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Immunoglobulin E–mediated hypersensitivity to amoxicillin: *in vivo* and *in vitro* comparative studies between an injectable therapeutic compound and a new commercial compound

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Clinical & Experimental Allergy

Summary

Background Skin testing with amoxicillin (AX) is necessary to diagnose immediate hypersensitivity reactions to this β-lactam. A commercial AX (DIA-AX) has recently become available for skin testing.

Objective The aim of this study was to compare DIA-AX with the injectable form (INJ-AX) in patients who have well-demonstrated IgE-mediated hypersensitivity to AX.

Methods Chemical characterization using high-performance liquid chromatography of both DIA-AX and INJ-AX reagents was performed. Patients diagnosed with an immediate allergic reaction to AX and a positive skin test to INJ-AX ($N = 55$) were re-evaluated within 6 months by performing skin testing with INJ-AX and DIA-AX. Basophil activation test (BAT) and Radioallergosorbent test (RAST) inhibition assay using both reagents were performed in a selected group of patients.

Results The chemical analysis indicated that both DIA-AX and INJ-AX contained an AX compound with a purity above 95%. In the re-evaluation, 53 (96.4%) cases maintained skin test positivity to INJ-AX and were also positive to DIA-AX. Comparison of the papule area between the two reagents showed no significant differences between both reagents. BAT was performed in 30 samples and was positive to both compounds in 15 cases; no patient had a positive result to just one reagent. RAST inhibition studies using three individual cases and a pool of positive sera showed that the percentage inhibition detected with DIA-AX and INJ-AX was parallel and almost exactly the same.

Conclusions This study shows that DIA-AX is equivalent to INJ-AX in terms of skin test response, as well as with *in vitro* immunochemical and biological tests. DIA-AX can therefore be used in the diagnosis of immediate hypersensitivity reactions.

Keywords amoxicillin, basophils, IgE, immunochemical, *in vitro* tests, skin test
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Introduction

Penicillins are the drugs that are more frequently involved in IgE-mediated hypersensitivity reactions [1, 2], with a diagnosis based mainly on the performance of skin testing. As for many years benzylpenicillin (BP) was the most relevant drug involved in this type of reaction [3], the diagnosis has been based on the use of the major and minor determinants of BP [1–7]. The major determinant is formed by the conjugation of BP to the polylysine reagent, forming penicilloyl-polylysine (PPL) [8]. Minor determinants (MDM) are breakdown products of BP, sodium benzylpenicilloate and benzylpenilloic acid [9].

In the late 1980s, a number of studies showed that amoxicillin (AX) was the drug most frequently involved in immediate reactions and that this reagent was required for skin testing [10, 11]. From that time on, data on the relevance of AX have also been reported in the United States [12], in different European countries [5, 10–13] and again more recently in the United States [14]. Therefore, AX is currently recommended by the European Network for Drug Allergy (ENDA) in routine skin testing [1].

Skin testing with PPL, MDM and AX is a safe, cheap and reliable diagnostic tool. Other diagnostic methods include *in vitro* tests, which are more expensive and less sensitive, and the drug provocation test (DPT), which is not free of

risk but is the gold standard. An oral DPT is still necessary to validate a negative skin test [1, 2, 15]. In recent years, PPL and MDM were withdrawn from the market, although they have recently been substituted with another kit, which has an equivalent sensitivity and specificity [16–18] and is now used in many countries. In the United States, PPL is back on the market since June 2010 and MDM has never been commercially available.

Concerning skin testing with AX, because of the lack of commercialized diagnostic reagents, in many countries, the injectable therapeutic form is currently used, with many studies proving the validity of this approach for diagnosing immediate hypersensitivity to penicillins [7, 10–16, 19]. In the search for additional AX determinants, other chemical structures, such as amoxicilloic acid and diketopiperazine, have been tested, although with no improvement in diagnosis [20], thus confirming the need for the use of AX itself in skin tests for the evaluation of subjects with immediate hypersensitivity reactions to this β-lactam. Nevertheless, the soluble form of AX for the intravenous route has now been substituted in many countries by the combination of AX-clavulanic acid, leading to a situation where clinicians no longer have AX available for diagnostic purposes. The lack of AX reagents is therefore a matter of concern.

