

Flow cytometric basophil activation test by detection of CD63 expression in patients with immediate-type reactions to betalactam antibiotics

M. L. Sanz, P. M. Gamboa*, I. Antépara*, C. Uasuf, L. Vila, C. Garcia-Avilés, M. Chazot and A. L. De Weck

Department of Allergology and Clinical Immunology, University Clinic of Navarra, School of Medicine, University of Navarra, Pamplona, and *Servicio de Alergología, Basurto Hospital, Bilbao, Basque Country, Spain

Summary

Background In this study, we used flow cytometry to determine the percentage of activated basophils that expressed the CD63 marker after *in vitro* stimulation by different betalactam antibiotics. The diagnostic reliability of the technique was assessed, as well as its correlation with specific IgE.

Methods Fifty-eight patients with clinical allergy to betalactam antibiotics and presenting positive skin tests to at least one of the allergens (minor determinant mixture (MDM), benzylpenicilloyl-polylysine (PPL), penicillin, ampicillin, amoxicillin, cephalosporins) were tested. Thirty subjects non-allergic to betalactams were also studied as controls. The flow assay stimulation test (FAST) uses flow cytometry to determine the percentage of basophils that express CD63 as an activation marker after *in vitro* stimulation with allergen. Double labelling with monoclonal antibodies anti-CD63-PE and anti-IgE FITC was used.

Results The allergic patients show a statistically greater number of activated basophils than the control subjects, after the incubation of cells with all the betalactams at various concentrations. The sensitivity of the technique is 50%, the specificity 93.3%, the likelihood ratio for a positive value 7.46 and the likelihood ratio for a negative value 0.54. In spite of having a greater sensitivity (37.9%) and specificity (86.7%) than CAP, differences between sensitivity and specificities of both techniques (CAP and FAST) do not reach statistical significance.

Conclusion The basophil activation test is a particularly useful technique in the diagnosis of patients with IgE-mediated allergy to betalactams and allows the identification of 50% of patients. Used in conjunction with CAP, it allows the identification of 65.5% of such patients.

Keywords betalactam allergy, CD63 expression, flow cytometric basophil activation test, *in vitro* diagnosis

Submitted 21 September 2001; revised 26 May 2001; accepted 31 August 2001

Introduction

Betalactam antibiotics are the drugs that most frequently produce adverse reactions mediated by a specific immunological mechanism. It is estimated that as many as 400–800 deaths due to anaphylaxis to this group of drugs occur annually [1].

The *in vitro* diagnosis of drug allergy represents a difficult problem, as to date there is no reliable technique that confirms diagnosis accurately. Recently, flow cytometry has been used as an *in vitro* diagnostic method for IgE-mediated allergic reactions to drugs [2]. Basophils are a minor fraction of blood leucocytes (less than 0.2%), able to release histamine and several other mediators, particularly in IgE-mediated allergic

processes. Flow cytometry allows the analysis of large numbers of cells and permits the simultaneous quantification of several parameters, such as cellular size, and several membrane markers.

The CD63 molecule is a 53-Kda protein expressed on cytoplasmic granules of resting basophils, monocytes, macrophages and platelets, that moves to the cellular surface upon cell activation [3].

In this study we applied the technique described by Sabbah and Sainte Laudy [4,5] with some modifications [6,7]. In this assay, basophil stimulation is detected by first identifying basophils carrying a high density of IgE molecules on their surface, followed by detection of the CD63 molecule expression subsequent to *in vitro* stimulation with penicillins or penicillin-derived allergens. Our goal was to determine the effectiveness of the basophil activation test in the diagnosis of patients with IgE-mediated hypersensitivity reactions to betalactam antibiotics. This test was compared with specific IgE determinations and skin tests with betalactam-derived reagents.

Correspondence: María L. Sanz, Department of Allergology and Clinical Immunology, University Clinic of Navarra, Apartado 4209, 31080 Pamplona, Spain. E-mail: mlsanzlar@unav.es

Table 1. Individual results

Patient number	Clinical reaction	FAST (*) (% antigen activated basophils/stimulation index)	Specific IgE (KU/L)	Positive skin tests (id = intradermal, pr = prick)
<i>Culprit drug: amoxicillin</i>				
1	Urticaria	Amoxicillin (12.4/6.5)	Negative	MDM (id)
2	Anaphylaxis	Negative	Negative	Penicillin (id) Amoxicillin (id)
3	Anaphylaxis	PPL (22.1/3.7)	Ampicillin (5.63) Amoxicillin (6.45) Penicillin G (14.8) Penicillin V (6.67)	Amoxicillin (pr)
4	Anaphylaxis	Penicillin (20.5/3.4) Ampicillin (23/3.8) Amoxicillin (28.2/4.7)	Ampicillin (0.59) Amoxicillin (1.29) Penicillin G (0.40) Penicillin V (0.40) Penicillin V (0.96)	Penicillin (id) Amoxicillin (id) PPL (id)
5	Anaphylaxis	Amoxicillin (9/9.1)	Negative	Penicillin (id) Amoxicillin (id)
6	Anaphylaxis	Negative	Ampicillin (1.01) Penicillin G (0.53) Penicillin V (0.49)	Ampicillin (pr) Amoxicillin (pr)
7	Anaphylaxis	PPL (8.3/3.5)	Negative	Amoxicillin (id)
8	Urticaria	MDM (7/6.2)	Negative	MDM (id)
9	Urticaria	Negative	Ampicillin (2.93) Amoxicillin (2.91) Penicillin G (3.11) Penicillin V (3.71)	MDM (pr)
10	Anaphylaxis	Ampicillin (22.2/6.3) Amoxicillin (27.4/7.7)	Not done	Ampicillin (pr) Amoxicillin (pr)
11	Urticaria	Penicillin (23.9/9.9) Ampicillin (41.7/17.4) Amoxicillin (46.7/19.5)	Negative	Ampicillin (id) Amoxicillin (id)
12	Anaphylaxis	Negative	Ampicillin (3.9) Amoxicillin (68) Penicillin G (39.5) Penicillin V (56.4)	Ampicillin (id) Amoxicillin (id)
13	Urticaria	Negative	Negative	Penicillin (id) Ampicillin (id) Amoxicillin (id) MDM (id)
14	Anaphylaxis	Negative	Negative	Ampicillin (id) Amoxicillin (id)
15	Anaphylaxis	MDM 19.8/2) Amoxicillin (20.8/2.1)	Negative	Amoxicillin (pr)
16	Anaphylaxis	Negative	Negative	MDM (pr)
17	Anaphylaxis	Ampicillin (13.9/2.1) Amoxicillin (15.6/2.4)	Negative	Amoxicillin (pr)
18	Anaphylaxis	Negative	Negative	Amoxicillin (id)

