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Diagnosis of Immediate-Type ß-Lactam Allergy In Vitro by Flow-Cytometric Basophil Activation Test and Sulfidoleukotriene Production: A Multicenter Study

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Abstract

Introduction: This multicenter study aimed to evaluate the diagnostic value of 2 cellular tests based on basophil reactivity—the basophil activation test (BAT, Flow-CAST) and the sulfidoleukotriene release assay (CAST-ELISA)—in immediate-type ß-lactam allergy, particularly in patients with a clinical history of allergy and a negative skin test result.
Material and Methods: In a multicenter study encompassing 10 European centers, 181 patients with a history of immediate-type ß-lactam allergy, and 81 controls, we evaluated the diagnostic efficiency of specific IgE determinations and of 2 cellular tests based on basophil reactivity, the BAT and the sulfidoleukotriene release assay.
Results: With Flow-CAST, sensitivity varied for individual ß-lactam allergens from 16% for penicilloyl-polylysine to 33% for amoxicillin, reaching 50% when all 5 allergens were considered. In ß-lactam–allergic patients with negative skin test results (22.8%), Flow-CAST showed positive results for at least 1 of the 5 allergens in 37%. Specificity varied from 89% to 97%, depending on the allergens used. In CAST-ELISA, the overall sensitivity in skin test–positive patients was 41.7%; in patients with negative skin test results it was 27.9%. Both tests were not absolutely correlated, so that when all the results were considered together, sensitivity increased to 64.3% and specificity varied for both tests combined from 73% to 92%. In contrast, specific IgE determinations in the same population yielded a lower sensitivity (28.3%).
Conclusions: A diagnostic algorithm including skin tests and specific IgE, followed by cellular tests in negative patients and controlled challenge enabled us to confirm ß-lactam allergy in 92% of cases. This procedure would also allow us to avoid two-thirds of the required controlled challenges.

Key words: Immediate-type ß-lactam allergy. In vitro diagnosis. Cellular tests. BAT. Sulfidoleukotriene production.

■ **Resumen**

Introducción: Este es un estudio multicéntrico enfocado a evaluar el valor diagnóstico de 2 pruebas celulares basadas en la reactividad del basófilo -el test de activación de basófilos (TAB, Flow-CAST) y el ensayo de liberación de sulfidoleucotrienos (CAST-ELISA)- en la alergia de tipo inmediato a ß-lactámicos, particularmente en pacientes con historia clínica de alergia y pruebas cutáneas negativas.
Material y métodos: En un estudio multicéntrico que abarca 10 centros europeos, 181 pacientes con historia de alergia de tipo inmediato a ß-lactámicos y 81 controles, hemos evaluado la eficiencia diagnóstica de las determinaciones de la IgE específica y de 2 tests celulares basados en la reactividad de los basófilos, el TAB y el ensayo de liberación de sulfidoleucotrienos.
Resultados: Con Flow-CAST, la sensibilidad varió para cada alérgeno ß-lactámico individualmente de 16% para el PPL a 33% para la amoxicilina, alcanzando el 50% cuando se consideraban los 5 alérgenos. En los pacientes alérgicos a los ß-lactámicos con resultados negativos en las pruebas cutáneas (22,8%), el Flow-CAST mostró resultados positivos para al menos 1 de 5 alérgenos en el 37% de los pacientes. La especificidad varió de 89% to 97%, dependiendo del alérgeno evaluado.
En el CAST-ELISA, la sensibilidad general en los pacientes con pruebas cutáneas positivas fue del 41,7%; en pacientes con pruebas cutáneas negativas fue del 27,9%. Estos dos test no se correlacionaban completamente, de manera que cuando todos los resultados se consideraban conjuntamente, la sensibilidad aumentó al 64,3% y la especificidad varió para la combinación de ambos test del 73% al 92%. Por otra parte, la determinación de la IgE específica en la misma población dio lugar a una menor sensibilidad del 28,3%.
Conclusiones: Un algoritmo diagnóstico incluyendo pruebas cutáneas e IgE específica, seguido de los test celulares y provocación controlada nos permitió confirmar la alergia a ß-lactámicos en el 92% de los casos. Este proceso podría también evitar dos tercios de las provocaciones controladas requeridas.

Palabras clave: Alergia de tipo inmediato a betalactámicos. Diagnóstico *in vitro*. Tests celulares. TAB. Producción de sulfidoleucotrienos.

Introduction

The drugs of the ß-lactam family, such as penicillins and cephalosporins, more commonly have allergic side effects than drugs from other families [1,2]. According to recent surveys, 5%-10% of the population in several countries are allergic to ß-lactams [3-8], and allergy to these drugs is still the most frequent cause of anaphylaxis, ahead of food and insect venoms [9-11]. Allergic reactions to ß-lactams may be immunoglobulin (Ig) E–mediated immediate-type reactions (anaphylactic shock, urticaria, or angioedema) [1,2,12-14], T cell–mediated delayed-type reactions (morbilliform exanthema), or, less often, other organic manifestations [15-18]. Some years ago, benzylpenicillin (BPN) and penicillin V were the most frequent culprit drugs, although today reactions are more common with amoxicillin and several cephalosporins [19-22]. Therefore, we can define 2 broad categories of ß-lactam–allergic patients: those who are broadly sensitive to the ß-lactam nucleus and those who are selectively sensitive to some ß-lactam side chains [20,21].

The clinical history is the first step in the diagnosis of ß-lactam allergy, although it is not always reliable. Some authors report that only 10%-20% of skin tests showed positive results for ß-lactams [1-4]. Positive skin test results are frequent, even in patients with vague histories [23,24] or in groups exposed to ß-lactams but with no history of adverse reactions [25,26]

Besides the clinical history, skin tests (prick, intradermal, or patch) with the drugs and their derivatives (penicilloyl-polylysine [PPL] and minor determinant mixture [MDM]) have been used, although a proven reaction to the drugs themselves remains the mainstay of diagnosis [4,27-31]. In vivo tests are not without side effects, particularly in patients with a history of anaphylactic shock and in those who must use the maximum recommended concentration [20,31-40]. Skin reactivity to ß-lactams often declines with time in allergic patients [41-45]. Furthermore, at least in Europe, a considerable number of patients with a history of allergy that has been confirmed by challenge tests may present negative skin test results [46-50]. In the USA, this phenomenon appears to be less common [51-56].

ß-Lactam–specific IgE tests are a new highly specific tool for confirming the clinical diagnosis [57-59]. Sensitivity is somewhat low, 30%-40% according to most reports [60-63], although it has been shown to be higher in some groups [60,63]. Furthermore, these tests are not commercially available for all ß-lactams, particularly cephalosporins. Finally, in most ß-lactam–allergic patients, the serum level of specific IgE can decline quite rapidly, the test often becoming negative within 6 months to 3 years after the last exposure [64], as confirmed by unpublished studies (de Weck, Blanca).

For these reasons, cellular tests based on basophil reactivity for diagnosis of immediate-type allergy to ß-lactams have been of interest for some time. Histamine release testing with various penicillins in allergic patients has been reported [65-71]; however, sensitivity is low [68-71], and other drawbacks have prevented the technique from becoming a routine or widely used diagnostic test in this allergy. Recently, a commercially available sulfidoleukotriene release test (CAST-ELISA, Bühlmann Laboratories AG, Allschwil, Switzerland), which has proved useful in the diagnosis of IgE-mediated allergies to inhalants, foods, insect venoms, and several drugs [72], has also been evaluated in ß-lactam allergy. After a number of anecdotal reports [73-87], 2 studies have shown a relatively high specificity but low sensitivity (47.7% [62] and 34.6% [78]).

As for basophil reactivity, early attempts involved microscopic evaluation of basophil degranulation [79,80], and this technique has also been applied to diagnosis of ß-lactam allergy [81], although with minimal success. The development of flow-cytometric techniques to follow the expression of

activation markers such as CD63 or CD203c on the membrane of activated basophils [82-85] has opened new perspectives. Following some anecdotal case reports, a first systematic study on 60 ß-lactam–allergic patients and 30 controls was published by Sanz et al in 2002 [86,87]. This report was soon confirmed by Torres et al [88] in a study involving 70 patients and 40 controls. Other preliminary reports, however, were less enthusiastic [89-91].

The European Network for Drug Allergy (ENDA) therefore felt it necessary to organize a multicenter study to clinically evaluate and, if possible, validate these 2 new tests in the diagnosis of ß-lactam allergy. The study was performed in 10 European allergology centers, most of which had broad experience in the diagnosis and management of this allergy. The study centers followed the same detailed protocol, and the individual clinical and laboratory data were reported on similar forms. To our knowledge, this is the first study of its kind on cellular diagnostic tests in allergy.

Materials and Methods

Patients

A total of 181 patients (88 males [48.6%] and 93 females [51.4%] aged between 16 and 81 years [mean 53.6 years]) with a history of immediate-type allergy to ß-lactams were recruited in 10 different groups between May 2003 and May 2006. Complete clinical and laboratory data were obtained according to the ENDA protocol for all the patients and are evaluated here. Detailed clinical information was obtained on atopic status (22/171, 12.8%), history of allergic reactions to ß-lactams, culprit drugs, presence of symptoms, and eventual therapy at the time of testing as well as the time elapsed since the last clinical reaction to ß-lactams. When appropriate, the results for re-exposure and provocation were also given. The history was considered as positive when the clinical reaction was documented by a physician, and when more than one event was recorded.