Recently, AX specifically designed for skin testing has been commercialized, with the manufacturer stating that it is equivalent to the injectable form of AX, although no comparative studies have been carried out as yet. Accordingly, we evaluated this newly available AX reagent in patients with well-demonstrated IgE-mediated hypersensitivity to AX and compared the results with those obtained with the injectable form that has been used for many years [1–3, 7, 10–14, 16, 19–21]. In addition to the chemical characterization, skin tests and *in vitro* tests including serum Radioallergosorbent test (RAST) inhibition studies and basophil activation tests (BATs) were performed with both reagents.

Patients and methods

Patients and controls

Patients ($N = 55$) diagnosed with an immediate allergic reaction to AX using the diagnostic procedure described in the ENDA protocol and skin test positive were evaluated [21]. A control group comprised 68 healthy volunteers who tolerated AX and were skin test negative to INJ-AX.

Patients were provided by two Spanish hospitals ($N = 20$ in Malaga and $N = 10$ in Madrid) and one Italian hospital ($N = 25$) over a 1-year period (January–December 2010). The inclusion criteria required a positive skin test to injectable AX (Glaxo Smithkline Beecham, Madrid, Spain) (INJ-AX) during the 6 previous months. Two clinical categories were established: anaphylaxis and urticaria. Three cases that met the entry criteria declined enrolment. None of the 55 cases have been reported on in previous publications. Another evaluation was performed, consisting of skin tests and *in vitro* studies using INJ-AX and the AX newly commercialized by Diater (Madrid, Spain) (DIA-AX).

The study was approved by the ethics and research committees of the three hospitals, and informed consent for the diagnostic procedures was obtained from the patients and controls.

DIA-AX preparation and stability

DIA-AX is obtained from sodium AX raw material (Sandoz, Les Franqueses del Valles, Spain), with an active substance purity of 95.2%, and total known and unknown impurities below 2.2%. This AX is dissolved and processed for its dosage at 20 mg/mL per vial. The subsequent lyophilization process yields a dry white powder. The vial containing the resulting product is closed and sealed under vacuum and with a relative humidity of 5%, until its reconstitution for skin tests.

The stability of the product (DIA-AX) stored in the vial at temperatures below 25 °C is 1 year. After its reconstitution in a physiologic pH aqueous solution, it has to be stored at 4 °C and its stability lasts 1 day. Therefore, it is recommended to use freshly prepared solutions.

Chemical characterization of the amoxicillin reagents

The purity of DIA-AX was analysed and compared with that of INJ-AX by reverse-phase HPLC (high-performance liquid chromatography) using a UV detector at 254 nm. The samples (DIA-AX and INJ-AX) and amoxicillin trihydrate (chemical reference substance) were dissolved in a mixture of (1/99 v/v) acetonitrile/0.05 M potassium dihydrogen phosphate adjusted to pH 5.0 with a dilute sodium hydroxide solution to a final concentration of 1.7 mg/mL. Then, 50 µL of each sample were injected into a Waters HPLC equipped with a Symmetry C18 5 µm (250–4.6 mm) chromatographic column. Samples were eluted with acetonitrile/0.05 M potassium dihydrogen phosphate, pH 5.0, starting with 2.5% acetonitrile isocratic elution for 5.20 min and using a linear 2.5–20% acetonitrile gradient for 22 min. The flow rate was 1 mL/min.

