized.

Discussion

Changes in the pattern of allergic reactions to betalactams are partly because of differences in the chemical structures involved. The most important consequence of this is that IgE now recognize different chemical structures, of which the side chain of AX is the most relevant and the best studied. As a consequence, the sensitivity of skin tests when PPL and MDM are used has changed. Studies in Spain have shown a decrease in sensitivity from 77.7% to 42.1% and 22.1% over the years (5, 6). Similar results have also been seen in other countries (7, 8, 16, 17). In the French study, for example, 46% of cases were positive to PPL and/or MDM, independently of the skin test response to the culprit drug, and 14% were exclusively positive to both haptens (7). Sensitivity has been improved by including skin testing with AX, and the diagnostic approach now recommended by the ENDA is to add commercially available penicillins to the skin testing panel (2, 8). However, skin test sensitivity is still not optimal, ranging from 50% to 70%, indicating that in the presence of a clear history of allergic reaction, up to 30% of cases can have a negative skin test.

Given that AX is the culprit drug involved, the search for new determinants generated from AX could help in the diagnosis of immediate allergic reactions to this antibiotic. We found that the three determinants examined here and at the

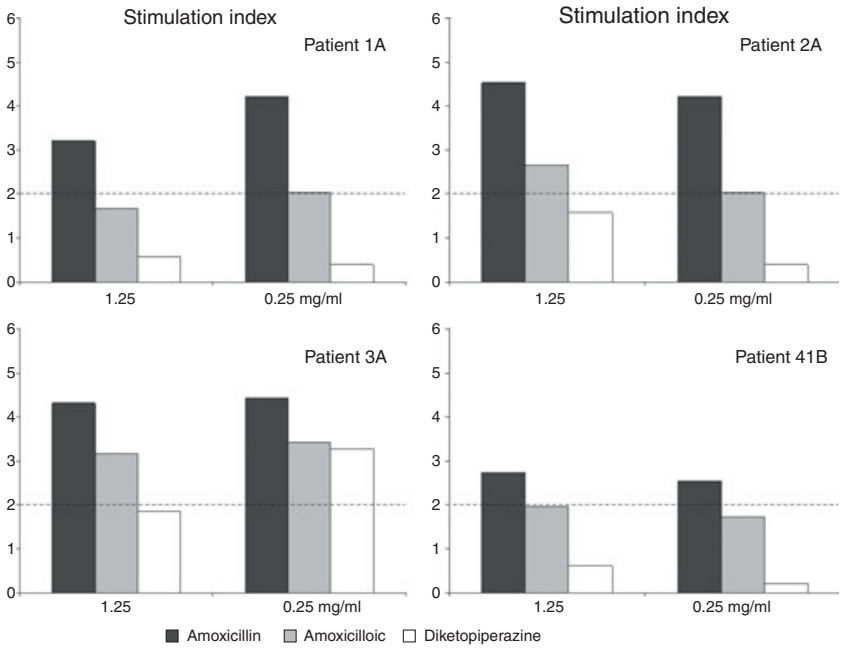

Figure 2 Basophil activation test using amoxicillin, amoxicilloic acid, and diketopiperazine, at two concentrations (1.25 and 0.25 mg/ml) in four cases, three from Group A and one from Group B.

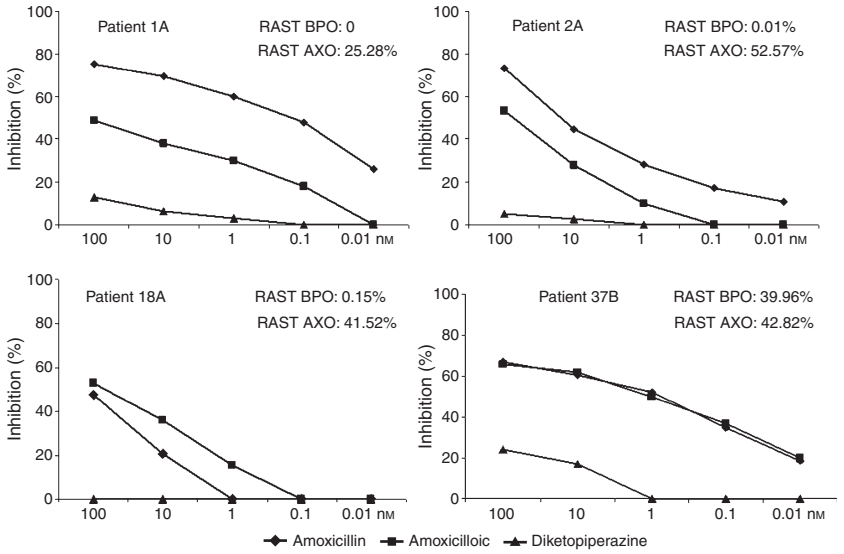

Figure 3 RAST inhibition assay using in the solid phase AX-PLL discs and in the fluid phase amoxicillin, amoxicilloic acid, and diketopiperazine, at tenfold concentrations in four cases, three from Group A and one from Group B. The upper right corner of each graph shows the RAST result using BPO-PLL and AX-PLL discs.

concentrations used (1, 5 and 10 mg/ml) were nonirritant in the control group and safe in the patient group. Analysis of the results in the three groups studied indicates that in those subjects with selective reactions to AX (Group A), 81.8% were positive to AX, 30.3% to amoxicilloic acid, and 6.1% to diketopiperazine. The reason why AX was lower at this point than in the initial evaluation was attributed to the varying time interval of the evaluation, because one of the inclusion criteria for this group was a compatible clinical history plus a positive skin test to AX. In those patients who also reacted to BP determinants (Group B), 50% were positive to AX, 44.4% were positive to amoxicilloic acid, and none to diketopiperazine. In both these groups, all the patients who were positive to amoxicilloic acid or diketopiperazine were also positive to AX, indicating that the use of these determinants did not, by themselves, contribute to improving sensitivity. This was supported by BAT and inhibition studies, as will be discussed later. Analysis of the results in Group C showed that, even though the inclusion criteria clearly required AX to be responsible for the reaction independently of a positive skin test, neither AX nor the other determinants tested induced any response.

These findings differ from those of previous reports using BP determinants in subjects allergic to penicillins, which detected that 5.12% of the patients were just positive to benzylpenicilloic acid (9), and 14% to 20% were positive to the mixture of benzylpenicilloic and benzylpenilloic acids (10, 11). However, although our Group B could be equivalent to the subjects included in these studies, the characteristics of our patients differed slightly, because, although they were also classified as allergic to penicillins, in our group, AX was the triggering agent and probably the sensitizer, although they were considered to be a cross-reacting group.

The fact that diketopiperazine is a ubiquitous substance present in nature and that it is also produced in humans (18), e.g. in thyroid hormones, might have influenced the low sensitization to this compound detected in our study. Moreover, we found no evidence of episodes of urticaria related to any known or unidentified agent in the three cases who were skin test positive to diketopiperazine.

To study the IgE recognition by these structures, we performed RAST inhibition studies and BAT assays. The RAST inhibition studies showed the best inhibition with AX and amoxicilloic acid, both greater than 50%. Diketopiperazine showed no inhibition in those cases from Group A, with very little inhibition detected in Group B. We detected no specific response to these determinants in the BAT assay.

We therefore conclude that in patients with immediate allergic reactions to amoxicillin, skin testing or *in vitro* testing with minor determinants of amoxicillin, amoxicilloic acid, and diketopiperazine does not improve the diagnosis. Further efforts are required to find new structures that can improve IgE recognition.

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Conflict of interest

The authors have no conflict of interest concerning the data reported in this study.

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