

Original article

Negativization rates of IgE radioimmunoassay and basophil activation test in immediate reactions to penicillins

Background: Skin test sensitivity in patients with immediate allergy to penicillins tends to decrease over time, but no information is available concerning *in vitro* tests. We analysed the negativization rates of two *in vitro* methods that determine specific immunoglobulin E (IgE) antibodies, the basophil activation test using flow cytometry (BAT) and the radioallergosorbent test (RAST), in immediate allergic reactions to penicillins.

Methods: Forty-one patients with immediate allergic reactions to amoxicillin were followed up over a 4-year period. BAT and RAST were performed at 6-month intervals. Patients were randomized into groups: Group I, skin tests carried out at regular intervals; Group II, skin tests made only at the beginning of the study.

Results: Differences were observed between RAST and BAT ($P < 0.01$), the latter showing earlier negativization. Considering different haptens, significant differences for the rate of negativization were only found for amoxicillin ($P < 0.05$). Comparisons between Groups I ($n = 10$) and II ($n = 31$) showed a tendency to become negative later in Group I with RAST.

Conclusions: Levels of specific IgE antibodies tended to decrease over time in patients with immediate allergic reactions to amoxicillin. Conversion to negative took longer for the RAST assay, although the differences were only detected with the amoxicillin hapten. Skin testing influenced the rate of negativization of the RAST assay, contributing to maintenance of *in vitro* sensitivity. Because of the loss of sensitivity over time, the determination of specific IgE antibodies to penicillins in patients with immediate allergic reactions must be done as soon as possible after the reaction.

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Immediate hypersensitivity reactions to penicillins are the most frequent cause of allergic reactions to drugs and are mediated by specific immunoglobulin E (IgE) antibodies (1). These antibiotics are able to generate hapten-carrier conjugates that are recognized by the immunological system (1). The most frequent and best identified structure recognized is benzylpenicilloyl (BPO), also known as the major antigenic determinant, which is formed by the nucleophilic attack of the betalactam ring by the amino group of proteins and which is used for skin testing coupled to poly-L-lysine (PLL), forming benzylpenicilloyl-poly-L-lysine (PPL) (2). In addition, though in fewer cases, application of pure crystalline penicillin and its hydrolysis products to the skin can also induce a positive response, these products being considered the minor determinants (3). PPL and the so-called minor determinant mixture (MDM), formed by benzylpenicilloic acid and benzylpenicillin (BP), have been used for skin testing (1).

The sensitivity of skin testing using PPL and MDM varies, ranging from 7% (4) to 63% (5), the differences

being due to the diagnostic criteria used and the time interval between the reaction and the study (1). These studies suggest that skin testing with PPL and MDM was considered safe and useful for diagnostic purposes and those cases with negative skin testing could safely receive penicillin treatment (1, 4, 6). However, it is important to consider that subjects can become skin test-negative over time, and although these data were originally provided from retrospective studies (5, 7, 8), they have been corroborated by prospective analyses (9, 10).

In addition to skin testing, *in vitro* methods for quantizing IgE antibodies recognizing the penicillin hapten were developed, consisting mainly of solid-phase immunosorbent assays, such as the radioallergosorbent test (RAST) (11, 12). Other methods used were based on the capacity of peripheral blood leukocytes to release histamine after incubation of blood with penicillins from patients sensitized to betalactams (13). This technique, although initially cumbersome, has been recently adapted, using flow cytometry, to diagnose patients with

immediate allergic reactions to allergens and haptens, such as penicillins, and is denominated the basophil activation test (BAT) (14, 15).

With allergens, skin tests have a similar sensitivity as RAST or BAT (16, 17) and this can be extended to other drug allergies, such as anaphylaxis to rocuronium bromide where a comparable sensitivity has been obtained between BAT and skin testing (18). This is not the case, though, with allergies to other drugs, such as betalactams, where some patients can be *in vitro* test-positive but skin test-negative (19).