Skin tests

Skin prick and intradermal tests were carried out as described previously [21], using 0.02–0.03 mL of solution prepared daily. The reagents were INJ-AX and DIA-AX, at a concentration of 2 mg/mL and, if negative, at 20 mg/mL. These AX consist of the sodium salt form that can be easily dissolved in a physiologic pH aqueous solution, although the acid form of AX is slightly soluble in water

and presents a U-shaped solubility curve as a function of pH [22], being only of 4 mg/mL at pH 7. When dissolving INJ-AX and DIA-AX in water (pH 7.4) using 20 mg/mL, the resulting solution reaches pH 7.7. In the first evaluation, PPL (5×10^{-5} M) and MDM (2×10^{-2} M) (Diater) were used. In skin prick testing, a weal larger than 3 mm surrounded by erythema, with a negative response to the control saline, was considered positive. In intradermal tests, the weal area was marked initially and 20 min after testing, and an increase in diameter >3 mm surrounded by erythema was considered positive. In an attempt to avoid systemic symptoms, skin testing with both reagents was performed at a 4-h interval.

Basophil activation test by flow cytometry

The BAT was performed as described [23], with a few modifications using INJ-AX and DIA-AX. The concentrations used for both determinants (1.25 and 0.25 mg/mL) were chosen based on dose–response curves and cytotoxicity studies. The cells were analysed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) by acquiring at least 1000 basophils per sample, and the 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.

Radio allergosorbent test Inhibition

This was carried out as reported [24] by incubating 30 μL of sera from patients allergic to AX (with RAST values higher than 7% label uptake) with INJ-AX and DIA-AX, at 200, 100, 50, 10, 5 and 1 mM in the fluid phase for 18 h at room temperature. We then added a cellulose disc bound with AX conjugated to poly-L-Lysine (AXO-PLL) (Sigma, St Louis, MO, USA) as the solid phase and incubated it for 3 h. After washing, radiolabelled anti-IgE antibody (kindly provided by ALK-Abello, Madrid, Spain) was added and incubated overnight. The discs were then washed and their radioactivity was measured in a gamma counter (Cobra II auto-gamma, Packard BioScience Company, Frankfurt, Germany). The results were expressed as percentage of inhibition with respect to the non-inhibited serum. Comparison of the inhibition capacity of the reagents was made at 50% inhibition.

Statistical studies

The coefficient of variation for each measurement for the RAST inhibition assays was assessed with a pool of sera in order to establish the minimum variation accepted as different cross-reactivity. Values higher than 15% were considered as different and the coefficient of variation of each sample was within 10% of the variation. Comparison of the mean area and the SI between the two determinants was performed using non-parametric analysis (Mann–Whitney test). All reported *P*-values represent two-tailed tests, with values <0.05 considered statistically significant.

Results

The purity of the AX samples was carefully analysed by HPLC. Figure 1 shows that the HPLC chromatograms were very similar for the DIA-AX and INJ-AX samples, revealing a main peak at a retention time of 7.8 min. These analyses showed that DIA-AX contained 95% AX and traces of non-identified products, whereas INJ-AX contained 99.6% AX and traces of non-identified products.

We evaluated a total of 55 patients, 22 men and 33 women, with histories of immediate reactions to AX and positive skin tests to INJ-AX carried out in the 6 months before this study. The clinical characteristics are shown in Table 1. The mean age at the time of study evaluation was 46.96 years (range 13–76) and the mean time interval was 117.31 days (range 7–690 days) between the reaction and the first skin test evaluation and 142.85 days (range 30–720 days) between the reaction and the second evaluation. Twenty-two patients had experienced urticarial reactions and 33 anaphylaxis. AX was the drug responsible in 16 patients and AX combined with clavulanic acid in 39, in all cases administered by the oral route. In the