Similar data were obtained from 81 control patients in 7 groups. Of these, 76 had no history of allergic reaction and 5 had a history of allergic reaction to other drugs. Twenty patients (24.7%) were atopic with the corresponding history, positive skin test results, and specific IgE to some inhalant allergens. They had tolerated ß-lactams in the past and 54 patients had negative challenge results at the time of the tests.

Skin Tests

Skin tests (prick and, when necessary, intradermal) were performed according to the usual techniques and recommendations [4,27]. The ß-lactam allergens used for all groups were PPL (max 5×10^{-5} mol/L; Allergopharma, Hamburg, Germany), MDM (max 2×10^{-2} mol/L; Allergopharma), BPN (max 1000 U/mL), amoxicillin (max 20 mg/mL), and ampicillin (max 20 mg/mL). In some cases, cephalosporins such as cefuroxime (max 1 mg/mL), cefazolin (max 1 mg/mL), or other cephalosporins were also used in skin tests. In all cases, the study was started with prick tests at the indicated concentrations or dilutions, followed, if a negative result was obtained, by intradermal tests. If the patient presented a positive prick test result to an allergen, the series of in vivo tests with that allergen was interrupted. Histamine (10 mg/mL) and saline solution (0.9%) were used as positive and negative controls, respectively. Wheals 3 mm greater than the negative control for prick testing and 5 to 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 most cases using the CAP FEIA technique (Pharmacia, Uppsala, Sweden) with BPN, penicillin V, and amoxicillin. In patients with a reaction to cephalosporins, cefaclor was also tested. All results higher than 0.35 kU_A/L were considered positive.

Flow-Cytometric Basophil Activation Assay (Flow-CAST)

For this study, all reagents (Flow-CAST) and ß-lactam allergens were provided by the manufacturer (Bühlmann Laboratories AG). The technique was performed following the manufacturer's instructions and has been fully described elsewhere [86,90]. Briefly, blood was collected in 6-mL EDTA tubes and stored at 2° C-8°C; the test was carried out within 24 hours of sample extraction. One 6-mL EDTA tube enables up to 5 allergens to be tested in 2 concentrations. The tubes were centrifuged at 200*g* for 5 min at 4°C. The supernatant (plasma leukocytes) was pipetted and centrifuged again at 500*g* for 10 min at 4°C. It was then decanted and the cell pellet was resuspended in 100 µL of HEPES/calcium buffer (stimulation buffer; HEPES 20 mM, NaCl 133 mM, KCl 5 mM, $CaCl_2$ 7 mM, $MgCl_2$ 3.5 mM, HAS 1 mg/mL, pH 7.4) containing interleukin (IL) 3 (20 ng/mL). Subsequently, 50 µL of reconstituted solutions of BPN (final concentrations 2 and 0.4 mg/mL), PPL (final concentrations 0.025 and 0.00 5 mg/mL), MDM (final concentrations 0.5 and 0.1 mg/mL), amoxicillin (final concentrations 1.25 and 0.25 mg/mL), and ampicillin (final concentrations 1.25 and 0.25 mg/mL) were added to 50 µL of cell suspension in microplate wells. Patients with reactions to cephalosporins were also tested with the culprit drug at various final concentrations that were usually no higher than 2 mg/mL. These final concentrations were chosen following preliminary assays and dose response-curves (data not shown). A monoclonal anti-IgE receptor antibody (Bühlmann Laboratories) at a concentration of 1 µg/mL was used as a positive control.

In order to evaluate baseline values without stimulation, 50 µL of stimulation buffer was added to another well and 50 µL of cell suspension was added to all wells. The microplate was covered with an adhesive plastic sheet and incubated for 40 min at 37°C. The reaction was stopped by adding 100 µL of HEPES buffer (pH 7.3) containing EDTA (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, EDTA 0.27 mM) but with no calcium or magnesium (washing buffer). The plates were then centrifuged at 1000*g* for 5 min at 4°C, and 100 µL of the supernatants was pipetted and saved for sulfidoleukotriene analysis by CAST-ELISA (see below). The basophils from the cell pellet were double-labeled by adding 20 µL of a mixture of anti-CD63 phycoerythrin-labeled antibody diluted at 1:80

and of anti-IgE fluorescein isothiocyanate (FITC)-labeled antibody diluted at 1:60 in washing buffer. After incubation for 30 min at 2°C-8°C (protected from light exposure), 4 mL of an erythrolytic reagent (lysing reagent) was added to each tube and left at room temperature for 5 min. Cell lysis was stopped with 1 mL of washing buffer. After centrifuging for another 5 min at 1000*g*, the supernatants were decanted and 500 µL of washing buffer added to each tube, which were then gently shaken before flow-cytometric analysis.

Flow-cytometric analysis was performed at 488 nm on a FACScan flow cytometer (Becton Dickinson, Madrid, Spain) or similar instrument equipped with one or more argon lasers. The results were analyzed using CellQuest (Becton Dickinson, Madrid, Spain) or an equivalent application. On the histogram (defined by forward scatter and side scatter), a first cell gate was defined by a bit map around the lymphocytes. A second gate was defined around cells showing high-density fluorescence with anti-IgE FITC, identifying them as basophils. In each assay, at least 500 basophils were counted. The other parameter analyzed on the identified basophils was CD63, as described elsewhere [82,86].

Sulfidoleukotriene Assay (CAST-ELISA)

The assay measures the amount of sulfidoleukotriene (LTC_4, LTD_4, LTE_4) produced by leukocytes after in vitro stimulation by allergens. Following isolation of leukocytes and incubation with various ß-lactam allergens, as described above, 100 µL of supernatant was collected from all wells and frozen at –20°C until analysis. Within one month, the supernatants were analyzed for sulfidoleukotrienes by ELISA according to the manufacturer's instructions (CAST-ELISA, Bühlmann Laboratories AG).

Statistical Analysis

Non-normally distributed variables were compared using the Mann-Whitney test. Qualitative data were compared using the chi-square test with a Yates correction when necessary. All *P* values were 2-tailed and a value of ≤.05 was considered statistically significant. The specificity and sensitivity values were obtained by analysis of different cut-off points in receiver operating characteristic (ROC) curves. Sensitivity was calculated as the number of positive cases detected by the respective techniques in the patient group, and specificity as the number of negative cases detected by the same techniques in the control group. The statistical analysis was performed using SPSS version 10.0 (SPSS Inc, Chicago, Illinois, USA).

Results

During this multicenter study, 181 case reports were collected from 10 groups. Seven groups contributed more than 10 patients and 10 controls each. Due to incomplete or unclear clinical data, 3 cases could not be evaluated and were excluded from the final analysis (178 cases) (Table 1). The most frequent clinical manifestations were anaphylactic shock (118, 56%), angioedema (28, 12%), urticaria (58, 28%), and morbilliform exanthema (4, 2%). The culprit drugs were BPN (11 cases, 6.2%), penicillin V (3 cases, 1.7%), amoxicillin (131 cases, 72.4%), ampicillin (13 cases, 7.2%), and some cephalosporins (17 cases, 9.4%). Fifty-three patients (29.2%) experienced more than 1 clinical allergic event following administration of ß-lactams and/or reacted to a challenge with a ß-lactam.

Positive skin test results to a ß-lactam allergen were reported in 132/170 cases (77.6%) (Table 1). Of the 132 patients with positive skin test results, BPN tests were positive in 26/138 cases (18.8%), PPL tests in 21/157 (13.4%), MDM

Table 1. Patient Groups and Results of Skin Tests, BAT, and CAST

Group	Patients Cases	ST-pos	ST-neg	Sensitivity	sIgE-pos	sIgE-neg	Sensitivity	BAT-pos	BAT-neg	Sensitivity	CAST-pos	CAST-neg	Sensitivy
AAChen	5					4		2	3		3	2	60.0%
ANCona	10	10	0	100%		10		6	4	60.0%	3	7	30.0%
ANGers	8	8	0	100%				2	6	25.0%	3	5	37.5 %
GRAz	1	1	0			1			1			1	
FLOrence	7	4						6	1	86.0%	5	2	71.4%
LIMoges	8	5	3	62.5 %		5		3	5	38.0%			
MALaga	28	20	7	74.1%	4	16	20.0%	12	14	46.0%	10	14	41.7%
NANcy	1	1							1				1
PAMplona	93	67	25	72.8 %	28	60	31.8 %	40	52	43.0%	34	55	38.2%
ROMe	20	16	3	80.0%	8	12	40.0%	15	5	75.0%	7	13	35.0%
Total	181	130	38	77.4 %	2	108	28.3 %	86	92	48.3 %	65	100	39.3%

Abbreviations: BAT, basophil activation test; CAST, cellular allergen stimulation test; neg, negative; pos, positive; sIg, specific immunoglobulin; ST, skin test.