Table 1. *continued*

Patient number	Clinical reaction	FAST (*) (% antigen activated basophils/stimulation index)	Specific IgE (KU/L)	Positive skin tests (id = intradermal, pr = prick)
19	Anaphylaxis	Negative	Negative	Amoxicillin (id)
20	Anaphylaxis	Negative	Negative	Ampicillin (id) Amoxicillin (id)
21	Anaphylaxis	Ampicillin (33.9/91.6) Amoxicillin (41.7/112.7)	Penicillin G (0.39) Penicillin V (0.57) Ampicillin (0.52) Amoxicillin (0.46)	Amoxicillin (id)
22	Anaphylaxis	Negative	Ampicillin (0.44) Penicillin G (0.46)	Amoxicillin (id)
23	Anaphylaxis	PPL (55.1/10.2)	Ampicillin (97.3) Amoxicillin (93) Penicillin G (> 100) Penicillin V (> 100)	Ampicillin (pr) PPL (pr) MDM (pr)
24	Anaphylaxis	Ampicillin (7.4/5.9) Amoxicillin (8.9/7.1)	Negative	Ampicillin (id) Amoxicillin (id) MDM (id)
25	Anaphylaxis	Negative	Negative	Amoxicillin (id)
26	Anaphylaxis	Amoxicillin (14.1/9.3)	Negative	Amoxicillin (pr)
27	Anaphylaxis	Penicillin (8.2/3.6)	Amoxicillin (0.6)	Amoxicillin (id)
28	Anaphylaxis	Negative	Ampicillin (9.38) Amoxicillin (8.58) Penicillin G (16.1) Penicillin V (3.4)	Penicillin (pr) Ampicillin (pr) Amoxicillin (pr) MDM (pr)
29	Anaphylaxis	Negative	Amoxicillin (0.67)	Amoxicillin (id)
30	Anaphylaxis	Negative	Ampicillin (1.58) Amoxicillin (0.99) Penicillin G (2.81) Penicillin V (1.85)	Amoxicillin (pr)
31	Anaphylaxis	Penicillin (7.6/6.2)	Ampicillin (3.04) Amoxicillin (3.33) Penicillin G (22.7) Penicillin V (3.66)	Ampicillin (id) Amoxicillin (id)
32	Urticaria	Negative	Ampicillin (5.63) Amoxicillin (1.38) Penicillin G (5.95) Penicillin V (5.19)	Amoxicillin (id)
33	Anaphylaxis	MDM (68.5/2.6) Ampicillin (64.7/2.4) Amoxicillin (67.8/2.5)	Negative	Penicillin (id) Ampicillin (id) Amoxicillin (id)
34	Urticaria	Negative	Negative	Penicillin (id) Ampicillin (id) Amoxicillin (id) MDM (id)
35	Urticaria	Negative	Negative	Amoxicillin (pr)
36	Anaphylaxis	Negative	Negative	Amoxicillin (pr)
37	Anaphylaxis	Negative	Negative	Ampicillin (id) Amoxicillin (id)
38	Anaphylaxis	Negative	Negative	Ampicillin (pr) Amoxicillin (pr)
39	Anaphylaxis	Amoxicillin (10.4/7.3)	Negative	Ampicillin (id) Amoxicillin (id)
40	Urticaria	PPL (17/16)	Negative	Amoxicillin (pr) PPL (pr)