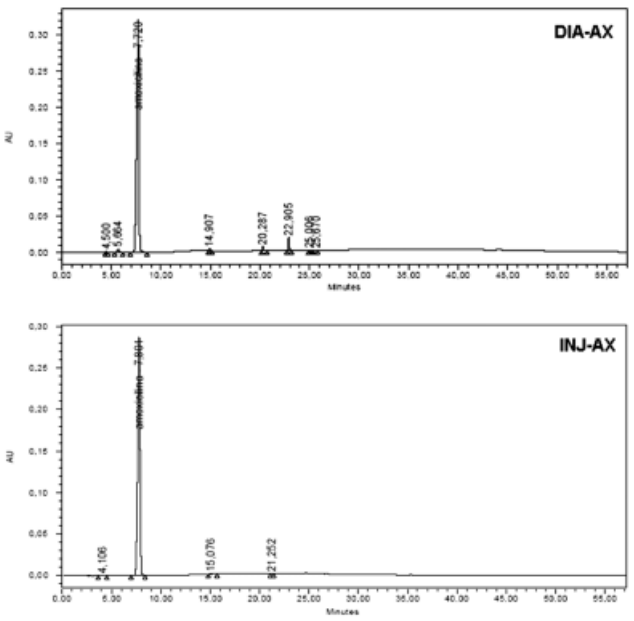

Fig. 1. HPLC chromatogram recorded at 254 nm of the AX released by Diater (DIA-AX) and the injectable AX (INJ-AX). HPLC, high-performance liquid chromatography; AX, amoxicillin; INJ-AX, injectable AX; DIA-AX, AX from Diater.

Table 1. Clinical characteristics of the group of patients evaluated and skin test (ST) results using injectable AX (INJ-AX) and AX from Diater (DIA-AX)