Table 2. Individual Case Results for Cephalosporins

ENDA No	Events	Time Elapsed	Skin Tests Betalactams BPN	PPL	MDM	AMX	AMP	Other	Spec IgE to BL	BAT CAST Baseline	CAST Positive Control	BPN 2	BPN 0.4	PPL 0.025	PPL 0.005	MDM 0.5	MDM 0.1	AMX 1.2	AMX 0.25	AMP 1.2	AMP 0.25	Cephalosporins	
14	URT AS CFT		Neg	Neg	Neg	Neg	Neg	CEFPR +		1	0	1	0	0	0	0	1	1	0	0	1	CEF +	
	Sept-02 Jan-03	8 mo								141	1496	176	162	159	131	130	147	124	167	164	140		
15	URT CEF	14 mo	Neg	ID +	ID +	Neg	ND	CEFID +		1	29	2	0	1	1	0	0	0	1	2	2	CEF 0	
	Apr-02									125	882	0	65	0	121	75	0	0	0	0	0	CEF 0	
16	AS CFT	3 mo	ID +	ND	ND	PR +	ID +	CEF PR +		0		0	0.9	1.5	0.2	0	0.7	0.9	0	0.5	0.7		
	Nov-03									170	1145	182	194	202	176	164	140	178	134	192	185		
32	AS CEF	9 mo		Neg	Neg	Neg	Neg	CEF PR +	Neg	1.3	92.8	0.6	0.9	2.8	1.9	1.4	0.8	5.3	2.1	1.6	0.8	CEF 1.7	
	Oct-02									282	3981	479	427	525	138	501	776	617	1585	95	339	CEF 191	
36	AS CEF	3 mo		Neg	Neg	Neg	Neg	CEF PR +	Neg	1.8	25.5	1.4	2.5	2.1	3.4	1.7	2,1	1.6	1.7	1.6	5.3	CEF 2.5	
	Jul-03									126	3548	155	69	72	166	107	141	148	138	105	148	CEF155	
40	AS CEF	2 mo		Neg	Neg	Neg	Neg	CEF PR +	Neg	5.7	8.1	1.4	3.0	3.5	3.4	2.4	1.7	1.6	24	1.8	2.2	CEF 1.8	
	May-03																						
47	MEX AMX	9 mo	+ late	Neg	Neg	+ late		CEF Neg		20.3	43.3	10.9	21.8	15.8	12.5	15.5	8.5	10.4	19.7	13.4	12.2	CEF 2.9	CEF 8.1
	May-03									1828	3200	1782	1280	2811	1714	2331	###	2194	2582	2560	2468	2628	2857
48	ZRT AMP	3 mo	ID +	Neg	Neg	Neg	+ID	CEF Neg		1.4	46.6	0.8	1.6	0.8	0.9	1.3	1.0	1.9	0.3	0.3	5.8	CEF 0.5	CEF 0.3
	Mar-04									331	3200	40	293	315	253	180	168	184	311	173	270	123	45
51	AS AX	4 mo				+		CEF Neg		0.8	26.0						20.0	2.0					
	Oct																						
52	URT AE AMP	36 mo	+				Neg	CEF Neg		0.9	8.6	0.4	0.8				0.4	0.4				CEF 0.4	
	2001																						
54	AS AMX	3mo	Neg			Pos		CEF Pos	Neg AX BPN	1.5	0.8						1.2	1.0				CEF 1.0	CEF 0.9
	Jun-04																						
55	URT CEF	12 mo	Neg			Neg		CEF Pos	Neg AX BPN	5.6	91.2						10.1	10.2				CEF16.8	12.2
	2003																						
56	URT CEF	4 mo	Neg			Neg		CEF Neg	Neg AX BPN	20.4	98.0						38.0	34.0				CEF 28.0	CEF 23.0
	May-04																						
57	URT AE	10 yrs	Neg			Neg		CEF Neg	Neg AX BPN	0.7	22.0	1.2	1.7				0.6	0.2				CEF 5.0	CEF 0.6
	1994																						
58	AS CFT	12 mo	Neg			Neg		CFT Neg	Neg AX BPN	0.7	20.4	0.9	0.2				2.4	1.7				CFT 1.5	CFT 0.5
	Mar-04																						
64	AS CEF	1 mo	ID +	Neg	Neg	Neg	Neg	CEF Neg	Neg	1.8	66.4	0.9	0.6	1.0	0.3	0.6	0.7	1.3	0.9	0.6	0.6	CEF 0.3	0.3
	Feb-99									4	1870	4	4	4	4	4	4	4	4	4	4	4	4
68	AS CEF	2 mo	Neg	Neg	Neg	Neg	Neg	CEF PR +	Pos	3.4	58.0	2.8	2.6	3.7	3.8	1.9	2.0	1.1	4.4	2.6	2.9	CEF 19.5	20.1
	Jun-99									107	831	100	59	4	4	4	4	4	4	93	100	4	4
85	URT AE CEF	1 mo	Neg	ID +	Neg	Neg	Neg	CEF Neg	Pos	4.4	45.9	6.7	7.0	5.4	7.6	6.1	4.8	2.7	6.4	7.4	4.4	CEF 5.4	12.6
	Oct-99									107	3032	52	52	52	127	25	25	52	195	196	25	25	25
99	AS CFT	1 mo	Neg	Neg	Neg	Neg	Neg	CFT ID +	Pos	1.9	72.7	12.6	2.2	0.7	0.8	1.9	1.9	3.7	11.7	2.7	8.9	CFT 1.0	0.8
	Jan-00									4	2520	168	25	59	114	4	107	4	4	4	4	189	93
109	URT AE CEF	1 mo	Neg	Neg	ID +	Neg	Neg	CEF Neg	Neg	14.3	27.9	0.4	0.2	0.2	0	0.3	0.2	0.6	0	0.2	0.3	CEF 0.2	0.5
	May-00									87	1206	184	145	92	206	150	192	95	77	222	115	265	267
114	AS CEF	8 mo	Neg	Neg	Neg	Neg	Neg	CEF Pos	Neg	3.2	78.3	0.8	1.7	1.8	1.8	1.4	2.1	4.5	2.1	0.9	2.3	CEF 3.8	3.5
	Feb-00									68	3751	93	80	52	21	40	20	117	82	46	78	182	103
140	AS CEF	1 mo	Neg	Neg	Neg	Neg	Neg	CEF pos		1.0	1.4	0	0	0	1.4	0.8	1.3	0.9	1.9	0.9	0	CEF 1.9	1.5
	Mar-04																						
157	AS AMX	1 mo		Neg	ID +	PR +				2.7	93.2	23.2	26.0	4.7	2.5	20.8	20	16.7	19.6	19.6	14.6	CFX 3.1	CFT 2.9
	Nov-05									299	3672	160	182	73	47	221	234	121	223	191	148	45	161
158	AS AMX	8 mo		Neg	Neg	PR+				3.5	46.0	3.7	4.9	5.6	5.3	3.8	4.2	4.5	4	3.7	3.4	CFX 5.6	CFT 4.2
	Jul-05									59	3184	58	54	67	37	60	52	62	41	54	57	50	55
159	AS Feb-04 BPN	6 mo		Neg	Neg	Neg		CFX PR+		4.2	20.3	3.8	3.9	7.2	3.2	4.6	6.2	4.9	3.7	4.9	4.8	CFX 3.6	CFT 3.7
	AS Aug-05 CFX									392	3324	330	312	295	290	300	320	403	387	311	217	276	353
160	AS AX-CLV	3 mo		Neg	Neg	ID +				3.6	95.3	3.6	2.7	3.7	2.8	3.4	2.9	3.1	3.1	3.4	3.4	CFX 2.8	CFT 2.8
	Dec-05									73.5	3441	64	163	80	63	59	58	322	88	59	71	66	62
161	AS Mar-99 AMX	12 mo		Neg	Neg	PR +	PR +			2.2	77.3	1.9	1.4	3	2.1	1	1.8	2.1	2.3	3.1	2.1	CFX 2.2	CFT 0.8
	AS Mar-05 AMX									110	3212	63	114	102	100	90	96	104	96	104	110	91	125

ST specific IgE–positive

Provocation positive or 2 recorded e

FLOW–CAST –positive

CAST–positive

Abbreviations: AE, angioedema; AMP, ampicillin; AMX, amoxicillin; AS, asthma; BPN, penicillin G; CEF, cefazolin; CFX, cefuroxime; CFT, ceftazidime; ID, intradermal; MDM, minor determinant mixture; ND, not determined; Neg, negative; Pos, positive; PPL, penicilloyl-polylysine; PR, prick test; URT, urticaria.

