Original Article

Basophil activation and sulfidoleukotriene production in patients with immediate allergy to betalactam antibiotics and negative skin tests

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Abstract. *Background:* New in vitro diagnostic methods for IgE-mediated drug allergic reactions, such as basophil activation test and antigen specific sulfidoleukotriene test, have proven their usefulness in patients with positive skin tests.
Objective: To assess the usefulness of basophil activation test and antigen specific sulfidoleukotriene test in the diagnosis of patients with IgE-mediated allergy to Betalactam antibiotics and negative skin tests.
Methods: The 23 patients included in the study underwent basophil activation test, antigen specific sulfidoleukotriene test and specific IgE. The patients were classified into three groups. *Group A:* patients with positive specific IgE. *Group B:* patients with a unique immediate reaction to Betalactams, negative specific IgE and positive oral provocation tests. And *Group C*: patients with at least two immediate reactions induced by Betalactams and negative specific IgE.
Results: The sensitivity/specificity of the different tests are: basophil activation test 39.1%/93.3%, antigen specific sulfidoleukotriene test 22.7%/83.3%, specific IgE 21.7%/86.7%. The joint use of the three tests allows diagnosis of 60.9% of the patients.
Conclusion: In vitro diagnostic tests, especially basophil activation test, are very important tools in the diagnosis of patients with IgE-mediated allergy to Betalactams and negative skin tests, avoiding performance of potentially dangerous oral provocation tests in a high percentage of cases.

Key words: Basophil activation test, Betalactams, Drug allergy, in vitro test, leukotriene, specific IgE, skin test.

Introduction

IgE-mediated reactions to betalactams are the most frequent drug allergic reactions mediated by a specific mechanism [1]. Their diagnosis is based on clinical history and performance of skin tests with betalactams. In patients with negative skin tests and suspected of allergic reaction to betalactams, we can perform antigen specific IgE as complementary diagnostic technique previous to the provocation test. Nevertheless, the sensitivity of specific IgE determination (CAP) is under 50% [2], even in patients with negative skin tests [3]. This is why the joint use of skin tests and antigen specific IgE determination does not allow detection of a high percentage of allergic patients, which could reach 30% [3,4]. These false negative results would require performance of provocation tests in a high percentage of patients with allergy to betactams with the risk of

potentially dangerous adverse reactions after administration of the drug. In addition to these limitations, skin tests are not free of side effects and can induce systemic reactions in 0.5% to 17% of the patients, especially in patients with anaphylaxis [4-7].

We intend to assess the usefulness of both in vitro tests, Basophil Activation Test (BAT) and Allergen-induced Sulfidoleukotriene Production (ASP), in a group of patients allergic to Betalactams (BL) with negative skin tests, since these patients are a diagnostic challenge in the clinical praxis.

The usefulness of BAT has been confirmed in the diagnosis of patients allergic to Betalactams with positive skin tests [2], as well as in the diagnosis of allergy to other drugs such as muscle relaxants [8], metamizol [9], omeprazol [10], etc. As far as ASP is concerned, this has also been observed to be a useful in vitro technique in immediate allergic reactions to these antibiotics [11,12], although the results are not as encouraging as in BAT; other authors, on the contrary, find little diagnostic utility with different drugs [13,14].

Patients and methods

Patients

Twenty-three patients (aged 45.7±13.9 years), 10 men and 13 women were included, who had presented anaphylaxis or urticaria/angioedema within 30 minutes after administration of Penicillin G (PENG), Ampicillin (AMP) or Amoxicillin (AMX) with negative skin tests, seen in our Departments in 2000-2003. All the patients underwent detailed clinical history, skin tests, specific IgE, BAT and ASP.

The 23 patients were divided into three groups: Group A: clinical history compatible with immediate type allergic reaction and positive specific IgE; Group B: clinical history compatible with immediate type allergic reaction, with negative specific IgE and positive drug provocation test. Group C: clinical history compatible with immediate type allergic reaction with negative specific IgE and two or more episodes or urticaria and/or anaphylaxis after betalactam administration. Due to the multiplicity of reactions induced by betalactams, once any other possible origin had been ruled out by the corresponding allergologic examinations (skin tests, in vitro specific IgE, etc), provocation was avoided due to ethical reasons.