ID	Sex	Age	Reaction	Culprit drug	INTER-1 (days)	ST INJ-AX-1	INTER-2 (days)	ST INJ-AX-2	ST DIA-AX-2
1	F	40	Urticaria	Amox	365	P20+(4×4 mm)	385	P20+(3×4 mm)	P20+(3×4 mm)
2	M	61	Urticaria	Amox	30	P20+(4×5 mm)	60	P20+(4×5 mm)	P20+(6×7 mm)
3	F	32	Anaphylaxis	Amox–Clav	15	ID20+(8×8mm)	60	ID20+(8×7 mm)	ID20+(12×9 mm)
4	F	45	Urticaria	Amox	255	P20+(6×6mm)	320	P20+(6×5 mm)	P20+(9×7 mm)
5	M	24	Urticaria	Amox–Clav	109	ID20+(5×4 mm)	135	ID20+(5×4 mm)	ID20+(5×5 mm)
6	M	54	Anaphylaxis	Amox–Clav	15	P20+(5×5 mm)	40	P20+(7×5 mm)	P2+(7×6 mm)
7	F	64	Urticaria	Amox–Clav	7	ID20+(5×5 mm)	30	ID20+(6×5 mm)	ID20+(6×7 mm)
8	F	55	Urticaria	Amox	41	ID20+(5×4 mm)	65	ID20+(5×4 mm)	ID20+(5×6 mm)
9	F	47	Urticaria	Amox–Clav	25	ID20+(6×5 mm)	45	ID20+(6×4 mm)	ID20+(7×5 mm)
10	M	39	Anaphylaxis	Amox–Clav	30	P20+(5×4 mm)	60	P20+(5×5 mm)	P20+(5×6 mm)
11	F	33	Anaphylaxis	Amox–Clav	30	ID20+(4×4 mm)	60	ID20+(3×4 mm)	ID20+(3×5 mm)
12	F	45	Anaphylaxis	Amox–Clav	340	ID2+(4×3 mm)	360	ID2+(4×3 mm)	P20+(3×4 mm)
13	F	42	Urticaria	Amox–Clav	300	ID20+(6×5 mm)	320	ID20+(6×4 mm)	ID20+(7×6 mm)
14	M	51	Urticaria	Amox–Clav	76	ID20+(3×4 mm)	110	ID20+(3×4 mm)	ID20+(4×5 mm)
15	M	38	Anaphylaxis	Amox–Clav	90	P20+(5×4 mm)	120	P20+(4×4 mm)	P20+(3×4 mm)
16	F	43	Urticaria	Amox–Clav	62	ID20+(3×2 mm)	75	ID20+(3×2 mm)	ID20+(4×3 mm)
17	F	47	Anaphylaxis	Amox	95	ID20+(4×5 mm)	125	ID20+(3×5 mm)	ID20+(5×4 mm)
18	F	60	Anaphylaxis	Amox–Clav	60	ID20+(6×5 mm)	77	ID20+(6×7 mm)	ID20+(6×9 mm)
19	M	64	Anaphylaxis	Amox–Clav	240	ID20+(5×4 mm)	250	ID20+(6×4 mm)	P20+(5×3 mm)
20	M	42	Anaphylaxis	Amox	97	ID20+(3×4 mm)	157	negative	negative
21	M	44	Anaphylaxis	Amox–Clav	360	ID20+(4×4 mm)	397	negative	negative
22	M	27	Urticaria	Amox	60	ID20+(3×3 mm)	85	ID20+(3×2 mm)	ID20+(3×3 mm)
23	F	39	Anaphylaxis	Amox	115	ID20+(4×5 mm)	125	ID20+(4×5 mm)	ID20+(3×5 mm)
24	F	51	Anaphylaxis	Amox–Clav	30	ID20+(4×4 mm)	60	ID20+(3×4 mm)	ID20+(3×3 mm)
25	M	64	Anaphylaxis	Amox–Clav	67	ID20+(3×5 mm)	90	ID20+(3×2 mm)	ID20+(4×3 mm)
26	M	32	Urticaria	Amox	120	ID20+(3×5 mm)	135	ID20+(4×5 mm)	ID20+(3×4 mm)
27	M	47	Anaphylaxis	Amox–Clav	30	P20+(3×4 mm)	60	P20+(3×4 mm)	P20+(3×4 mm)