Table 3. Examples of Individual Case Results

Group	ENDA No	Events	Time Elapsed	Provocation	Skin Tests Betalactams BPN	PPL	MDM	AMX	AMP	Spec IgE to BL	FLOW CAST Baseline	Pos control	BPN 2	0.4	PPL 0.025	0.005	MDM 0.5	0.1	AMX 1.2	0.25	AMP 1.2	0.25
A. Multiple FLOW- and CAST –positive																						
ANG	13	AS AMX 2001. Aug-03	2 mo	ND	ID +	ND	ND	PR +	ND		1	24	31	17	2.0	0.3	5.0	8	30	21	25,0	23
											68	887	549	624	65	28	184	160	1323	1704	1860	1556
PAM	101	AS AMX 1996	4 yrs	ND	Neg	Neg	Neg	ID +	Pos	Neg	26.5	53.1	36.3	31.3	41.8	48.5	68.5	47.8	67.9	65.8	64.7	50.9
											43	2779	216	109	784	770	907	269	1654	1584	1819	1551
B. Multiple FLOW -positive, CAST-negative																						
FLO	43	URT AMX Jul-02	13 mo	ND		ND	ND	ND	ND		2.9	71.4	45.5	20.3	27.7	34.0	21.6	11.4	18.4	2.5	5.7	2.1
											269	2300	231	200	213	213	345	372	369	446	390	337
MAL	75	AS BPN 1974	25 yrs	ND	Neg	ID +	Neg	Neg	Neg	Neg	5.4	67.4	19.7	24.7	0.6	1.6	10.4	14.2	15.3	23.6	14.4	14.3
											4	2478	4	4	4	4	4	4	4	4	4	4
C. Multiple CAST -positive, FLOW -negative																						
PAM	113	URT AMX Jun-00	3 mo	AX 100 URT	Neg	Neg	Neg	PR +	Neg	Neg	0.9	10.9	0.1	0.1	0	0.2	0.1	0.2	0.1	0.3	0.1	0.5
											167	170	350	330	530	3640	1050	930	270	750	300	325
PAM	97	AS AMX Oct-99	4 mo	ND	Neg	Neg	Neg	ID +	ID +	Pos	1.2	70.6	7.6	1.3	2.1	1.9	1.5	1.7	2.0	1.4	2.0	2.2
											4	647	1330	756	879	564	599	694	585	606	742	524
D . One to two FLOW - and CAST -positive																						
PAM	78	AS AMX Apr-99	2 mo	ND	Neg	Neg	Neg	PR +	Neg	Neg	6.6	56.6	8.7	8.9	6.4	8.6	6.9	4.8	15.6	13.2	12.3	13.9
											4	6080	66	59	18	32	4	4	141	79	189	66
MAL	37	AS AMX Jul-03	3 mo	ND		Neg	Neg	PR +		Neg	6	45.4	2.9	16.4	12.3	4.1	9.4	1.3	1.9	1.0	0.2	0.2
											83	5754	178	479	912	525	138	229	182	132	155	204
E. One or two FLOW -positive, CAST -negative																						
PAM	61	AS AMX Jan-00	2 mo	ND	Neg	Neg	Neg	PR +	Neg	Pos	5.9	44.7	2.5	3.6	22.1	18.6	4.3	7.1	3.8	4.5	2.5	2.0
											4	681	4	4	4	4	4	4	4	4	4	4
PAM	63	AS AMX Feb-99	1 mo	ND	ID +	Neg	Neg	Neg	Neg	Neg	0	65.4	0.4	0.6	2.4	2.1	1.2	1.2	8.8	9.1	1.2	2.1
											4	1166	4	4	4	4	4	4	4	4	4	4
F. One or two CAST -positive, FLOW -negative																						
PAM	130	AS AMX Jan-02	2 mo	AX 500 Pos UR T AS	Neg	Neg	Neg	Neg	Neg	Neg	10.7	92.6	2.7	6.5	5.4	7.5	4.3	6.3	7.3	7.1	5.0	4.8
											45	7923	44	77	44	29	63	200	86	13	47	183
	72	AS AMX Mar-99	2 mo	ND	Neg	Neg	Neg	ID +	Neg	Pos	4.5	74.7	2.9	3.9	5.9	3.7	3.0	3.2	3.1	2.8	3.5	4.4
											45	6000	175	134	45	189	134	45	120	120	86	93
G. All FLOW- and CAST -negative																						
	65	AS AMX Jan-99	3 mo	ND	Neg	Neg	Neg	PR +	PR +	Pos	3.0	81.6	3.7	4.2	3.4	2.8	1.5	2.4	1.9	2.8	1.2	2.2
											53	2048	121	107	148	86	107	66	127	86	80	114
	103	URT AMX Mar-00	1 mo	AX 500 Pos UR T	Neg	Neg	Neg	ID +	Pos	Neg	9.0	76.6	6.3	4.4	1.3	0.9	0.7	0.2	6.4	2.9	6.6	2.5
											59	4901	55	34	41	17	0	0	89	55	67	19
	127	URT AE AMX Jul-94 Nov-00	5 mo	AX 100 Pos UR T	Neg	Neg	Neg	Neg	Neg	Neg	2.3	71.3	1.1	1.4	1.9	1.1	2.6	1.9	1.3	2.2	2.3	1.7
											199	2536	206	304	282	0	360	268	395	280	299	0

Skin test specific IgE–positive

Provocation positive or 2 recorded events

FLOW–CAST–positive

CAST–positive

Abbreviations: AE, angioedema; AS, asthma; AMX, amoxicillin; BPN, penicillin G; ID, intradermal; MDM, minor determinant mixture; ND, not determined; Neg, negative; PPL, penicilloyl-polylysine; PR, prick test; URT, urticaria.

tests in 31/153 (20.3%), amoxicillin tests in 90/162 (55.6%), and ampicillin tests in 41/147 (27.9%). In addition, the results of skin tests with cephalosporins (Table 2) were positive in 11 out of 16 patients (68.7%) for whom a cephalosporin was the culprit drug. All patients with positive skin test results for ampicillin had positive results for amoxicillin.

Of the 170 patients with skin test records, 38 (22.4%) had negative results for BPN, PPL, MDM, amoxicillin, and ampicillin, despite a clinical history of allergy. Eight additional patients had positive skin test results, although only with the culprit cephalosporin. Of the 38 patients whose skin test results were negative to the 5 standard ß-lactams, 19 (50%) had a proven clinical allergy, as demonstrated by a positive challenge result and/or a record of multiple clinical events upon exposure to ß-lactams.

Among the 148 patients for whom results of specific IgE determinations were available, only 40 (27.0%) (Table 1) were found to be positive (> 0.35 kU_A/L) to BPN, PNV, amoxicillin, or ampicillin.

The results for Flow-CAST are presented in Table 1. Some individual examples are shown in Table 3. The optimal cut-off points in terms of sensitivity and specificity to distinguish positive from negative results were established by ROC curves for each ß-lactam allergen, each allergen concentration, and all the possible allergen concentrations, using either net basophil activation values from 3% to > 5% or gross values from > 5% to > 15% and stimulation indexes (SI, test value/baseline value) varying between 1.2 and 3. This very extensive analysis (results not shown) revealed that the optimal cut-off values for Flow-CAST were found at gross activation values > 5% and SIs of around 2 (see the example for amoxicillin in the Figure). These cutoffs were used for further analysis of the results.

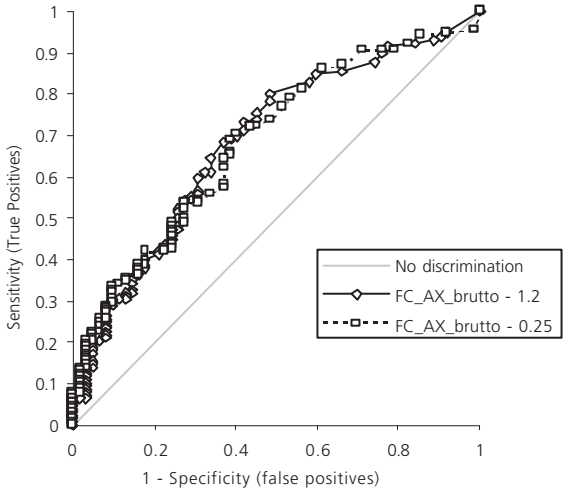

Figure. Determination of receiver operating characteristic curve - Example Determination of sensitivity and specificity in basophil activation test for amoxicillin at concentrations of 1.25 and 0.25 mg/mL.

According to these criteria, 86 of the 178 (48.3%) patients tested (Table 1) were considered positive, since they reacted to at least 1 concentration of any of the 5 allergens tested. In 52 (60.5%) of these Flow-CAST–positive cases, the patient reacted to more than 1 concentration and/or 1 ß-lactam allergen. Of 121 skin test–positive patients, 65 (53.7%) were Flow-CAST-positive. Of the 46 skin test–negative results, 17 (37.0%) were BAT-positive. BPN was positive in 20%, PPL in 16%, MDM in 19%, amoxicillin in 33%, and ampicillin in 21% of all cases (Table 4). When the allergens were in combination, sensitivities increased, reaching 40% for PPL and amoxicillin, 44% for PPL and MDM and amoxicillin, and 51% for all 5 allergens together (Table 4). For the cephalosporins, only 2 of 13 patients with a positive skin prick test result were BAT-positive (Table 2), although 5 were also positive to at least 1 of the 5 standard allergens.