Patients with a unique episode of urticaria or no life-threatening anaphylaxis who did not accept to undergo the challenge test were not included in the study. Also, patients with background of urticaria/angioedema or anaphylaxis of any other etiology were discarded.

In order to avoid bias, the four diagnostic tests were performed by different persons, none of whom knew the results of the other tests.

We included 30 non-allergic to BL adult subjects, 11 males and 19 females, with a mean age of 44.66±14.97 years as controls; eight of them were atopic. 30% of the subjects in this group came to consultation after having suffered immediate apparently allergic reactions to betalactams (urticaria, angioedema, exanthema) but in whom an allergic mechanism had been ruled out. All of them had negative skin tests to Betalactams, and their tolerance was checked by means of oral provocation tests and re-provocation one month later. Allergy to other drugs was discarded in all the control subjects.

All subjects included in the study were informed orally about the study and signed the corresponding informed consent. The study obtained approval from the Ethics Committee.

Skin tests

Skin tests were performed according to the usual techniques. Prick tests were performed using ALK Abelló (Madrid, Spain) lancets, followed by intradermal tests. Benzyl penicilloyl polylisine (PPL) (5 x 10^{-5} mol/L; Allergopharma, Hamburg, Germany), minor determinant mixture (MDM) (2 x10^{-2} mol/L, Allergopharma, Hamburg, Germany), sodium penicillin G (1000 UI/ml), AMP (20 mg/ml, Antibióticos SA, León, Spain), AMX (20 mg/ml, Beecham, Toledo, Spain) were used. Histamine hydrochloride (10 mg/ml) as positive control and NaCl 0.9% as negative control were used. Wheals 3 mm greater than the negative control for prick testing and 5 mm greater than the negative control for intradermal testing were considered positive.

Drug provocation test

A single blind, placebo-controlled provocation test was performed in all the cases. Amoxicillin was given orally at the following dosages: 10, 25, 50, 100, 250 and 500 mg at hour intervals.

In vitro tests

Flow cytometry stimulation test (FAST)

Blood was collected in 6 ml ACD tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France) and stored at 4ºC; the test was carried out within 24 hours of blood sampling. The tubes were centrifuged for 10 min at 4ºC, 550xg. Cells from the layer above the red blood cells were collected and centrifuged again for 10 min at 4ºC, 550xg. The supernatant was decanted and 500 µl of HEPES calcium buffer, called stimulation buffer (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, $CaCl_2$ 7 mM, $MgCl_2$ 3.5 mM, HSA 1 mg/ml, pH 7.4), containing IL3 (2ng/ml) and 10 µl of heparin (5000 UI /ml (ROVI,

Madrid, Spain) were added to each tube, mixed and gently shaken.

Final concentrations of betalactams used in the assay were the following: PEN G (final concentration 2 and 0.5 mg/mL), AMP (1.2 and 0.3 mg/mL), AMX (1.2 and 0.3 mg/mL), MDM (0.5 and 0.25 mg/mL) and PPL (25 and 12.5 μg/mL). As a positive control, we used a monoclonal anti-IgE antireceptor antibody (Bühlmann, Allschwil, Switzerland) at a final concentration of 1 μg/ml. In order to evaluate basal values without stimulation, we added merely 50 μl of stimulation buffer in another well.

The working dilutions of drugs used in the test were freshly prepared each day. Cellular viability studies were previously performed in cells from four healthy individuals that were incubated for six hours at 37ºC with the different betalactam antibiotics included in this study at both concentrations tested. The results were analysed by staining with Trypan blue. We observed a cellular viability over 93% in all cases. We placed 50 μl of the patient's cell suspension in each well and covered the plate with an adhesive plastic sheet. The plates were incubated at 37ºC for 40 min.

The reaction was then stopped by adding 100 μl of HEPES buffer ph 7.3 without calcium or magnesium but containing EDTA (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, EDTA 0.27 mM), denominated washing buffer. Soon afterwards, plates were centrifuged for 5 min at 4ºC, 1000xg.

The basophils from the pellet were double labelled by adding 20 μl of anti-CD63 PE-labelled antibody (Caltag Laboratories, Burlingame, USA), diluted 1:80 in tubes Cytometer Falcon and anti-IgE FITC-labelled antibody (Caltag Laboratories, Burlingame, USA) diluted 1:60 in washing buffer. After incubation for 30 min at 4ºC (protected from light exposure), we added 4 ml of erythrolytic reagent (Ortho-Mune lytic reagent, Ortho Diagnostic System Inc., San Fernando de Henares, Madrid) in each tube and left them at room temperature for 5 min. The cell lysis was stopped with 1 ml of washing buffer. After centrifuging another 5 min at 1000xg, the supernatant was decanted and 500 μl of washing buffer added to each tube, which were then gently shaken before flow cytometric analysis.