28	F	28	Urticaria	Amox	43	P20+(3×4 mm)	60	P20+(5×4 mm)	P20+(6×4 mm)
29	F	55	Urticaria	Amox–Clav	95	ID20+(5×4 mm)	122	ID20+(5×3 mm)	ID20+(4×4 mm)
30	F	34	Anaphylaxis	Amox	340	ID20+(3×5 mm)	360	ID20+(3×5 mm)	ID20+(3×5 mm)
31	F	47	Anaphylaxis	Amox–Clav	30	P2+(6×8 mm)	60	P2+(7×8 mm)	P2+(7×10 mm)
32	F	51	Urticaria	Amox–Clav	240	ID2+(5×5 mm)	265	ID2+(5×5 mm)	ID2+(4 5 mm)
33	M	61	Anaphylaxis	Amox	690	P2+(4×4 mm)	720	P2+(4×4 mm)	P2+(4×4 mm)
34	M	76	Anaphylaxis	Amox	120	P2+(4×6 mm)	135	P2+(4×5 mm)	P2+(5×6 mm)
35	M	32	Urticaria	Amox	30	ID2+(5×6 mm)	60	ID2+(5×6 mm)	ID2+(5×7 mm)
36	F	53	Anaphylaxis	Amox	30	P2+(3×5 mm)	60	P2+(5×5 mm)	P2+(5×5 mm)
37	F	51	Urticaria	Amox	300	ID2+(4×4 mm)	323	ID2+(4×4 mm)	ID2+(4×4 mm)
38	F	67	Urticaria	Amox–Clav	150	ID20+(6×4 mm)	170	ID20+(3×4 mm)	ID20+(4×5 mm)
39	F	67	Anaphylaxis	Amox–Clav	30	P2+(6×6 mm)	60	P2+(6×8 mm)	P2+(5×6 mm)
40	F	54	Anaphylaxis	Amox–Clav	30	ID2+(6×7 mm)	60	ID2+(5×7 mm)	ID2+(5×5 mm)
41	M	32	Anaphylaxis	Amox–Clav	30	P2+(6×8 mm)	59	P2+(6×8 mm)	P2+(6×7 mm)
42	M	55	Anaphylaxis	Amox–Clav	60	P2+(7×98 mm)	82	P2+(7×9 mm)	P2+(7×7 mm)
43	F	18	Urticaria	Amox–Clav	120	P20+(4×4 mm)	135	P20+(3×4 mm)	P20+(4×5 mm)
44	M	13	Urticaria	Amox–Clav	60	P20+(5×6 mm)	75	P20+(5×6 mm)	P20+(5×6 mm)
45	F	67	Urticaria	Amox–Clav	30	P20+(5×7 mm)	60	P20+(5×7 mm)	P20+(5×7 mm)
46	M	24	Anaphylaxis	Amox–Clav	60	ID2+(6×5 mm)	85	ID2+(4×5 mm)	ID20+(5×6 mm)
47	F	51	Anaphylaxis	Amox–Clav	30	P2+(8×10 mm)	59	P2+(8×12 mm)	P2+(10×12 mm)
48	F	57	Anaphylaxis	Amox–Clav	30	P20+(4×6 mm)	60	P20+(4×6 mm)	ID2+(4×5 mm)
49	F	47	Anaphylaxis	Amox–Clav	120	P20+(5×6 mm)	135	P20+(5×6 mm)	ID2+(4×5 mm)
50	M	71	Anaphylaxis	Amox–Clav	30	ID2+(6×5 mm)	52	ID2+(4×5 mm)	ID20+(4×4 mm)
51	F	39	Anaphylaxis	Amox–Clav	300	P2+(4×5 mm)	320	P2+(4×5 mm)	P2+(4×5×mm)
52	M	50	Anaphylaxis	Amox–Clav	30	ID2+(7×6 mm)	59	ID2+(7×8 mm)	P20+(5×5 mm)
53	F	54	Anaphylaxis	Amox–Clav	30	P20+(5×5 mm)	55	P20+(5×5 mm)	P20+(6×6 mm)
54	F	65	Urticaria	Amox–Clav	300	ID2+(4×5 mm)	315	ID2+(4×5 mm)	ID2+(5×5 mm)
55	F	34	Anaphylaxis	Amox–Clav	30	P20+(4×5 mm)	45	P20+(4×5 mm)	P20+(5×9 mm)