The overall results for CAST-ELISA are given in Table 1. The optimal cut-off point was also established by extensive ROC analysis, using net sulfidoleukotriene values of between 70 pg/mL and 300 pg/mL, and SIs of between 1.3 and 3, and investigating each ß-lactam allergen separately or in combination (results not shown). The net optimal cutoff was found to be 100 pg/mL to 130 pg/mL for sulfidoleukotriene release, depending on the allergen, and adding an SI did not improve the results. For practical reasons, a net cut-off of 100 pg/mL was chosen for all further evaluations. Patients were considered positive when they reacted to at least 1 concentration of any of the 5 allergens tested; however, in 34 (52.2%) of these 65 positive cases, the patient reacted to more than 1 concentration and/or 1 allergen**.** Overall, of the 115 skin test–positive patients tested with CAST-ELISA, 48 (41.7%) had positive results. Of the 43 skin test–negative patients tested, 12 (27.9%) had positive results with CAST-ELISA. Positive results with CAST-ELISA were observed for BPN in 19/152 patients (12.5%), for PPL in 34/149 patients (22.8%), for MDM in 27/154 (17.5%), for amoxicillin in 36/159 patients (22.6%), and for ampicillin in 33/159 patients (20.8%). CAST-ELISA with a cephalosporin allergen was positive in 17 cases (23.5%).

Of 157 patients tested with Flow-CAST and CAST-ELISA, 31 (19.7%) had positive results for both tests, 43 (27.4%) for Flow-CAST alone, and 27 (17.2%) for CAST-ELISA alone. Accordingly, the addition of CAST-ELISA to Flow-CAST increases the sensitivity of these in vitro tests to 101/157 (64.3%) (Table 5). Optimal sensitivity is achieved by testing with more than 1 allergen (Table 4), since, in various combinations, it raises sensitivity from 10%-25% for individual allergens to about 50%. The combination of Flow-CAST and CAST-ELISA, known as CAST-Combi (Bühlmann Laboratories AG), further increases sensitivity by about 10%-15%, irrespective of the combination of allergens used (Table 6).

Of the 171 patients with a history of clinical allergy to the ß-lactams tested, 21 (12.8%) had a documented personal history of atopy. This, however, does not seem to influence the sensitivity of the different diagnostic tests used.

The results for specificity with Flow-CAST and CAST-ELISA in control patients are shown in Table 7. Of the 81 controls, 20 (24.7%) had a personal history of atopic disease,

Table 4. Sensitivity and Specificity of BAT (Flow-CAST) for Each Allergen and Combinations Thereof

Cutoff >5%	SE	SP	SE	SP	SE	SP	SE	SP	SE	SP
Allergen	BPN		PPL		MDM		AMX		AMP	
Higher concentration positive	15	97	11	96	13	94	25	96	15	97
Lower concentration positive	10	96	11	96	13	94	26	96	17	95
Both positive (duo)	5	99	5	99	6	97	17	96	11	99
E/O positive	20	95	16	93	19	91	33	96	21	93
SE with skin test positive (Pts)	20		14		20		35		20	
SE with skin test negative (Pts)	18		19		9		22		17	

	SE	SP	SE	SP	SE	SP	SE	SP
Allergens	BPN+PPL		PPL+MDM		BPN+MDM		BPN+PPL+MDM	
Duo for ≥1 allergen	9	98	9	96	8	96	11	95
E/O for ≥1 allergen	30	91	27	89	29	90	35	87
SE with skin test positive (Pts)	31		28		30		38	
SE with skin test negative (Pts)	25		22		24		28	

	SE	SP	SE	SP	SE	SP	SE	SP
Allergens	BPN+AMX		PPL+AMX		MDM+AMX		AMX+AMP	
Duo for ≥1 allergen	18	96	20	95	18	95	18	96
E/O for ≥1 allergen	38	93	40	93	37	91	37	94
SE with skin test positive (Pts)	41		43		40		37	
SE with skin test negatve (Pts)	28		30		26		28	

	SE	SP	SE	SP	SE	SP	SE	SP
Allergens	BPN+PPL+AMX		PPL+AMX+MDM		PPL+AMX+AMI		AMX+AMP+MDM	
Duo for ≥1 allergen	20		21	94	21	95	20	95
E/O for ≥1 allergen	95		44	89	43	90	40	89
SE with skin test positive (Pts)	49		47		44		41	
SE with skin test negative (Pts)	33		35		35		33	

	SE	SP	SE	SP
Allergens	BPN+PPL+AMX+MDM		BPN+PPL+AMX+AMP	
Duo for ≥1 allergen	22	94	22	95
E/O for ≥1 allergen	48	87	47	88
SE with skin test positive (Pts)	53		50	
SE with skin test negative (Pts)	37		37	

	SE	SP
Allergens	BPN+PPL+AMX+AMP+MD	
Duo for ≥1 allergen	23	94
E/O for ≥1 allergen	51	84
SE with skin test positive (Pts)	54	
SE with skin test negative (Pts)	41	

Abbreviations: AMP, ampicillin; AMX, amoxicillin; BPN, penicillin G; Duo, both positive; E/O, either/or positive; MDM, minor determinant mixture; PPL, penicilloyl-polylysine; Pts, patients; SE, sensitivity; SP, specificity.

Table 5. Correlations: BAT and CAST Results

Group	Patients, Cases	BAT-pos CAST-pos	BAT-pos CAST-neg	BAT-neg CAST-post	BAT-neg CAST-neg
AAChen	5	1	1		3
ANCona	10		6		2
ANGers	8	2		1	5
GRAz1					1
FLOrence	7	4	2	1	
MALaga	20	3	5	4	8
PAMplona	86	17	18	17	34
ROMe	20	4	11	2	3
Total	157	31	43	27	56

BAT- and/or CAST-pos,101/157 (64.3%); CAST-pos alone, 27/157 (17.2%); CAST-pos, 58/157 (36.9%); BAT-pos, 74/157 (47.1%).
Abbreviations: BAT, basophil activation test; CAST, cellular antigen stimulation test; neg, negative; pos, positive.

Table 6. Sensitivity and Specificity in Function of Culprit Drug

178 Pts. (All) 81 Ctrls.	AMX		AMX+PPL+MDM		BPN+PPL+MDM		AMX+AMP+BPN+PPL+MD	
	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)
Flow-CAST	25.4	96.3	41.6	88.9	33.7	87.3	47.8	82.7
CAST	24.3	94.5	32.9	84.9	29.7	83.6	41.8	76.7
CAST COMBI	37.3	92.6	52.8	81.5	47.8	81.0	63.5	72.8
131 Pts. (AMX) 81 Ctrls.	AMX (Culprit Drug)		AMX+AMPI		AMX+PPL+MDM		AMX+AMP+BPN+PPL+MD	
	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)
Flow-CAST	27.3	96.3	31.1	91.4	41.7	88.9	46.2	82.7
CAST	28.4	94.5	32.2	90.4	37.3	84.9	44.1	76.7
CAST COMBI	44.9	91.8	47,0	85.2	55.3	81.5	66.1	69.9
13 Pts. (AMP) 81 Ctrls.	AMP (Culprit Drug)		AMP+AMX		AMP+PPL+MDM		AMX+AMP+BPN+PPL+MD	
	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)
Flow-CAST	33.3	90.7	42.9	91.4	57.1	82.7	64.3	82.7
CAST	9.1	90.4	18.2	90.4	8.0	82.2	41.7	76.7
CAST COMBI	41.7	84.0	42.9	85.2	57.1	76.3	64.3	72.8
14 Pts. (BPN) 81 Ctrls.	BPN (Culprit Drug)		BPN+AMX		BPN+PPL+MDM		AMX+AMP+BPN+PPL+MD	
	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)
Flow-CAST	20.0	94.9	26.7	92.6	46.7	87.3	53.3	82.7
CAST	13.3	91.8	26.7	87.7	40,0	83.6	46.7	76.7
CAST COMBI	20.0	88.6	33.3	84,0	66.7	81.0	73.3	72.8
25 Pts. (Ceph) 81 Ctrls.	AMX		AMX+PPL+MDM		BPN+PPL+MDM		AMX+AMP+BPN+PPL+MD	
	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)	SE (%)	SP (%)
Flow-CAST	19.2	96.3	30.8	88.9	23.1	87.3	38.5	82.7
CAST	5.0	94.5	9.1	84.9	13.6	83.6	27.3	76.7
CAST COMBI	19.2	92.6	30.8	81.5	26.9	81.0	42.3	72.8

Abbreviations: AMP, ampicillin; AMX, amoxicillin; BAT, basophil activation test; BPN, benzylpenicillin; CAST, cellular antigen stimulation test; Ctrls, controls; MDM, minor determinant mixture; Pts, patients; PPL, penicilloyl-polylysine; SE, sensitivity; SP, specificity.