Flow cytometric analysis of surface markers was performed at 488 nm on a FACScan flow cytometer equipped with a 15 nW argon ion laser (Becton Dickinson) and analysed by CellQuest software.

On the histogram defined by forward scatter and side scatter, the initial cell gate was defined by a bit map around lymphocytes. The second gate was defined around cells showing high density anti-IgE-label, identifying them as basophils. We assessed at least 500 basophils in each assay. The other parameter analyzed on the identified basophils was CD63.

In order to rule out small unspecific activations, we also considered the results positive when the stimulation index (percentage of activated basophils with BL/ percentage of activated basophils in basal conditions) was equal to or greater than 2 at any of the two concentrations of betalactams and activation percentages equal to or greater than 5%.

Antigen specific sulfidoleukotriene production (ASP)

For allergen-induced sulfidoleukotriene determination, we followed the instructions of the assay's manufacturer (CAST-ELISA, Bühlmann Laboratories, Allschwil, Switzerland), using the same BL concentrations as for BAT. As in BAT, these concentrations were selected after being previously tested on a pool of control subjects in order to exclude concentrations that cause unspecific leukotriene release.

In order to rule out small unspecific leukotriene releases, releases over 100 pg/ml were considered significant. A stimulation index (percentage of leukotrienes released after contacting with the antigen/ percentage of leukotrienes released without stimulus) greater than 3 at any of the two concentrations of BL was considered positive.

In vitro specific IgE determination

In vitro specific IgE determinations were performed in all cases by the CAP technique (Pharmacia Uppsala, Sweden) with penicilloyl G, penicilloyl V, ampicillin and amoxicillin. All results higher than 0.35 kU/l were considered positive.

Statistical analysis

The data were analysed by means of the statistical program SPSS 10.0.

Sensitivity (true positive/total number of patients tested) and specificity (true negative/total number of healthy controls tested) values of the different diagnostic tests were obtained. We could not calculate the predictive values of the different tests, since there are not enough studies to allow assessment of real allergic subject prevalence among the patients with really suspected reactions but with negative skin tests.

Concordance between the different diagnostic tests was assessed by the Kappa test. Comparison between qualitative variables was done by Fisher´s exact test. All tests were two-sided. Values $p<0.05$ were considered statistically significant.

Results

Clinical data of the patients, together with their specific IgE, BAT and ASP results are shown in Table I. Thirteen patients had urticaria/angioedema and ten anaphylaxis. The median lapse of time from the clinical reaction to the moment of the study was 3 months.

Table 1. Characteristics of the patients and results of the *in vitro* tests.