Table 1. continued

Patient number	Clinical reaction	FAST (*) (% antigen activated basophils/stimulation index)	Specific IgE (KU/L)	Positive skin tests (id = intradermal, pr = prick)
41	Urticaria	Amoxicillin (59.5/6.1)	Negative	Ampicillin (id) Amoxicillin (id) MDM (id)
42	Anaphylaxis	Negative	Negative	Ampicillin (pr) Amoxicillin (pr)
43	Anaphylaxis	Penicillin (5.8/2.1) Ampicillin (10.5/3.8) Amoxicillin (12.2/4.4)	Negative	Amoxicillin (id)
44	Urticaria	Negative	Negative	Penicillin (id)
45	Urticaria	Negative	Negative	Amoxicillin (pr)
<i>Culprit drug: penicillin G</i>				
46	Anaphylaxis	Penicillin (24.6/4.5) MDM (14.2/2.6) Ampicillin (14.4/2.6) Amoxicillin (23.6/4.4)	Negative	PPL (id)
47	Anaphylaxis	Negative	Negative	MDM (id)
48	Anaphylaxis	Penicillin (23.8/2.3) PPL (63.9/4.7) MDM (50.6/4.8) Amoxicillin (49.5/4.7)	Penicillin G (0.53) Penicillin V (0.64)	PPL (id)
49	Anaphylaxis	PPL (35.1/175.4)	Ampicillin (0.28) Penicillin G (0.85) Penicillin V (1.02)	MDM (id)
50	Anaphylaxis	MDM (10.5/5.7)	Negative	Amoxicillin (pr and id)
51	Anaphylaxis	Amoxicillin (5.2/2.6)	Penicillin G (1.27)	PPL (pr and id)
52	Anaphylaxis	Negative	Ampicillin (0.51) Penicillin G (0.85) Penicillin V (0.38)	Amoxicillin (id) PPL (id) MDM (id)
<i>Culprit drug: cephalosporin (cefuroxime patients 53–57, cefazolin patient 58)</i>				
53	Anaphylaxis	Negative	Negative	Cefuroxime (id)
54	Anaphylaxis	Cefuroxime (20.1/5.8)	Ampicillin (0.15) Penicillin G (1.42) Penicillin V (1.38)	Cefuroxime (pr)
55	Urticaria	Cefuroxime (12.6/2.8)	Penicillin G (0.53) Penicillin V (0.64)	PPL (id)
56	Anaphylaxis	Penicillin (12.6/6.6) Ampicillin (8.9/4.6) Amoxicillin (11.7/6.1)	Penicillin V (0.52)	Cefuroxime (id)
57	Urticaria	Negative	Negative	MDM (id)
58	Urticaria	Negative	Negative	Cefazolin (id)

Id = intradermal skin test; Pr = skin prick test; PPL = benzylpenicilloyl polylysine; MDM = minor determinant mixture. (*)The highest percentage and SI of activated basophils obtained in each patient with one of the penicillin reagents tested.

Material and methods

Patients

Fifty-eight patients were studied, 21 men and 37 women (average age 51.5 years; SD 15.2) selected for immediate-type clinical reaction following betalactam therapy. Their clinical features are shown in Table 1. Thirty non-allergic patients (13 men and 17 women) (52.5 years; SD 14.9) who visited us after presenting non-allergic adverse reactions to other drugs were chosen as controls. All control patients had negative skin tests to betalactams and tolerated systemic betalactams. Allergy to the other drugs was ruled out in all control patients.

Patients with suspected betalactam allergy were examined at the Departments of Allergology of the University Clinic of Navarra and Basurto Hospital. The inclusion criteria were: history of anaphylaxis or urticaria-angioedema immediately following administration of betalactams and at least one positive skin test with one or more of the betalactam-derived reagents used. Patients attended because of a reaction caused by amoxicillin or amoxicillin-clavulanic acid in 46 cases, penicillin G in six cases, cefuroxime in five cases and cefazolin in one case ($n = 58$).

We considered that the patients had suffered from anaphylaxis if they had presented several of the following symptoms: palm/sole or systemic pruritus, generalized erythema, difficulty in breathing, speaking or swallowing, marked cardiovascular symptoms (hypotension) and/or loss of consciousness. If they had merely weals or localized oedema in one or several parts of the body we diagnosed them as having urticaria-angioedema.

All the patients underwent skin tests, *in vitro* specific IgE determination and basophil activation tests. In order to avoid bias, the three diagnostic tests were performed by different persons, none of whom knew the results of the other tests.

Skin tests

Skin tests were performed using the usual techniques [8]. Prick tests were performed with ALK-ABELLO lancets, and the intradermal tests with 26½-gauge hypodermic needles, injecting 0.05 mL of the antigen in the volar surface of the forearms. Benzylpenicilloyl-polylysine (PPL) (5×10^{-5} mol/L, Allergopharma, Hamburg, Germany), minor determinant mixture (MDM) (2×10^{-2} mol/L, Allergopharma, Hamburg, Germany), sodium penicillin G (1000 UI/mL; Laboratorio Level, Barcelona, Spain), ampicillin (20 mg/mL, Antibióticos SA, León, Spain) and amoxicillin (20 mg/mL, Beecham, Toledo, Spain) were used. In patients who reported reactions to cefuroxime (Glaxo-Wellcome, Madrid, Spain) or cefazolin (Lilly, Madrid, Spain), these compounds were tested at a maximum concentration of 10 mg/mL. In all cases, the study was started with prick tests in which all the previously mentioned antigens were tested in each patient, followed by intradermal tests. If the patient presented a positive skin prick test to some allergens, the series of *in vivo* tests was interrupted. Histamine (10 mg/mL) and NaCl 0.09% were used as positive and negative controls, respectively. Weals 3 mm greater than the negative control for prick testing and 5–10 mm greater than the negative control for intradermal testing were 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. In those patients who reported a reaction to cephalosporins, cefaclor was also tested. All results equal to or higher than 0.35 kU/L were considered as positive.

Flow cytometry stimulation test (FAST)

The technique used has been described in a previous paper [6]. Blood was collected in 6-mL ACD tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France) and stored at 4 °C; the test was carried out within 24 h of blood sampling. For testing up to three allergens, one ACD tube was required. The tubes were centrifuged for 10 min at 4 °C, 550 g. Cells from the layer above the red blood cells were collected and centrifuged again for 10 min at 4 °C, 550 g. The supernatant was decanted and 500 µL of HEPES calcium buffer, denominated stimulation buffer (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, HSA 1 mg/mL, pH 7.4), containing IL3 (2 ng/mL) and 10 µL of heparin (5000 UI/mL (ROVI, Madrid, Spain)) were added to each tube, mixed and gently shaken.