Table 7. Skin Tests, Specific IgE, BAT, and CAST in Controls

Group	No.	ST-pos	ST-neg	IgE-pos	IgE-neg	BAT-pos	BAT-neg	C AST-pos	CAST-neg
ANCona	10		10		9		10		10
ANGers	2		2				2		2
GRAz	10		10	5	5	3	7	5	5
FIOrence	3		3			1	2		3
LIMoges	6		2				6		
MALaga	20		10		10	3	17	5	15
PAMplona	30		30	3	27	2	28	6	24
Total	81		67	8	51	9	72	16	59
Specificity			100%		86.5%		88.9%		78.7%

Abbreviations: BAT, basophil activation test; CAST, cellular activation stimulation test; Ig, immunoglobulin; neg, negative; pos, positive; ST, skin test.

although this did not seem to influence their reactivity to ß-lactams. All the control patients had negative skin test results and a negative history of clinical allergy to ß-lactams. When challenged with ß-lactams (53/81, usually with 1 g of amoxicillin), they showed no reaction at all. Regardless of the concentration tested, Flow-CAST was positive in 9/81 controls, that is, an overall specificity of 88.9%. As for the individual drugs, BPN was positive in 3/81 controls, PPL in 4/81, MDM in 3/81, amoxicillin in 2/81, and ampicillin in 5/81, resulting in specificities of 96.3%, 95.1%, 96.3%, 97.5%, and 93.8%, respectively. For CAST-ELISA, the overall rate of positive reactions in controls was 16/81, resulting in a specificity of 78.7% (Table 7). However, most of these reactions involved only 1 concentration of a single allergen. For individual allergens, the specificities were 92% (6/75) for BPN, 91.2% (6/69) for PPL, 91.2% (6/69) for MDM, 92% (6/75) for amoxicillin, and 90.7% (7/75) for ampicillin.

As expected, specificity decreases slightly when the combined results for the 5 allergens are considered together (Table 6). Depending on the combination of allergens tested, the specificity for Flow-CAST ranges from 83% to 96%, for CAST-ELISA from 77% to 95%, and for both tests combined from 73% to 92%. Specificity may be slightly higher, since our analysis includes 5 control patients with a negative history, negative skin test results, negative provocation results, and slightly positive specific IgE to BPN. Of these 5 controls, 4 had some positive results for Flow-CAST and/or CAST-ELISA, a much higher proportion than in the other controls, suggesting that these controls are sensitized to ß-lactams, albeit without symptoms. If these controls are excluded, specificity increases by 3% to 5%.

The results were also analyzed in terms of diagnostic efficiency, namely, to evaluate which combination of ß-lactam allergens and which combination of diagnostic tests provide optimal confirmation of the clinical diagnosis of ß-lactam allergy. For practical and financial reasons, combinations with the lowest number of ß-lactam allergens and highly sensitive and specific tests would be desirable. An analysis including all patients tested with 5 ß-lactam allergens (BPN, PPL, MDM, amoxicillin, ampicillin) or 3 (PPL, MDM, amoxicillin) is shown in Table 8. The main finding was that skin test sensitivity was around 70%; the addition of specific IgE determinations increases the percentage of positive patients by only 5%. On the other hand, the addition of Flow-CAST to skin tests increases sensitivity by about 10%. The use of all tests improves sensitivity to about 85%. Cellular basophil tests seem to be particularly informative in ß-lactam–allergic patients with negative skin test results, since they show a sensitivity of 28% to 51% whether they are used alone or in combination. Similar results are found when amoxicillin-sensitive patients are tested with 3 reagents only (Table 9). The sensitivities and specificities obtained are in the same order of magnitude. Many other combinations of ß-lactam allergens used have been analyzed; only the most clinically relevant are shown.

This analysis provides us with a logical sequence and algorithm for investigating patients with a clinical history of immediate-type allergic reaction to ß-lactams. A possible algorithm is shown for 124 patients in Table 10. Skin tests are the first measure needed; they are positive in 70.2% of patients. Determination of specific IgE confirms allergy in an additional 5.6% or 18.8% of patients with a negative skin-test result. Flow-CAST yields positive results in an additional 9.7% or 40% of the skin tests and specific IgE–negative patients. CAST-ELISA brings in an additional 4.8% of positive results, yielding a total of 112/124 patients (90.3%) with some objective confirmation of their clinical history of ß-lactam allergy. Of the 12 patients who were negative to all tests, 8 (66.7%) tested positive to a challenge. Undoubtedly, targeted cellular basophil tests would provide objective confirmation of the clinical diagnosis in many cases and reduce the number of challenges required.

One disadvantage of cellular basophil tests is the presence of patients who did not respond to the positive control with anti-IgE or anti-IgFcεR1. As shown in Table 11, nonresponders are present in both positive patients and in controls. In the present study, the simultaneous use of Flow-CAST and CAST-ELISA enabled us to distinguish between 2 categories of nonresponders: those who had negative results to both tests and those who had negative results to Flow-CAST but positive results to CAST-ELISA. The first are obviously true nonresponders. In the second group, however, we must ask how the 2 outcomes (flow-cytometric activation and sulfidoleukotriene production) can be different in the same incubation well and setup. It is noteworthy that of the 5 true nonresponders to anti-IgFcεR1,

Table 8. Evaluations With Different Test Combinations

Pts/Ctrls Total	181 Pts	81 Ctrls		
Flow-CAST results	178 Pts	81 Ctrls		
CAST results	158 Pts	73 Ctrls		
ST results	167 Pts	77 Ctrls		
sIgE results	150 Pts	58 Ctrls		
Culprit Drug(s) of 179 Pts	131 AX	13 AMPI	14 PenG/V	25 CEF & Others

Allergens used for CAST/Flow-CAST/ST : AMX + AMP + BPN + PPL + MDM Cutoffs: Flow-CAST : >5% CD63 / Stim. Index >2 /// CAST : >100 pg/mL Net Stimulation				
All Patients (n=178)	Pos. Result	Neg. Result	Sensitivity	Specificity
ST	121	46	72.5%	100%
sIgE	45	105	30.0%	86%
CAST	66	92	41.8%	77%
Flow-CAST	85	93	47.8%	83%
CAST-COMBI	113	65	63.5%	73%
ST + sIgE	109	35	75.7%	
ST + Flow-CAST	138	29	82.6%	
ST + CAST -COMBI	144	24	85.7%	
sIgE + Flow-CAST	91	59	60.7%	
sIgE + CAST -COMBI	110	40	73.3%	
ST + sIgE + Flow-CAST	122	22	84.7%	
ST + sIgE + CAST -COMBI	127	17	88.2%	
Negative Patients	n	Positive in:	Flow-CAST	CAST-COMBI
ST-neg	46		37.0%	50.0%
sIgE-neg	105		43.8%	61.9%
ST-neg & sIgE-neg	35		37.1%	51.4%

Allergens used for CAST/Flow-CAST/ST : AMX + PPL + MDM Cutoffs: Flow-CAST : >5% CD63 / Stimulation index > 2 /// CAST : >100 pg/mL Net Stimulation				
All Patients (n=178)	Pos. Result	Neg. Result	Sensitivity	Specificity
Skin Test (ST)	115	50	69.7%	100%
sIgE	45	105	30.0%	86%
CAST	52	106	32.9%	85%
Flow-CAST	74	104	41.6%	89%
CAST-COMBI	94	84	52.8%	82%
ST + sIgE	105	35	75.0%	
ST + Flow-CAST	134	33	80.2%	
ST + CAST -COMBI	139	28	83.2%	
sIgE + Flow-CAST	87	63	58.0%	
sIgE + CAST -COMBI	99	51	66.0%	
ST + sIgE + Flow-CAST	119	25	82.6%	
ST + sIgE + CAST -COMBI	114	20	85.1%	
Negative Patients	n	Positive in:	Flow-CAST	CAST-COMBI
ST-neg	51		27.5%	41.2%
sIgE-neg	105		40.0%	51.4%
ST-neg & sIgE-neg	39		28.2%	43.4%

Abbreviations: AMP, ampicillin; AMX, amoxicillin; BAT, basophil activation test; BPN, benzylpenicillin; CAST, cellular antigen stimulation test; Ctrls, controls; MDM, minor determinant mixture; neg, negative; pos, positive; PPL, penicilloyl-polylysine; Pts, patients; sIgE, specific immunoglobulin E; ST, skin test.

Table 9. Sensitivity and Specificity for Diagnostic Tests and Combinations of Tests in Patients Taking Amoxicillin

Example: Diagnostic Workup With AMX Patients (Allergens: AMX+PPL/MDM) Cutoffs: Flow-CAST: >5% CD63 / Stimulation index >2 /// CAST: >100 pg/mL Net Stimulation				
AMX Patients (n=131)	Pos. Result	Neg. Result	Sensitivity	Specificity
Skin Test (ST)	91	34	72.8%	100%
sIgE	32	81	28.3%	86%
CAST	43	74	36.8%	85%
Flow-CAST	54	77	41.2%	89%
CAST-COMBI	72	59	55.0%	82%
ST + sIgE	81	29	73.6%	
ST + Flow-CAST	105	20	84.0%	
ST + CAST-COMBI	111	14	88.8%	
sIgE + Flow-CAST	67	46	59.3%	
sIgE + CAST -COMBI	82	31	72.6%	
ST + sIgE + Flow-CAST	94	16	85.5%	
ST + sIgE + CAST -COMBI	99	11	90.0%	
Negative Patients	n	Positive in:	Flow-CAST	CAST-COMBI
ST-neg	34		35.3%	50.0%
sIgE-neg	81		39.5%	54.3%
ST-neg & sIgE-neg	29		37.9%	51.7%

Abbreviations: AMX, amoxicillin; CAST, cellular antigen stimulation test; Ig, immunoglobulin; MDM, minor determinant mixture; neg, negative; pos, positive; PPL, penicilloyl-polylysine; sIgE, specific immunoglobulin E.