Diagnosis using *in vitro* testing, RAST and/or BAT, is currently gaining relevance for several reasons: skin testing with minor determinants can sometimes induce systemic reactions (19, 20); some patients have important risk factors like age or cardiovascular diseases, certain countries have legal regulations making it difficult to perform drug provocation tests, or even skin testing, and finally some drugs, such as certain cephalosporins, are either not or only poorly soluble at the optimal concentration recommended for skin testing, thereby rendering impossible the performance of this test (20, 21). However, the question remains as to whether these methods lose sensitivity as the time interval between the reaction and the study increases, as happens with skin testing (10). The importance of the time interval for detecting IgE antibodies has also been shown in hypersensitivity to neuromuscular blocking agents where BAT sensitivity decreases 3 years after the reaction (22). Accordingly, in the present study we followed prospectively a group of subjects with immediate allergic reactions to amoxicillin (AX) to determine the negativization rate of IgE levels in blood samples using both RAST and BAT assays. In order to establish whether skin testing during the study influenced the delay to negativization of the *in vitro* tests, subjects were divided into two groups: those in whom skin testing was performed at regular intervals and those in whom skin testing was only performed at the start of the study to establish the diagnosis.

Patients and methods

Patients

We assessed 41 patients with an immediate allergic reaction to AX and with RAST- and BAT-positive at the start of the study. The diagnostic procedure was basically as described in the European Network of Drug Allergy (ENDA) protocol, based on skin testing, *in vitro* tests and a drug provocation test if necessary (20). The criterion for inclusion in the study was to have both RAST- and BAT-positive. Three clinical categories were established: anaphylactic shock, anaphylaxis or urticaria. Anaphylaxis was considered to be the presence of several of the following symptoms: pruritus on palms or soles, later becoming generalized, generalized erythema, urticaria, dyspnoea, difficulty in speaking or swallowing and/or tachycardia. If hypotension and/or loss of consciousness also appeared an anaphylactic shock was considered. Urticaria was defined as rapidly evolving and transient pruriginous wheals occurring at different body sites.

All patients were followed up at 6-month intervals for 4 years. In all cases a blood sample was obtained for both RAST and BAT assays and a clinical history was done in order to assess whether a new reaction or inadvertent contact with penicillin had occurred. Patients were randomized into two groups: those who underwent a skin test (one test per year, independently of the result of the skin testing) (Group I) and those who underwent no further skin testing during the 4-year study period (Group II). The institutional review boards approved the study, and informed consent for the diagnostic procedures was obtained from all patients.

Skin test

Skin testing was carried out as previously described (19). The freshly prepared reagents were PPL (Allergopharma Merck, Reinbeck, Germany) (5×10^{-5} M), MDM (Allergopharma) (2×10^{-2} M) and AX (SB Smithkline Beecham, Madrid, Spain) (20 mg/ml). In the skin prick tests, a wheal larger than 3 mm with a negative response to the control saline was considered positive. In the intradermal tests the wheal area was marked initially and 20 min after testing, and an increase in diameter greater than 3 mm was considered positive (19, 20).

In vitro-specific IgE antibody determination

This was performed by RAST using BP and AX conjugated to PLL (Sigma, St. Louis, MO, USA) in the solid phase, as described (11, 12). Briefly, 30 µl of the patient's sera was incubated with the disc with different penicillin-PLL conjugates. Washed radiolabelled anti-IgE antibody [Diagnostic Products Corporation (DPC), Los Angeles, CA, USA] was then added and incubated overnight. The discs were then washed and their radioactivity was measured in a gamma counter (Packard BioScience Company, Frankfurt, Germany). Results were calculated as a percentage of the maximum. Samples were considered positive if they were higher than 2.5% of label uptake, which was the mean + 2SD of the negative control group.

Basophil activation by flow cytometry

BAT (BASOTEST®, Orpegen Pharma, Heidelberg, Germany) was done as described (15). Briefly, 100 µl of heparinized whole blood was aliquoted per test and 20 µl of stimulation buffer was added and incubated for 10 min at 37°C in a water bath. After this, 100 µl of the washing solution was added to the negative control tube, 100 µl of the fMLP (chemotactic peptide *N*-Formyl-Met-Leu-Phe) to the positive control tube and 100 µl of two drug concentrations to the different samples. The concentrations used were: BP at 2 and 0.4 mg/ml and AX at 1.2 and 0.25 mg/ml. These optimal concentrations were chosen on the basis of dose-response curves and cytotoxicity studies. The samples were incubated for 20 min at 37°C in a water bath. The degranulation was stopped by incubating the samples on ice for 5 min and then 20 µl of staining reagent containing two monoclonal antibodies, anti-IgE-PE and anti-gp53-FITC (gp53 is a glycoprotein expressed on activated basophils) was added to each tube and incubated for 20 min in an ice bath covered to prevent exposure to light. Then, 2 ml of prewarmed lysing solution was added and incubated for 10 min at room temperature. After centrifuging and washing, the cells were analysed in a flow cytometer by acquiring at least 1000 basophils per sample. Results were considered as positive when the percentage of CD63 was 5% over spontaneous activation observed for the negative control and the stimulation index (SI), calculated as the ratio between the percentage