Nº	SEX AGE	CLINICAL REACTION	BAT (% Antigen Activated Basophils/Stimulation index)	ASP (% Antigen Activated Basophils/Stimulation index)	Specific IgE (kU/L)	Culprit Drug	Months since last reaction (number reactions)
PATIENTS WITH POSITIVE CAP (Group A)							
1	Female 38	Anaphylaxis	MDM (0.5 mg/mL) 5.8/4.1	Negative	PG 0.45 PV 0.55 Am 0.45 AX 0.55	Am	9 (1)
2	Male 65	Urticaria	MDM (0.25mg/mL) 6.6/6.4	Negative	PG 0.55 PV 0.36	PG	2 (1)
3	Female 38	Urticaria	Negative	Negative	PG 0.49 PV 2.89 Am 0.58	Ax	27(1)
4	Male 69	Urticaria	Negative	Negative	PV 0.86 Am 1.43	PG Ax	12(2)
5	Male 72	Urticaria	MDM (0.5 mg/mL) 7.9/10	Negative	PG 0,39 PV 0,37	Ax	1(2)
PATIENTS WITH NEGATIVE CAP AND POSITIVE ORAL PROVOCATION TEST (Group B)							
6	Female 39	Anaphylaxis	Negative	Negative	Negative	Ax	1 (1)
7	Female 63	Anaphylaxis	Negative	Am (0.3 mg/mL)183/4.1 MDM (0.25 mg/mL) 200/4.4	Negative	Ax	1 (1)
8	Male 40	Urticaria	Negative	Negative	Negative	Ax	120 (1)
9	Female 38	Urticaria	Am (1.2 mg/mL) 7.6/3.5 PPL (12.5 µg/mL) 8.8/4 MDM (0.5mg/mL) 10.1/4.6 MDM (0.25 mg/mL) 6.6/3	Negative	Negative	Ax	3 (1)
10	Male 34	Urticaria	Negative	PPL (25 µg/mL) 130/3.3	Negative	Ax	1 (1)
11	Male 59	Urticaria	Negative	Negative	Negative	Ax	1 (1)
12	Female 38	Urticaria	Negative	Negative	Negative	Ax	2(1)
13	Female 45	Anaphylaxis	Negative	Negative	Negative	Ax	24 (1)
14	Male 39	Urticaria	Negative	Negative	Negative	Ax	4 (1)
15	Female 33	Urticaria	Negative	Negative	Negative	Ax	1 (1)
16	Female 46	Anaphylaxis	PG (2 mg/mL) 5.2/10.5	Negative	Negative	Ax	1 (1)
17	Female 22	Anaphylaxis	Ax (1.2 mg/mL) 7.6/12.7 Ax (0.3 mg/mL) 6.3/10.5	PG (2 mg/ml) 202/14,4 Ax (1.2 mg/mL) 160/11.4 Ax (0.3 mg/mL) 116/8.3	Negative	Ax	1 (1)
PATIENTS WITH NEGATIVE CAP AND VARIOUS EPISODES OF URTICARIA/ANAPHYLAXIS AFTER ADMINISTRATION OF AMOXICILLIN (Group C)							
18	Male 43	Urticaria	Negative	Negative	Negative	Ax	4 (2)
19	Female 45	Urticaria	Ax (0.3 mg/mL) 7.6/3.5	Negative	Negative	Ax	2 (3)
20	Female 22	Anaphylaxis	PPL (25 µg/mL) 6.2/3.9	Negative	Negative	Ax	6 (2)
21	Female 46	Anaphylaxis	Negative	N.D.	Negative	Ax	2 (2)
22	Male 55	Anaphylaxis	PPL (25 µg/mL) 23.2/92.8	PG (2 mg/mL) 218/6.1 Am (1.2 mg/mL) 110/3.1 Ax (1.2 mg/mL) 151/4.2 Ax (0.3 mg/mL) 162/4.5 PPL (25 µg/mL) 886/25.3 PPL (12.5 µg/mL) 235/6.5	Negative	Ax	120 (2)
23	Male 62	Anaphylaxis	Negative	Am (1.2 mg/mL) 216/216	Negative	Ax	1 (3)

BAT: Basophil activation test; ASP: Antigen specific sulfidoleukotriene production

Am: Ampicillin, Ax: Amoxicillin, PG: Penicillin G, PV: Penicillin V, PPL: Benzylpenicilloyl polylisine.

MDM: Minor determinant mixture, ND: Not done.

In vitro Specific IgE

Five patients (Group A) had positive specific IgE (21.7% sensitivity), four with penicilloyl G, five with penicilloyl V, one with AMX and three with AMP. Specifity of specific IgE was 86.7% (26 true negative/ 30 control subjects). False positive results were one with penicilloyl G, three with penicilloyl V, five with AMP and two with AMX.

Basophil activation test

Nine patients had positive BAT (39.1% sensitivity): one with PENG, three with PPL, four with MDM, two with AMX and one with AMP. Specificity of BAT was 93.3% (28 true negative/30 control subjects). Sensitivity of the different groups was 60% in group A and 50% in group C. In patients from group B, sensitivity was 25%, showing no significant differences with the other two groups (Fisher´s exact test $p>0.3$ in both comparisons).

Antigen specific sulfidoleukotriene production

Five patients had positive ASP (22.7% sensitivity): two with PENG, two with PPL, one with MDM, two with AMX and three with AMP. Specificity of ASP was 83.3% (25 true negative/30 control subjects).

Sensitivity in the different groups varied, being 40% in group C and 25% in group B. Patients from group A had negative ASP. No significant differences were found between the three groups (Fisher´s exact test $p>0.4$ in all the comparisons).