In this study, during the stimulation phase and as indicated later in the text, we also added C_{5a} 10^{-8} M as previous studies demonstrate that C_{5a} acts at low concentration on human basophils, priming these cells to produce mediators [9,10]. In fact, in the presence of non-stimulating concentrations of C_{5a}, we observed a better activation of basophils in response to specific stimuli (results not shown).

Firstly, 10 µL of 10^{-8} M C_{5a} (Sigma, Madrid, Spain) were put in all wells of a polystyrene microtitre U plate (Greiner Microdon, Zaragoza, Spain). Subsequently, 50 µL of 1/40 and 1/160 dilutions of clinical intravenous preparations of benzylpenicillin (3.9 and 0.975 mg/mL), ampicillin (2.5 and 0.625 mg/mL), amoxicillin (2.5 and 0.625 mg/mL) or MDM (27.5 and 6.87 ng/mL) and PPL (0.5 and 0.125 mg/mL), were put in the corresponding wells. Patients with a reaction to cefuroxime or cefazolin were also tested with these substances (cefuroxime at the concentrations of 0.83 and 1.2 mg/mL, and cefazolin at the concentrations of 0.4 and 0.16 mg/mL). The working dilutions of drugs used in the test were freshly prepared each day. These dilutions were considered as optimal in the light of previous assays (data not shown). As a positive control, we used a monoclonal Le27 anti-IgE antibody solution (Bühlmann, Allschwil, Switzerland) at a final concentration of 1 µg/mL. In order to evaluate basal values without stimulation, we added 50 µL of stimulation buffer to another well. 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 37 °C 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 4 °C, 1000 g.

The basophils from the pellet were double labelled by adding 20 µL of anti-CD63 PE-labelled antibody (Caltag Laboratories, Burlingame, CA, USA), diluted 1:80 in tubes (Cytometer Falcon) and anti-IgE FITC-labelled antibody (Caltag Laboratories) diluted 1:60 in washing buffer. After incubation for 30 min at 4 °C (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 (18–20 °C)

for 5 min. The cell lysis was stopped with 1 mL of washing buffer. After centrifuging for another 5 min at 1000 g, the supernatant was decanted and 500 µL of washing buffer added to each tube, which was then gently shaken before flowcytometric 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. In each assay, we assessed at least 500 basophils. The other parameter analysed on the identified basophils was CD63. In ten cases all samples were run in duplicate, in order to assess the reproducibility of the technique.

Statistical analysis

Mean comparisons for variables without normal distribution were performed by the Mann–Whitney test. Comparison between qualitative data was performed by the squared chi-test with Yates correction when necessary. All reported *P*-values represented two-tailed tests, with values 0.05 or less considered statistically significant. The specificity and sensitivity values were obtained by analysis of different cut-off points in ROC curves. Sensitivity was calculated by the number of positive cases detected by the technique in the group of patients, and specificity by the number of negative cases detected by the technique in the control group. Reproducibility of FAST results was assessed by intraclass correlation coefficient. The statistical analysis was performed with the program SPSS version 9.0.

IgE-mediated allergic reactions to betalactams are low prevalence processes [8]. For such processes new diagnostic tests must have a high specificity in order to be considered useful in clinical practice. This is why we selected a value that resulted in a high specificity when choosing a cut-off point by ROC curves.

We analysed the likelihood ratio of the FAST technique. The likelihood ratio for a positive test is the probability of finding a positive result in patients in relation to the probability of finding the same result in healthy subjects. The likelihood ratio for a negative result is the probability of finding a negative result in healthy subjects in relation to the probability of finding this negative result in patients.

Results

Intraclass correlation coefficient in FAST with betalactams was 0.89 ($P < 0.0001$), which demonstrates an excellent reproducibility of this technique.

In order to consider a result as positive, we consider that the percentage of basophils activated after incubation with antigen should be at least double the percentage of basophils activated spontaneously (SI: stimulation index as a ratio of the percentage of activated basophils upon drug incubation vs. percentage of basophils activated spontaneously). When the percentage of basophils activated spontaneously was under 2.5% we required an additional condition, namely that the percentage of basophils activated after contact with the antigen should be equal to or greater than 5%.

In 95% of all assays with betalactam antibiotics performed on control individuals, basophil activation after incubation with drugs or drugs-derived reagents was negative.