of degranulated basophils with the different haptens and the negative control, was ≥ 2 to at least one of the dilutions mentioned before.

Statistical studies

The evolution of BAT and RAST tests was analysed by survival analysis (Kaplan-Meier test using log-rank statistics). Comparisons for quantitative variables without a normal distribution were done by the Mann-Whitney and Kruskal-Wallis tests. All reported *P*-values represented two-tailed tests, with values ≤ 0.05 considered statistically significant. The statistical analysis was performed using the spss program, version 11.5.

Results

The study included 41 patients with immediate allergic reactions induced by AX and with RAST- and BAT-positive at the start of the study (Table 1). Twenty-three patients were men and eighteen women, with a mean age of 43.49 ± 14.37 years and a mean interval of 174.8 ± 278.6 days between the reaction and the start of the study. The clinical reactions were anaphylactic shock in 12 cases (29.3%), anaphylaxis in 21 (51.2%) and urticaria in 8 (19.5%). Thirty-one patients were skin test-positive

Table 1. Clinical characteristics, skin tests, radioallergosorbent test (RAST) and basophil activation test (BAT) results at the beginning of the study in the group of patients evaluated with immediate allergic reactions to amoxicillin

	Group	Sex	Age	Reaction	Interval (days)	Skin test			BAT		RAST	
						PPL	MDM	AX	BP	AX	BPO	AXO
1	II	F	38	Anaph	90	—	—	+	0.90	2.98	0.20	3.10
2	II	M	67	Anaph	30	—	—	+	1.37	7.14	0.00	4.24
3	I	F	33	Anaph	270	—	—	—	1.03	3.37	0.06	3.87
4	I	M	59	Anaph	60	—	—	+	1.77	2.40	0.81	3.09
5	II	F	39	Urt	180	—	—	—	1.05	2.30	0.00	5.37
6	II	M	38	A. shock	90	—	—	+	0.99	2.13	0.33	3.24
7	II	M	39	A. shock	38	—	—	+	4.08	1.18	1.47	3.17
8	II	F	30	Anaph	60	—	—	+	2.05	2.86	0.14	4.69
9	II	F	58	A. shock	120	—	—	+	4.89	0.93	0.00	3.15
10	II	M	77	A. shock	20	—	—	+	16.68	0.87	0.00	5.00
11	I	F	54	Urt	1385	+	—	—	0.92	9.41	3.18	0.00
12	I	F	35	A. shock	6	—	—	+	1.02	7.03	0.05	4.90
13	II	F	55	Anaph	30	—	—	+	1.53	4.26	0.14	3.13
14	II	M	30	A. shock	6	+	+	+	2.33	4.02	5.00	3.71
15	I	F	32	Anaph	122	—	—	+	0.92	3.39	0.13	5.75
16	I	M	28	Anaph	160	—	—	+	3.13	2.21	0.43	3.44
17	I	F	55	Anaph	1030	+	+	—	0.98	3.37	4.49	0.00
18	II	M	21	Anaph	150	—	—	+	2.81	1.67	0.00	2.87
19	II	M	40	A. shock	365	—	—	+	1.32	13.83	0.10	4.04
20	II	M	28	Anaph	20	—	+	+	1.08	5.50	0.00	2.90
21	II	F	50	A. shock	120	—	—	+	22.07	1.64	4.45	3.18
22	II	M	27	Anaph	90	—	—	+	9.21	0.82	2.66	0.77
23	I	F	43	Anaph	2	—	—	+	2.26	2.46	0.00	3.85
24	II	F	56	A. shock	365	—	—	—	0.84	3.07	4.19	0.70
25	II	M	37	Anaph	15	—	—	+	2.21	3.67	0.00	3.71
26	II	M	59	Anaph	21	—	—	—	3.18	0.79	7.31	23.22
27	II	M	48	Urt	365	—	—	+	0.91	12.32	0.00	3.62
28	II	F	36	Anaph	120	—	—	+	1.61	2.21	0.26	4.71
29	II	M	46	Anaph	30	—	—	—	0.77	2.97	17.58	1.36
30	II	M	40	A. shock	300	—	+	+	2.48	1.01	0.00	6.65
31	I	M	65	Urt	180	+	—	—	3.56	1.45	19.71	0.14
32	II	F	49	Anaph	24	—	—	+	1.32	4.39	0.72	15.41
33	II	M	50	A. shock	30	—	—	—	3.67	2.20	16.85	17.13
34	II	F	28	A. shock	270	—	—	—	2.43	1.58	1.28	3.46
35	II	M	40	Anaph	20	—	—	+	3.98	1.94	5.24	2.33
36	II	M	40	Anaph	60	—	—	—	0.44	2.58	10.19	0.77
37	II	M	53	Urt	65	—	—	+	0.85	2.11	0.25	3.54
38	I	M	55	Urt	60	—	—	+	4.32	3.55	0.00	29.80
39	II	F	29	Urt	60	—	—	—	1.53	2.97	4.76	4.77
40	II	F	41	Anaph	20	+	—	—	3.76	2.13	18.15	10.88
41	II	M	35	Urt	720	—	—	—	3.76	0.99	0.34	11.65