Table 10. Summary of Diagnostic Workup According to Proposed Algorithm

Patients tested with ST, sIgE, and CAST-COMBI	
AMX	105
AMP	5
BPN	10
AMX+AMP	3
AMX+BPN	1
Total	124

Diagnostic Workup

	Positive result	Negative result	Positive result	Negative result	Positive result	Negative result	Positive result	Negative result	Positive result	ND
1. Skin test	87	37								
2. sIgE			7	30						
3. Flow-CAST					12	18				
4. CAST							6	12		
5. Provocation									8	4
Positive Diagnosis	87		94		106		112		120	

Abbreviations: AMP, ampicillin; AMX, amoxicillin; BPN, penicillin G; CAST, cellular antigen stimulation test; Ig, immunoglobulin; MDM, minor determinant mixture; ND, not determined; neg, negative; pos, positive; PPL, penicilloyl-polylysine.

2 had positive Flow-CAST results to a ß-lactam allergen. Of the 27 patents with a negative Flow-CAST control and a positive CAST-ELISA result, 10 had a positive Flow-CAST result to a ß-lactam allergen. This was observed mainly at the beginning of the study, and was apparently due to a 15% lower Ca^{2+} concentration in the reconstituted anti-IgFcεR1 antibody solution (used as the positive control) and to the fact that membrane expression of CD63 is more sensitive in some individuals than sulfidoleukotriene production (results not shown). This discrepancy was remedied in the later phase of the study.

The controversy surrounding the inclusion of nonresponders in the global evaluation of BAT studies led us to perform an evaluation based on different cutoffs for the positive control. As shown in Table 12, the cutoff chosen between 0% for basophil activation (inclusion of all nonresponders) and 15%

Table 11. Summary of Positive Control (Anti-IgE R1) Results in Patients and Controls

Group	Patients	Controls	Responders		Nonresponders		
			FLOW -pos CAST -pos	FLOW -pos CAST -ND	FLOW -neg CAST -pos	FLOW-neg CAST-neg	FLOW-neg CAST-ND
AAChen	5		1		4		
ANCona	10	10	15	1	3	1	
ANGers	8	2	5		5		
GRAz	1	10	11				
FLOrence	7	3	10				
LIMoges	8	6		12			2
MALaga	28	20	34	5	3	1	2
NANcy	1		1				
PAMplona	93	30	105	5	11		2
ROMe	20		16		1	3	
Total	180	81	200	23	27	5	6

FLOW-neg: 38/272 (13.9%)
CAST-neg: 5/232 (2.2%)
FLOW- and CAST-neg: 5/232 (2.2%)
Abbreviations: CAST, cellular antigen stimulation test; Ig, immunoglobulin; ND, not determined; neg, negative; pos, positive.

Table 12. Patients and Controls: SE and SP Are Not Dependent on Yield of Stimulation With a Positive Control

Stimulation With a Positive Control	0%		5%		8%		10%		15%	
AMX	SE	SP	SE	SP	SE	SP	SE	SP	SE	SP
Higher concentration pos.	25	96	26	96	27	96	27	96	29	95
Lower conc. pos.	26	96	26	96	27	96	26	96	28	95
Both pos.	17	96	18	96	19	96	19	96	20	95
Either neg or pos.	32	96	33	96	34	96	34	96	36	95
All 5 allergens										
Both pos.	23	94	24	93	26	93	26	93	27	92
Either neg or pos.	51	84	52	83	53	83	54	83	56	81
No. of patients/controls	178/81		166/76		157/72		150/69		140/63	

Abbreviations: neg, negative; pos, positive; SE, sensitivity; SP, specificity.

(exclusion of nonresponders) for positivity of the anti-IgE R1 control had very little effect on the sensitivity and specificity of the BAT reactions to ß-lactam allergens.

Discussion

To our knowledge, this is the first multicenter study on the diagnosis of ß-lactam allergy using not only skin tests and determination of specific IgE, but also 2 cellular tests, namely, the sulfidoleukotriene release test (CAST-ELISA) and the flow-cytometric BAT (Flow-CAST). The 10 groups participating in the study followed a common protocol. Eight of these groups each contributed at least 10 cases and/or controls, although 1 group alone contributed about 45% of the total number of patients and controls.

Almost all of the 181 penicillin-allergic patients included in the study had presented physician-documented immediate-type clinical allergic manifestations such as anaphylactic shock, urticaria, and angioedema. Only 3 patients were excluded because of an unclear or insufficiently documented clinical history. Almost 30.0% of the patients presented more than 1 clinical reaction after taking ß-lactams or reacted with an immediate-type clinical allergic reaction to a controlled ß-lactam challenge.

Of the culprit drugs, BPN was involved in only 6.2%, amoxicillin in 72.4%, ampicillin in 7.2%, and some cephalosporins in 9.4% (drug not reported 5.6%). This reflects the shift in ß-lactam prescriptions that has occurred in industrialized countries since the 1970s [19].

Skin tests were performed according to the ENDA protocol and recommendations [4,27] with 5 ß-lactam allergens (BPN, PPL, MDM, amoxicillin, and ampicillin), starting with skin prick tests followed, when negative, by intradermal skin tests. Skin tests were positive in 77.4% of those cases with a history of penicillin allergy; this rate is similar to that reported elsewhere in Spain [31], France [5], Italy [39], and Greece [49]. In North America, several studies report a lower proportion of penicillin-allergic patients showing positive skin test results [23,28,29,37,41,45,53,55]. This may be due to different inclusion criteria, the reliability of the patient's allergic history [23], and the ß-lactam reagents used in skin tests. Nowadays, it is necessary to add amoxicillin to the classical BPN, PPL and MDM set. The addition of ampicillin, on the other hand, is questionable: in our study, of the 40 patients with positive skin test results to ampicillin, 37 were also positive to amoxicillin, and only 3 to ampicillin alone. Very specific sensitization restricted to ampicillin seems rare, but has been reported [22].

Of considerable interest are patients with a positive and convincing history but negative skin test results; in our series, they amounted to 22.6%. Similar figures have been reported in cohorts from Spain (27-30%) [31,46], Italy (40.5%) [39], France (38.1%) [5], and Greece (27.8%) [49]. These European data contrast with those reported from North America, where patients with a clinical history of allergy but negative skin test results rarely, or never, respond with a clinical reaction to a ß-lactam challenge [51,52,56]. The reasons for this discrepancy remain unclear. Nevertheless, European experience shows that a convincing history of allergy to ß-lactam and negative skin test results does not rule out the need for further testing with techniques such as determination of specific IgE antibodies, cellular tests, or both. In the United States, no such recommendation has been made; therefore, American allergists make no or very little use of these additional tests.

Determination of specific IgE antibodies was positive in our study in 28.3% of patients with a clinical history of ß-lactam allergy. This appears to fall within the range reported in other European cohorts [20]. Since most ß-lactam–treated patients nowadays receive amoxicillin and a number of these patients become selectively sensitized to the amoxicillin side chain, it is important to include amoxicillin-derived reagents in the determination of specific IgE. In our patients with a positive clinical history but negative skin tests, specific IgE was only positive in 11.4% (4/35) of the cases.

The overall results of basophil activation testing using Flow-CAST are shown in Tables 1 and 4. When only the results to a single allergen are considered, the rate of positivity varies from 16% for PPL to 33% for amoxicillin. However, when all 5 ß-lactam allergens are used, an overall sensitivity of 48.3% is reached. This falls slightly to 44% when only PPL, MDM, and amoxicillin are used. These results emphasize the need to test more than one ß-lactam allergen and at least 2 concentrations in order to obtain optimal results. Ampicillin seems to be redundant, since all cases that are positive to ampicillin are also positive to amoxicillin. As shown in Table 1, the results are relatively homogeneous, since the sensitivities of the 4 groups contributing 10 or more allergic patients are 60%, 46%, 43%, and 75%. These results are obtained using a gross positivity cutoff of 5% for basophil activation and an SI ≥ 2, determined as optimal from ROC curves (see example for amoxicillin in the Figure). Slight variations in the cutoff of between 3% and 8% and/or an SI of between 1.3 and 3 had only minor effects on sensitivity in several allergen combinations (results not shown).

Interestingly, Flow-CAST was positive in 37% of 45 patients with a positive clinical history but negative skin test results. Of the 14 skin test–negative and Flow-CAST–positive patients challenged with ß-lactams, all had a positive result, but only 1 also had a positive specific IgE test result. This supports the advantage of BAT over specific IgE in this subgroup of patients.

The results shown in Table 3 emphasize the importance of Flow-CAST in the increasing number of patients for whom a cephalosporin is the suspected culprit drug. As shown in Table 3, Flow-CAST with a cephalosporin was positive in 6 cases. While some may be selectively sensitive to the culprit cephalosporin, quite a number yield a positive result to 1 or more of the 5 classic allergens, varying between 19% and 38%, depending upon the combination used (Table 6).