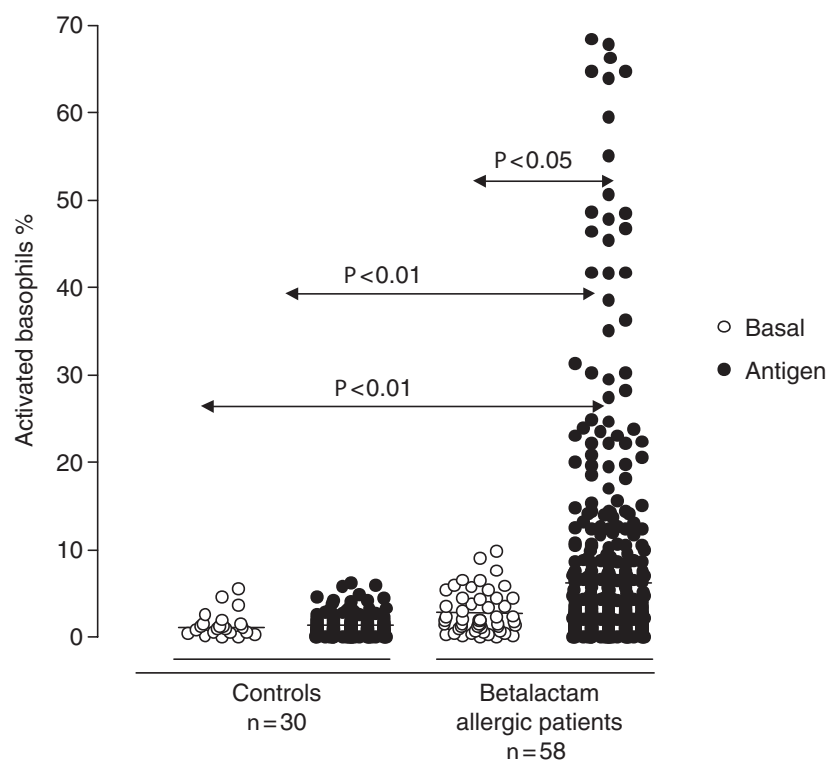


Fig. 1. Flow cytometric allergen stimulation test (FAST) in betalactam allergy. Basal and antigen-specific response in allergic patients and in controls. Each point represents the highest percentage of activated basophils obtained in each patient with all the penicillin reagents tested (antigen) or without stimulation (basal).

Out of the 58 patients included in the study, 29 were positive in at least one basophil flow cytometry stimulation test (FAST) performed with the different drug reagents tested (range 5.2–63.2%) and 29 were negative (Table 1). Out of the 30 controls, only two showed a positive basophil flow cytometry stimulation test. These data are summarized graphically in Fig. 1.

The group data for the basophil activation test with each betalactam-derived antigen tested are shown in Table 2. There are significant differences between the control group and the betalactam allergic group for each drug and concentration tested. In Fig. 2(a, b and c) the results of three positive cases ($n = 41$, $n = 23$ and $n = 11$) are shown.

When we analysed the patients in relation to the clinically responsible drug, we observed that in patients allergic to amoxicillin, positive FAST ($n = 13$), CAP ($n = 12$) and skin tests ($n = 39$; 13 of them prick tests) were most frequently produced by amoxicillin. In six patients allergic to penicillin G the response pattern was different. The most frequently positive allergens were PPL in four skin tests and penicilloyl G and V in three CAP determinations, whereas penicillin G, PPL and amoxicillin were positive in two FAST determinations. Finally, in the five patients allergic to cefuroxime (within the group of patients allergic to cephalosporins), the greatest responses were to cefuroxime in skin tests (four cases) and in FAST (two cases), and to penicilloyl G in CAP (two cases).

According to our results and using clinical history plus at least one positive skin test as positive criteria, the sensitivity of the FAST test was 50% (CI 37.1–62.9%), and the specificity 93.3% (CI 86.9–99.7%). For an approximate prevalence of 3% [8] of IgE-mediated sensitivities to penicillins, the positive predictive value was 18.9% and the negative predictive value 98.4%, respectively. The likelihood ratio for positive test result was 7.46

(CI 3.7–14.9) and the likelihood ratio for negative test 0.54 (CI 0.27–1.08).

In our study population, 22 patients had at least one positive specific IgE determination by CAP, as well as four of the controls. Sensitivity and specificity of the CAP were therefore 37.9% (CI 20.5–55.3%) and 86.7% (CI 74.5–98.9%), respectively. Although the proportion of patients detected by FAST is greater than by CAP, that difference does not reach statistical significance ($\chi^2 = 1.424$; $P = 0.233$).

The sensitivity of both tests in the 43 patients who reported anaphylaxis (FAST + 23, CAP + 19) was similar to the 15 patients who presented urticaria (FAST + 6, CAP + 3) and no significant differences between the two groups were found.

In summary, with the use of both techniques (FAST and specific IgE) we managed to objectively confirm the diagnosis of immediate type allergy to betalactams *in vitro* in 38 patients (65.5%). Only 20 patients (34.5%) allergic to betalactams with positive skin tests presented negative results in both *in vitro* techniques (FAST and CAP).

Discussion

In vitro diagnosis of drug allergy is a difficult challenge that has in most instances not yet been met. Previous studies have suggested that *in vivo* tests, such as skin test to penicillin, benzylpenicillin G and minor determinants of penicillin (MDM), can identify up to 95% of patients with a history of allergy to betalactams [11]. Some authors [12] affirm that patients with negative skin tests to PPL, benzyl penicillin G and a minor determinant mixture, can receive betalactam antibiotics safely. Nevertheless, 5% of patients with positive history but negative

Table 2. Percentage of basophils activated with the different drugs tested

Drug	Patients with FAST positive Median (25th–75th percentile)	Patients with FAST negative Median (25th–75th percentile)	Non-allergic controls Median (25th–75th percentile)	Probability (Mann–Whitney's *Allergic FAST positive vs. allergic FAST negative **Allergic FAST positive vs. controls)
Penicillin 1/40	20.1 (11.4–23.8)	1.5 (0.4–3.3)	0.5 (0.2–1.5)	* < 0.001 ** < 0.001
Penicillin 1/160	9.3 (6.4–21)	1.3 (0.6–3.6)	0.6 (0.2–1.9)	*0.006 **0.001
Ampicillin 1/40	22.6 (10.5–38.9)	1.9 (0.9–2.9)	0.8 (0.4–1.6)	* < 0.001 ** < 0.001
Ampicillin 1/160	14.1 (9.3–28.6)	1.8 (0.8–3.3)	1 (0.3–1.7)	* < 0.001 ** < 0.001
MDM 1/40	15.2 (7–53.6)	1.3 (0.7–2.8)	0.7 (0.4–2.1)	*0.004 ** < 0.001
MDM 1/160	10.7 (7.1–41.6)	1.7 (0.7–2.8)	0.6 (0.3–1.7)	* < 0.001 * < 0.001
PPL 1/40	22.1 (8.3–55.1)	1.6 (0.6–2.9)	0.8 (0.3–1.7)	* < 0.001 ** < 0.001
PPL 1/160	26.8 (12.3–50.8)	1.5 (0.7–2.8)	0.5 (0.1–2.6)	* < 0.001 ** < 0.001
Amoxicillin 1/40	15.6 (11.4–36.9)	1.3 (0.7–2.8)	0.7 (0.2–1.6)	* < 0.001 ** < 0.001
Amoxicillin 1/160	13.8 (9.3–36.1)	1.2 (0.7–2.9)	0.6 (0.3–1.2)	* < 0.001 ** < 0.001