F, female; M, male; Anaph, anaphylaxis; Urt, urticaria; A. shock, anaphylactic shock; PPL, penicilloyl-polylysine; MDM, minor determinants mixture; AX, amoxicillin; BPO-PPL, benzylpenicilloyl-polylysine; AXO-PLL, amoxicilloyl-polylysine.

to at least one of the haptens tested (24 to AX, 3 to PPL, 2 to MDM and AX, 1 to PPL and MDM and 1 to PPL, MDM and AX) and 10 negative to the three haptens tested. As a criterion for inclusion was to have a RAST- and BAT-positive, no drug provocation test was required in any of our patients.

For BAT, 13 (31.7%) patients were positive just to BP, 20 (48.8%) to AX and 8 (19.5%) to both drugs. With RAST, 8 (19.5%) patients were positive just to BPO-PLL, 27 (65.8%) to AXO-PLL and 6 (14.6%) to both haptens. The median (interquartile range) for BAT using BP was 1.77 (1.02–3.58) and using AX was 2.43 (1.66–3.58), and for RAST it was 0.29 (0.00–4.265) with BPO-PLL and 3.62 (3.09–4.90) with AXO-PLL.

The course of the RAST and BAT over the follow-up period of 4 years showed that with BAT five patients (12.2%) remained positive after one year, 2 (4.9%) after 2 years, one (2.4%) after 3 years and one (2.4%) after 4 years. With RAST, the corresponding figures were 9 (22%), 4 (9.8%), 2 (4.9%) and 1 (2.4%), respectively. There were no losses during the follow-up period. Considering as positive a RAST or BAT with at least one positive result to one hapten, a survival analysis for both RAST and BAT was done. Differences were seen in the rate of negativization between both tests (log-rank test = 7.41; $P = 0.0065$), with BAT being the test that became negative sooner (Fig. 1).

We also determined whether the rate of negativization of both BAT and RAST was different depending on the hapten analysed. We performed the same comparison described before but differentiating haptens; we found no differences in the rate of negativization between BAT and RAST for BP (log-rank test = 1.09; $P = 0.295$) whereas differences were found in the rate of negativization in the case of AX (log-rank test = 4.03; $P = 0.0446$). These

data indicate that AX is the hapten that declines faster in the BAT assay. These results can be seen in Fig. 2A,B.

We also performed survival analysis for both tests depending on the different clinical manifestations but found no differences in the negativization rate between RAST and BAT in patients with urticaria, anaphylaxis or anaphylactic shock (log-rank test = 3.52; $P = 0.0688$, log-rank test = 1.58; $P = 0.2088$ and log-rank test = 0.25; $P = 0.6181$, respectively) (data not shown in figures).