M, male; F, female; Amox, amoxicillin; Amox–Clav, amoxicillin–clavulanic acid; INTER-1, interval in days between the reaction and the initial evaluation; ST INJ-AX-1, skin tests with INJ-AX at the initial evaluation; INTER-2, interval in days between the reaction and the second evaluation; ST INJ-AX-2, skin tests with INJ-AX at the second evaluation; ST DIA-AX-2, skin tests with DIA-AX at the second evaluation; P20+, prick test positive at 20 mg/mL; P2+, prick test positive at 2 mg/mL; ID20+, intradermal test positive at 20 mg/mL; ID2+, intradermal test positive at 2 mg/mL.

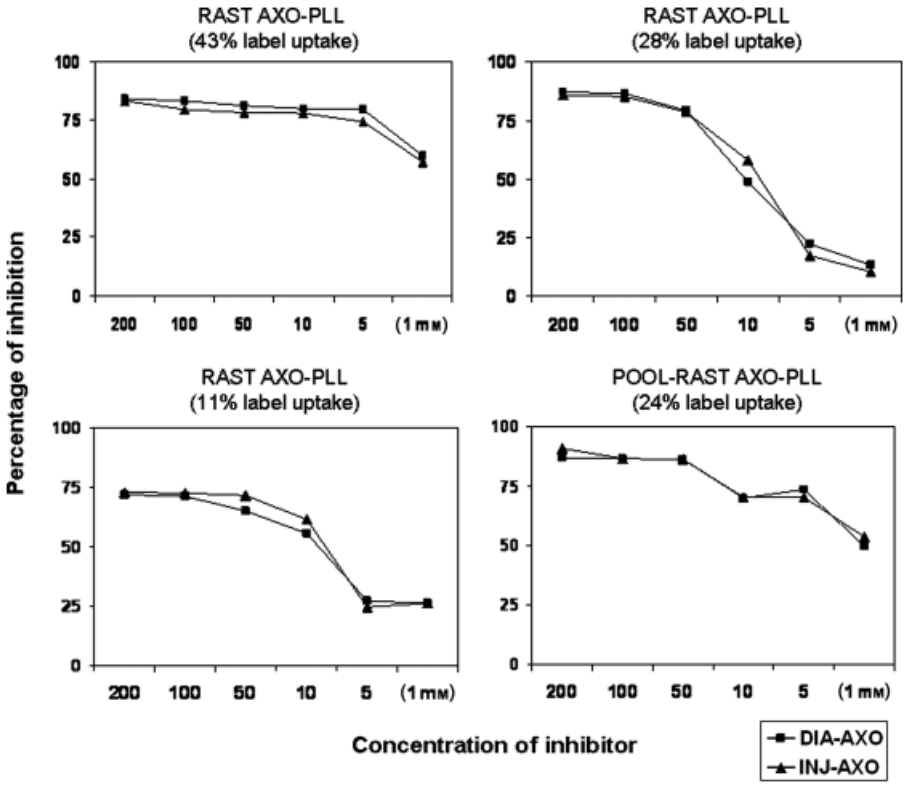

Fig. 2. RAST inhibition assays using AXO-PLL in the solid phase and injectable AX (INJ-AX) and AX from Diater (DIA-AX) in the fluid phase. Three cases are shown with different RAST results (low, medium and high) and with a pool of sera. AX, amoxicillin; AXO-PLL, AX conjugated to poly-L-lysine.

initial evaluation cases 2, 7, 13, 18, 34, 38, 47 and 50 ($N = 8$; 14.54%) were also positive to either PPL or MDM, or both determinants.

Patients were tested with both INJ-AX and DIA-AX (Table 1). Of the total experimental group, 53 (96.4%) subjects remained positive to INJ-AX and also displayed positive responses to DIA-AX, while 2 (3.6%) became negative to INJ-AX and were also negative to DIA-AX. With the INJ-AX, 24 (43.6%) subjects were positive to prick tests (15 at 20 mg/mL and 9 at 2 mg/mL) and 29 (52.7%) to intradermal tests (20 at 20 mg/mL and 9 at 2 mg/mL). With DIA-AX, 25 (45.4%) subjects were positive to prick tests (15 at 20 mg/mL and 10 at 2 mg/mL) and 28 (50.9%) to intradermal tests (21 at 20 mg/mL and 7 at 2 mg/mL). One patient (case 17) developed systemic symptoms with both AX determinants in intradermal testing.

Comparison between the two reagents showed almost identical results, except for cases 6, 12, 19 and 52, who presented a larger response to DIA-AX, and cases 46, 48, 49 and 50, who reacted more strong to INJ-AX (Table 1). Comparison of the area of the papule in mm^2 between the two reagents in those cases that were positive at the same concentration and skin test method ($N = 45$) showed no statistical differences, although these areas were slightly larger with DIA-AX (31 mm^2) than with INJ-AX (25.4 mm^2). Skin tests with both reagents were negative in all the control group subjects ($N = 68$).

We performed the BAT in 30 representative samples, and 15 cases (50%) were positive to both reagents. No patient showed a positive result to just one reagent. With INJ-AX, the mean SI was 1.56 at the concentration of 1.25 mg/mL and 1.24 at 0.25 mg/mL, and with DIA-AX, it was 1.71 at 1.25 mg/mL and 1.65 at 0.25 mg/mL. Comparison of the mean values of the two reagents at different concentrations showed no significant differences.