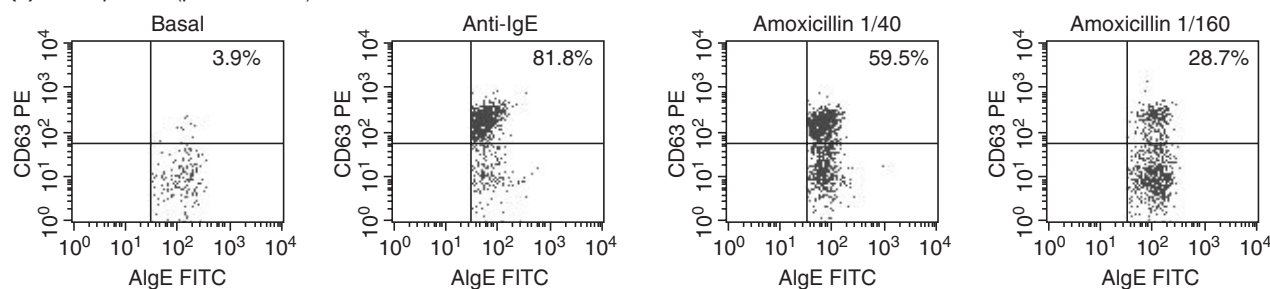
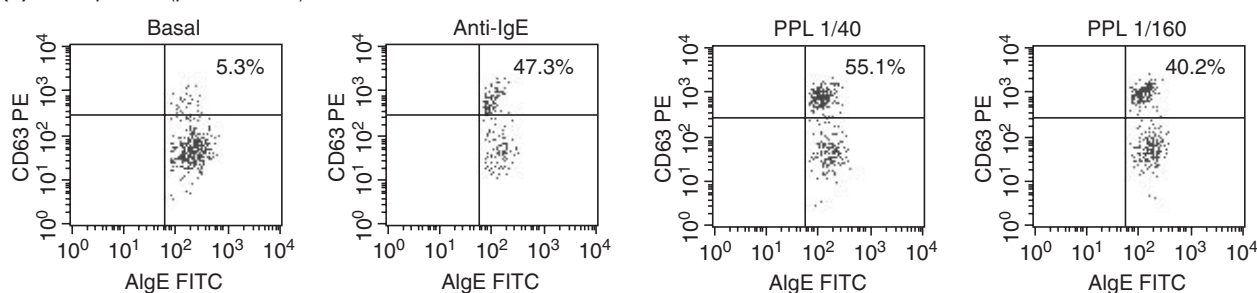
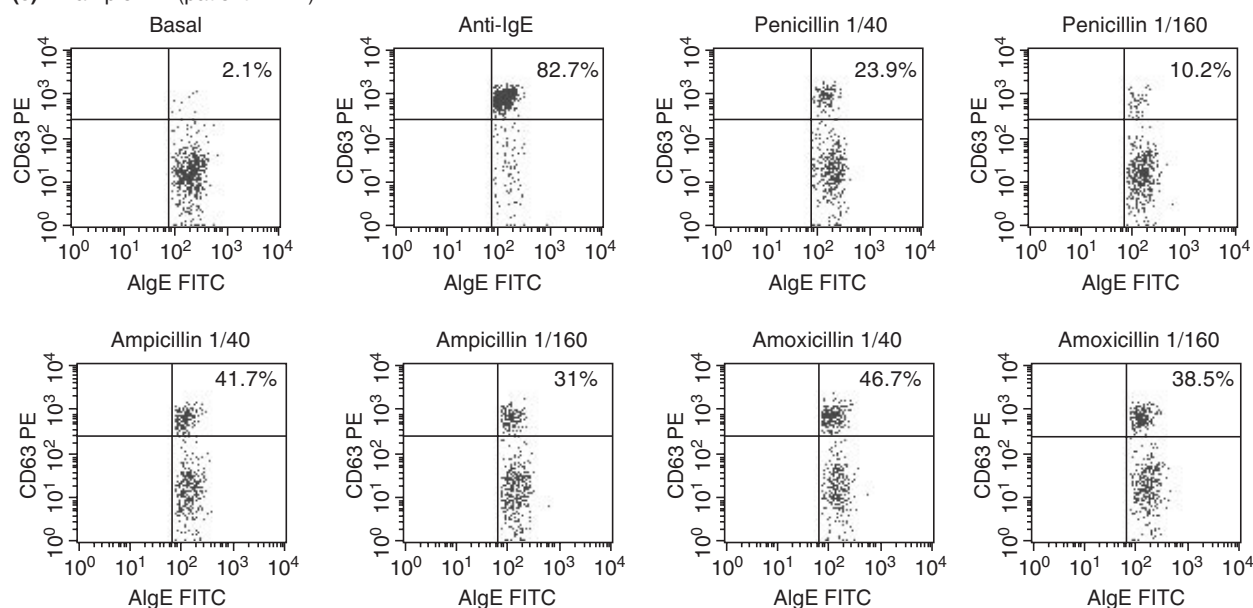
(a) Example n°1 (patient n=41)**(b) Example n°2 (patient n=23)****(c) Example n°1 (patient n=11)**