Comparison of the three techniques

Concordance among the three techniques is low (Kappa<0.22 in all the comparisons).

BAT had a higher sensitivity than the rest of techniques tested; however it does not reach statistically significant differences with the other two techniques, probably due to the scarce number of patients.

In none of the three techniques studied did we find statistically significant differences according to the symptoms presented by the patients: urticaria or anaphylaxis (Fisher´s exact test $p>0.6$ in all the comparisons). Nor did we find any significant differences when considering the time lapse since the reaction, established the cut-off point in 6 or in 12 months (Fisher´s exact test $p>0.6$ in all the comparisons). However, we wish to highlight that BAT detects 42.1% of patients with reactions having taken place less than twelve months before, vs 25% when the reaction has taken place more than twelve months before. These results are very similar when the cut-off point is established in six months (43.8% when the lapse of time is equal to or less than six months vs 28.6% when it is longer than six months). In both cases, the difference is not statistically significant.

The joint use of the three techniques allows identification of 14 patients (sensitivity 60.9%).

Discussion

The diagnosis of patients with immediate allergic reactions to betalactams is based on the clinical history corroborated by skin tests. These relatively simple and fast methods have two limitations. The first one is that skin tests are negative in a variable, but significant percentage of patients allergic to betalactams, ranging from 10% to 36% depending on the series [6,15,16]- especially in patients allergic to AX (4)- and their sensitivity decreases as the time lapse from the clinical reaction until the allergologic study increases [4,17]. The second limitation is that it is not a risk-free process, as it is able to induce severe systemic reactions in a high percentage of patients (from 0.5 to 17%), especially in patients with anaphylaxis [4-7], and even more in patients allergic to Amoxicillin [4]. Besides, skin tests cannot be used in patients with dermographism, extensive severe dermatitis or taking drugs with antihistamine activity.

This is why, the diagnosis of these patients with a betalactam allergic reaction and negative skin tests, is based on the in vitro tests. Up to now, we could only use specific IgE determination on a routine basis. In our series, this technique detects less than 22% of patients with these reactions who are studied for the first time. We studied the reliability of two new diagnostic tests, the basophil activation test and the antigen-specific sulfidoleukotriene determination in the diagnosis of Betalactam allergy. BAT shows a global sensitivity of 39.1%. The sensitivity is greater in patients with positive specific IgE or in patients who have suffered several BL induced reactions (50-60%) than in patients who have suffered a unique episode (25%), probably due to a greater sensitisation degree or a more intense humoral immune response. The basophil activation test has shown its usefulness in the diagnosis of patients allergic to betalactams with positive skin tests in a recently published validation study of this technique [2]. In this group of patients it had a sensitivity of 50%, slightly higher than in the present study (39.1%) probably due to the characteristics of the group of patients with negative skin tests and in most cases negative in vitro specific IgE, as well as to the different size of the sample. We found more positive results in BAT if the lapse of time between the reaction and the blood extraction is less than 12 months, as refered with skin tests [4]. Although these differences are not statistically significant (42% vs 25% 12 months since the reaction), this fact is in our opinion relevant because it would avoid performance of provocation tests in 17% of these patients.

In the control group, we included a heterogeneous population of subjects in order to increase the

specificity of the technique, 26.7% of them were atopic in order to further validate the diagnostic capacity.

Concerning the antigen-specific sulfidoleukotriene determination test, it shows a low sensitivity of 22.7%, similar to CAP.

Even though the three tests try to identify IgE-mediated reactions, their degree of correlation is low. With these data we conclude that they are complementary since they do not measure exactly the same events within the IgE-mediated reactions.

The joint use of the three in vitro diagnostic techniques allows identification of 60.9% of these patients. This fact is particularly interesting considering that we could avoid performance of dangerous in vivo examinations, such as oral provocations, in a high number of patients.

As a conclusion, the three in vitro tests analysed in this study and especially BAT (due to its high sensitivity and specificity), can be considered as valuable tools in the diagnosis of this subgroup of patients allergic to betalactams with negative skin tests, being in most cases the alternative to the risky provocation test.

Abbreviations:

AMX: Amoxicillin
AMP: Ampicillin
ASP: Allergen-induced sulfidoleukotriene production
BAT: Basophil activation test
BL: Betalactams
MDM: Minor determinant mixture
PENG: Penicillin G
PPL: Benzyl penicilloyl polylisine

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