RAST to AXO-PLL was performed in all patients, and was positive in 19 (34.5%). RAST inhibition studies using three individual cases, with a low (case 1, 11% label uptake), medium (case 19, 28% label uptake) and a high (case 6, 43% label uptake) RAST value using AXO-PLL, as well as a pool of positive control sera are shown in Fig. 2. Using the sera with the highest RAST value (top left), more than 50% inhibition was obtained, even with the lowest molar concentration of AX. The sera with the intermediate RAST value (top right) showed a typical and parallel curve, with values of 50% inhibition with equivalent molar concentrations in both INJ-AX and DIA-AX. Very similar data were also seen with the lowest RAST value sera. The results with the pool of sera (bottom right) confirmed the data obtained with the individual sera, with patterns of recognition very similar to those obtained with the highest RAST value sera. Comparison between the reagents showed that the percentage inhibition detected was parallel and almost exactly the same for all the sera evaluated.

Discussion

AX, either alone or more recently combined with clavulanic acid, is now the most frequent penicillin prescribed and therefore the most frequently involved in immediate allergic reactions [1, 2]. In fact, in our study, AX combined with clavulanic was responsible for 71% of the reactions. Many studies have shown that AX is a mandatory determinant when evaluating patients with immediate hypersensitivity reactions to penicillins [1–3, 7, 10–14, 16, 19, 20]. The use of AX is also recommended by the ENDA [1, 21] and some American [16] groups for diagnosis, although the injectable form is no longer available in many countries. Moreover, we have to take into account that the current commercial anti-penicillin IgE *in vitro* test has a low sensitivity [25]. The availability of a new product released into the market for use in skin testing (DIA-AX), coupled with the great experience gained with INJ-AX, necessitate comparative studies in order to provide clinicians with adequate information. The present study shows that DIA-AX is equivalent to INJ-AX in terms of skin-test responses as well as with *in vitro* immunochemical and biological test results. The chemical analysis using HPLC showed similar chromatograms with only one main peak, which indicates that both samples, DIA-AX and INJ-AX, contained AX compound with a purity above 95%.

In skin testing, all positive cases were positive to both reagents. Although some discrepancies existed in the method or the concentration at which the patients reacted, there was no tendency for a better result with either reagent. Nor were any differences detected in the area of the papule between INJ-AX and DIA-AX. The specificity was 100%, with all the controls presenting a negative response to both reagents. As described previously [1, 21, 26], skin testing was really safe, with just one patient (case 17) developing mild systemic symptoms with both AX reagents in the intradermal test. Two subjects (3.6%) became negative to INJ-AX and were also negative to DIA-AX, indicating just a loss of sensitivity of skin testing when repeated after 6 months [27]. Moreover, no cases developed a delayed positive response in skin testing.

AX can induce selective responses in a significant proportion of cases, with a tendency of BP determinants to become less relevant over the years [10, 11]. Detailed immunochemical studies have shown that AX generates a unique determinant, specifically the side chain, which is recognized by IgE antibodies. This is particularly important because, in this case, patients can only be diagnosed by skin testing with AX itself, as other AX determinants have been shown not to be useful [20]. Further studies showed that this penicillin derivative was also useful in *in vitro* diagnosis, with both RAST and BAT [11, 23]. In the present study, the RAST inhibition assays confirmed that both reagents are equivalent, with a percentage inhibition that was nearly identical, independent of the direct RAST value (high, medium or low) and in a pool of sera. Moreover, as far as BAT is concerned, even though both reagents yielded the same results, the activation produced by DIA-AX appeared to be slightly higher than that obtained with the INJ-AX. Specificity with both reagents was exactly the same.

Therefore, these results indicate that the AX that has been recently commercialized for skin test diagnosis of hypersensitivity reactions to β-lactams could be equivalent to the injectable form of AX is terms of skin test responses as well as with *in vitro* immunochemical and biological test results for diagnosing immediate hypersensitivity reactions to AX. However, more studies are necessary.

Clinical relevance

The reagent DIA-AX is a safe and useful tool for diagnosing IgE-mediated hypersensitivity to AX.

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