Fig. 2. Flow cytometric allergen stimulation test (FAST) in betalactam allergy. Example of positive cases: basal (negative control without stimulation), anti-IgE (positive control) and Ag-specific-responses. **(a)** Case number 41, positive to amoxicillin. **(b)** Case number 23, positive to PPL. **(c)** Case number 11, positive to penicillin, ampicillin and amoxicillin.

skin tests to these test allergens present positive provocation tests. On the other hand, there is a subgroup of patients with allergy to ampicillin and amoxicillin who tolerate penicillin G [13]. Most real allergic reactions to betalactams are the result of specific IgE antibodies against the betalactam ring [14]. *In vitro* tests to confirm penicillin allergy by measuring IgE antibodies to various penicillins are not considered to be reliable [15].

Flow cytometry is a suitable analytical technique to quantify the expression of cellular membrane markers, such as CD63, on basophils. This technique was previously described and developed for the diagnosis of allergy to various allergens [16–18]. Some technical aspects have been modified by us, such as the use of allergens in fluid phase in inhalant allergy [6]. In this study we also added C_{5a} 10⁻⁸ M as a priming agent.

Double-labelling fluorescence allows the selection of first, the basophil population with monoclonal antibodies against IgE or against the high affinity IgE receptor and then, the activated basophils with monoclonal antibodies directed against CD63. Other authors [19,20] use Ab anti-CD45 as an additional marker. In our experience this addition does not improve the results (results not shown).

The number of basophils evaluated is equal to or larger than 500 and represents a higher number of cells than those investigated in previous reports [21].

Previous studies performed by us and other authors show that the basophil activation test is a highly reliable procedure for detection of IgE-mediated allergy to inhalant allergens such as *Der p*, *Lol p* [6], cypress pollen [22], latex [17,23] and food allergens [24,25].

In patients sensitized to betalactam antibiotics, we observed a significant activation of basophils after stimulation with different betalactams in 50% of patients with a clinical history of immediate-type reaction and positive skin test to at least one penicillin-derived test reagent.

There are still few published papers demonstrating the validity of this technique in allergy to drugs [2,26] and none that documents the clinical sensitivity and specificity of this technique in the diagnosis of allergy to betalactams. Our results confirm that FAST sensitivity is higher than the sensitivity of specific IgE determination, the other *in vitro* test used in routine diagnosis of betalactam allergy. Indeed, FAST was able to detect a higher number of positive cases with the same specificity as specific IgE determination by CAP.

A new diagnostic test must have a high specificity in order to be considered useful in clinical practice. This is why, when choosing a cut-off point by ROC curves, we selected a value that resulted in a high specificity.

In allergy to betalactam antibiotics, another *in vitro* test based on the release of sulphidoleukotrienes (sLT) after stimulation of leucocytes by allergen *in vitro* (CAST) has been reported to yield positive results in immediate-type allergic reactions, but only in those patients with anaphylaxis [27]. The use of both techniques (FAST and CAP) allowed the detection of 65.5% of the allergic patients.

A possible reason for the discrepancy between results of *in vitro* serological and cellular tests, on the one hand, and skin tests, on the other hand, in betalactam allergy is that in such patients detectable serological IgE decreases much faster than skin test and tissue bound IgE.

Regarding cross-reactivity studies performed by several authors [13,14,28,29], it is well known that the betalactam nuclear ring structure is the main source of the immunodominant epitope in betalactams allergy. The patterns of cross-reactivity are almost complete between semi-synthetic penicillins, partial and incomplete between penicillins and cephalosporins, and almost non-existent with monobactams. On the other hand, there is also an immune response against the lateral chains. Although the structure of the amoxicillin lateral chain is what induces the major proportion of antibodies, other antibiotic structures are also required for optimal recognition [30].

In our study we observed patterns of IgE binding-penicillins similar to those described previously. The reactions in FAST among our patients allergic to amoxicillin or penicillin can be divided as follows: (a) patients who seem to recognize essentially the lateral chain and who react in FAST to amoxicillin,

sometimes together with ampicillin; (b) patients who recognize the penicilloyl group and react to PPL and/or penicillin; and (c) patients with other patterns of response who usually recognize different antigenic structures. The poor concordance between data from FAST and CAP is probably due to the use of different antigenic structures in each test [28].

In our experience the joint use of *in vivo* and *in vitro* tests permits an efficient and objective diagnosis of clinical reactions caused by immediate hypersensitivity to betalactam antibiotics. The basophil activation test has an excellent specificity and a moderate sensitivity; its combination with specific IgE determinations improves the efficacy of *in vitro* diagnosis of betalactam allergy.

Acknowledgement

This work has been partly supported by a grant from the Spanish Society of Allergology and Clinical Immunology (SEaic) (Research Funds-First Semester 2000). We thank Gema Betelu and Lesley Brain for proof-reading the manuscript, and Sonia Ariz for her technical support.