As for CAST-ELISA, the global results are analyzed in Tables 1 and 7. A positive CAST-ELISA result was obtained in 39.3% of the cases. This is consistent with 2 previous studies [62,78], in which the reported sensitivities were 47.7% and 34.6%, respectively. With CAST-ELISA, increasing the number of ß-lactam allergens tested also increases sensitivity: from 24.3% with amoxicillin alone to 41.8% with all 5 allergens combined (Table 6). Of 30 patients with a positive clinical history and negative skin test result, CAST-ELISA was positive in 9 (30%), thus confirming the diagnostic role of CAST-ELISA in these patients.

One may wonder whether both Flow-CAST and CAST-ELISA should be performed or whether one is redundant, since both are based on a basophil activation mechanism. Somewhat surprisingly, both in allergic patients (Table 5) and in controls (Table 11), CAST-ELISA and Flow-CAST can be dissociated. Accordingly, diagnostic sensitivity increases from 47.1% to 64.3% when CAST-ELISA is added to Flow-CAST (Table 5). The same phenomenon is observed in clinical history–positive/skin test–negative patients in whom the addition of CAST-ELISA to Flow-CAST increases sensitivity by about 10%-15% (Tables 7 and 8).

Diagnostic tests seldom reach 100% sensitivity; therefore, specificity becomes an important criterion, since high specificity guarantees the clinical significance of a positive test result. As indicated in Table 7, skin tests have an optimal specificity of 100%. However, the specificity for specific IgE, Flow-CAST, and CAST-ELISA reaches 86.5%, 88.9%, and 78.7%, respectively. The true specificity of BAT and CAST-ELISA may be slightly higher, since these controls include 5 individuals with no history of ß-lactam allergy and positive IgE results. Table 7 also shows that the results for controls in the groups that provided 10 controls or more are reasonably homogeneous.

Increasing the number of diagnostic tests used to confirm a suspected clinical allergy history improves diagnostic sensitivity and efficiency. The sensitivity and specificity results of the diagnostic tests used, either alone or in combination, considering all patients or only those with negative skin test results or only those in which amoxicillin is the culprit drug, are shown in Tables 8 and 9. A maximum sensitivity of 85%-90% is reached when all 4 tests (skin tests, ß-lactam–specific IgE, Flow-CAST, and CAST-ELISA) are used. The results of a virtual workup using the sequence skin tests→ specific IgE → Flow-CAST → CAST-ELISA (always using the next test on patients with negative results to the previous one) on 124 patients with a history of allergy to amoxicillin are shown in Table 10. At the end of this sequence, a positive test confirming the history of allergy was obtained in 112 of 124 cases (90.3%). Even then, 8 cases with entirely negative results had positive results to a controlled challenge. If only skin tests had been available, it would have been necessary to challenge 30 cases (skin test and specific IgE). The use of Flow-CAST and CAST-ELISA (18 positive cases together) would have enabled us to avoid about two-thirds of these challenges, thus reducing costs and patient discomfort.

Tables 8 to 10 show that the use of 4 tests and 5 ß-lactam allergens delivers the highest sensitivity, but also some decrease in specificity. Both the combinations shown and many others that are not shown deliver results that vary only slightly. The number of tests and ß-lactam allergens used are not indifferent in terms of work and costs involved in a routine diagnostic workup. Therefore, skin tests and ß-lactam–specific IgE, followed by BAT with 3 ß-lactam allergens (PPL, MDM, and amoxicillin), may represent a suitable practical compromise when both previous tests are negative and the clinical history is reliable. In patients for whom ß-lactam therapy is mandatory or desirable, a further workup with CAST-ELISA and/or challenge tests could be envisaged. In groups where BAT technology is not available, CAST-ELISA may be considered an alternative.

Since both skin hypersensitivity to ß-lactams [41,42] and specific IgE [43,64] decline with time, it was interesting to observe whether the time elapsed since the allergic reaction influences the results of the tests. This has been shown to be the case for BAT tests to metamizol [92] and neuromuscular blocking agents [93]. After a 12-month interval in our series, we observed that there was no marked difference in the percentages of positivity between tests performed less than 12 months after the last clinical reaction and those performed after 12 months for skin tests (< 12 months, 69 positive/96 [71.8%] vs > 12 months, 51/62 [82.2%], specific IgE (< 12 months, 25/83 [30.1%] vs > 12 months, 14/53 [26.4%]), or BAT (< 12 months, 47/92 [51.5%] vs > 12 months, 35/64 [54.6%]). For CAST-ELISA, there appears to be a trend for more positive results when the interval between the test and the last clinical reaction is less than 12 months (< 12 months, 36/84 [42.8%] vs > 12 months, 16/57 [28.0%]). As stated above, the optimal time to perform diagnostic tests in drug allergy is between 1 and 6 months after the clinical reaction. Tests performed within the first 4 weeks run the risk of falling within a postreaction refractory period [1].

One problem in the interpretation of cellular tests is that of nonresponders. In cellular tests based on IgE mechanisms, the basophils of some individuals do not respond by mediator release or expression of activation markers. For histamine release, the proportion of nonresponders amounts to 15%-25% in some reports [68,69], thus making it difficult to interpret negative results and determine diagnostic efficiency. The reason why nonresponse to anti-IgE in histamine release appears to be a deficiency in some of the enzymes required for intracellular signal transmission (syk) [90,94,95] and may be corrected by long (18 hours) incubation with IL-3 [94].

In the CAST-ELISA assay, the proportion of nonresponders to the anti-IgE positive control has usually been somewhat lower (6%-8%) [72]. In Flow-CAST, some authors have also reported 8%-10% of nonresponders [84]. Our multicenter study compared Flow-CAST and CAST-ELISA for the first time in a large number of patients and stressed the need for a careful approach. First, particularly at the start of the study, and usually only in a few groups, a number of anti-IgE–positive controls were negative in Flow-CAST (and would have been classified as nonresponders in a strictly Flow-CAST study), but were clearly positive in CAST-ELISA (Table 11). A closer investigation of this phenomenon revealed that it occurred when the lyophilized anti-IgE reagent was reconstituted in water instead of stimulation buffer, resulting in a final Ca^{2+} concentration about 15% lower than the optimal concentration required for basophil stimulation. Formal experiments using stimulation buffers with various Ca^{2+} concentrations have confirmed that CD63 expression in BAT is more sensitive to Ca^{2+} than sulfidoleukotriene release (CAST-ELISA) (results not shown). These experiments also revealed that the difference in sensitivity to external Ca^{2+} is not constant but specific to each individual cell population. When this was taken into account, the number of dissociated BAT-negative/CAST-ELISA–positive results almost disappeared, as most of these cases occurred during the first 10 months of the study. A second point to consider is the nature of the anti-IgE reagent used as a positive control. It has been reported that polyclonal anti-IgE antibodies are usually more efficient than monoclonal anti-IgE antibodies [96]. It has also been shown for CAST-ELISA that a monoclonal anti-IgFcεR1 antibody (clone 22E7) [97], is more efficient than anti-IgE antibodies [98]. All monoclonal anti-IgFcεR1 antibodies are not equal; monoclonal anti-IgFcεR1 CRA1 apparently yields a much higher proportion of nonresponders [99].

If only patients and controls not reacting to anti-IgFcεR1 both in BAT and CAST-ELISA are considered as true nonresponders (Table 11), the proportion of nonresponders becomes very low (5/232, 2.2%), while for BAT alone, including the dissociated BAT-negative/CAST-ELISA–positive cases discussed above, it would amount to 14.7% (38/259).

It is also noteworthy that some of these apparent nonresponders reacted positively to some ß-lactam allergens (10/27). Therefore, in the present study at least, the inclusion or exclusion of nonresponders seems to have little effect, despite warnings on the correct interpretation of the results [88] (Table 12). As shown in the example with amoxicillin or all 5 allergens combined, the sensitivity and specificity of BAT seem to vary little, regardless of whether the cutoff point for the positive anti-IgFcεR1 control is set at 0% (negative) or at 5% or more (positive).

Our study reached several conclusions. First, a detailed protocol must be prepared and participants must agree to follow it closely. Second, participating investigators should provide data on at least 10-15 patients and 10 controls. Only then can the groups establish a reproducible routine, identify possible technical drawbacks, and assess the homogeneity of the results. Third, soon after start of the study in each group, a bench scientist should determine whether the study protocol is being followed.

In the past, both BAT [91] and CAST-ELISA [62] have been claimed to be positive in about 50% of cases with a history of ß-lactam allergy, using isolated plasma leukocytes. This has been confirmed for BAT, using whole blood [88]. Somewhat lower results (34.6% sensitivity, 83% specificity) have been reported for CAST-ELISA [78] using only 1 allergen (BPN). Lower sensitivities have also been reported for BAT in preliminary form [89-91]. The results of our study confirm that both BAT and CAST-ELISA have a diagnostic value in numerous cases of immediate-type allergy to ß-lactam antibiotics, provided several allergens are used at appropriate concentrations. As with all in vitro tests, a negative BAT and/or CAST-ELISA does not exclude ß-lactam allergy, although positive results make the diagnosis very likely. Skin tests remain the main diagnostic approach, although cellular tests have a useful complementary role.

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