References

- DeShazo RD, Kemp SF. Allergic reactions to drugs and biological reagents. *JAMA* 1987; 278:1895–906.
- Abuaf N, Rajoely B, Ghazouani E et al. Validation of flow cytometry assay detecting *in vitro* basophil activation for the diagnosis of muscle relaxant allergy. *J Allergy Clin Immunol* 1999; 10:411–8.
- Knol EF, Mul FP, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol* 1991; 88:328–38.
- Sabbah A, Sainte Laudy J. Flow cytometry applied to the analysis of lymphocyte and basophil activation. *ACI Int* 1996; 8 (4):116–20.
- Sainte Laudy J. Application of flow cytometry to the analysis of activation of human basophils. Immunologic validation of the method. *Allergie Immunologie* 1998; 30:41–3.
- Sanz ML, Sánchez G, Vila L et al. Ag-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to *Dermatophagoides pteronyssinus* and *Lolium perenne*. *Clin Exp Allergy* 2001; 31:1007–13.
- De Weck AL, Sanz ML, Gamboa P et al. Determination by flowcytometry of activated basophils in patients allergic or pseudo-allergic to various allergens and drugs: a new diagnostic test (FAST). *Allergy Supplement* 2000; 63 (55):125.
- Dreborg S, Backman A, Basomba A et al. Skin tests used in type I allergy testing. *Allergy* 1989; 44 (Suppl. 10):1–59.
- Burgi B, Otten UH, Ochensberger B, Ibañez CF, Dahinden CA. Basophil priming by neurotrophic factors. Activation through the trk receptor. *J Immunol* 1996; 157:5582–8.
- Dahinden CA, Bischoff SC, Brunner T et al. Regulation of mediator release by human basophils: importance of the sequence and time of addition in the combined action of different agonists. *Int Arch Allergy Appl Immunol* 1991; 94:161–4.
- Gadde J, Spence M, Wheeler B, Adkinson F. Clinical experience with penicillin skin testing in a large inner-city STD clinic. *JAMA* 1993; 270:2456–63.
- Macy E, Richter PK, Falkoff R, Zeiger R. Skin testing with penicilloate prepared by an improved method: amoxicillin oral challenge in patients with negative skin-test responses to penicillin reagents. *J Allergy Clin Immunol* 1997; 100:586–91.

- 13 Blanca M, Vega JM, Garcia J. Allergy to penicillin with good tolerance to other penicillin: study of the incidence in subjects allergic to beta-lactams. *Clin Exp Allergy* 1990; 20:475–81.
- 14 Romano A, Fernandez J, Vega JM et al. Allergic reactions to ampicillin: studies on the specificity and selectivity in subjects with immediate reactions. *Clin Exp Allergy* 1997; 27:1425–31.
- 15 Sanz ML, Garcia BE, Prieto I, Oehling A. Specific IgE determination in the diagnosis of beta-lactam allergy. *J Invest Allergol Clin Immunol* 1996; 6 (2):89–93.
- 16 Sabbah A, Lauret MG, Maillard H. Preliminary study of basophil activation test for drug allergy using anti-membrane antibodies and flow cytometry. *Allergie Immunologie* 1995; 27:276–6.
- 17 Sainte Laudy J, Vallon C, Guerin J-C. Diagnosis of latex allergy. Comparison of histamine release and flow cytometric analysis of basophil activation. *Inflamm Res* 1996; 45 (Suppl. 1):35–6.
- 18 Sainte Laudy J, Drouet M, Lauret MG, Loiry M, Sabbah A. Test d'activation des basophiles par cytométrie en flux dans l'allergie aux venins d'hyménoptères. *Allergie Immunologie* 1999; 31:11–4.
- 19 Monneret G, Gutowski MC, Bienvenu J. Detection of allergen induced basophil activation by expression of CD 63 antigen using a tricolour flow cytometric methods. *Clin Exp Immunol* 1999; 115:393–6.
- 20 Gane P, Pecquet C, Crespeau H et al. Flow cytometric monitoring of allergen induced basophil activation. *Cytometry* 1995; 19:361–5.
- 21 Sainte Laudy J, Sabbah A, Vallon C, Guerin JC. Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release. *Inflamm Res* 1998; 47:401–8.
- 22 Pâris-Köhler A, Demoly P, Persi L, Lebel B, Bousquet J, Arnoux B. In vitro diagnosis of cypress pollen by using cytofluorimetric analysis of basophils (Basotest). *J Allergy Clin Immunol* 2000; 105:339–45.
- 23 Sánchez-López G, Sanz L, García-Avilés C et al. Flow-cytometric cellular allergen stimulation test (FAST) in latex allergy. *Allergy Supplement* 2000; 63 (55):22.
- 24 García-Avilés C, Sánchez-López G, Sanz María L et al. Flow-cytometric cellular allergen stimulation test (FAST) in the *in vitro* diagnosis of food allergy. *Allergy Supplement* 2000; 63 (55):128.
- 25 Moneret-Vautrin DA, Sainte Laudy J, Kanny J, Fremont S. Human basophil activation measured by CD 63 expression and LTC4 release in IgE-mediated food allergy. *Ann Allergy Asthma Immunol* 1999; 82:33–40.
- 26 Monneret G, Dumenil V, Benoit Y et al. Biological diagnosis of immediate drug allergy. Comparative study of histamine liberation tests and CD63 expression by flow cytometry. *Allerg Immunol (Paris)* 1999; 31:307–10.
- 27 Bircher AJ, Haldiman D, Weber J. CAST-ELISA testing for the diagnosis of immediate-type allergic reactions to betalactam antibiotics. *ACI Int* 1996; 8:109–10.
- 28 Baldo BA. Penicillins and cephalosporins as allergens: structural aspects of recognition and cross-reactions. *Clin Exp Allergy* 1999; 29:744–9.
- 29 Adkinson NF Jr. Beta-lactam crossreactivity. *Clin Exp Allergy* 1998; 28:37–40.
- 30 Moreno F, Blanca M, Mayorga C et al. Studies of specificities of IgE antibodies found in sera from subjects with allergic reactions to penicillin. *Int Arch Allergy Appl Immunol* 1995; 108:74–81.