To determine the role of skin testing in the maintenance of the response, we compared those patients who underwent skin testing during the study (Group I, $n = 10$) and those in whom just blood samples were obtained (Group II, $n = 31$). Both groups were randomly assigned at the start of the study and comparisons of different variables (age, sex, type of reaction, time interval between the reaction and the study, skin testing and initial RAST and BAT results) showed no significant differences between the two groups. We found no differences in the rate of negativization in the BAT assay between the two groups (log-rank test = 0.74; $P = 0.39$). However, differences were detected in the RAST assay (log-rank test = 4.39; $P = 0.036$), with a faster negativization in those patients in whom no skin testing was performed (Fig. 3A,B).

Discussion

Current evidence indicates that patients with immediate allergic reactions to penicillins may convert from skin test-positive to -negative after a variable period of time (5, 8, 9) and results indicate that in penicillin allergy the rate of negativization differs between patients with cross-reactivity and those with a selective IgE response (10). Because *in vitro* tests can be relevant for the diagnosis of immediate hypersensitivity reactions to penicillins, and they can sometimes be used as an alternative to skin testing, we considered it important to establish the interval of negativization of the two common *in vitro* methods available, RAST and BAT, in a prospective study. Additionally, in order to verify whether the repetition of skin testing could have any influence on the persistence of sensitivity, detected by the *in vitro* tests mentioned before, the patients were randomized into two groups: those with skin testing repeated at regular intervals of time and those in whom skin testing was only done at the start of the study.

During the sensitization process to an allergen, IgE is bound to the basophil FcεRI, which has a half-life of just a few days and is retained there for the entire the basophil life; in contrast to tissue mast cells, for which bound IgE have an unknown half-time, IgE bound to basophils have a similar half-time as the basophil. This sensitization can even promote basophil survival by autocrine or unknown mechanisms (23, 24). It is therefore tempting to expect

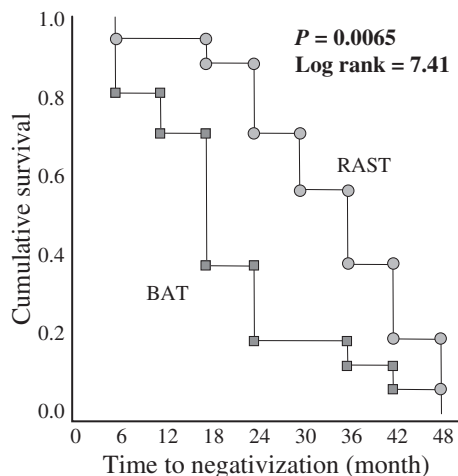


Figure 1. Comparison of radioallergosorbent test and basophil activation test survival analysis during a follow-up period of 4 years.

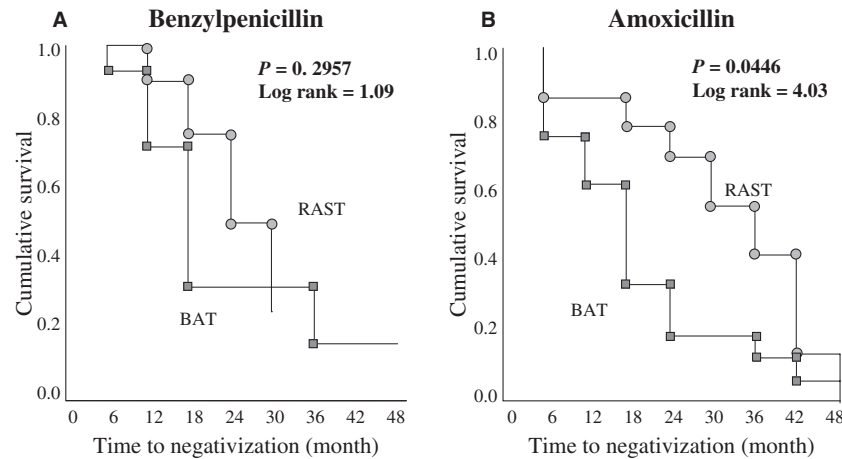


Figure 2. Comparison of radioallergosorbent test and basophil activation test survival analysis for each hapten: (A) benzylpenicillin; (B) amoxicillin.

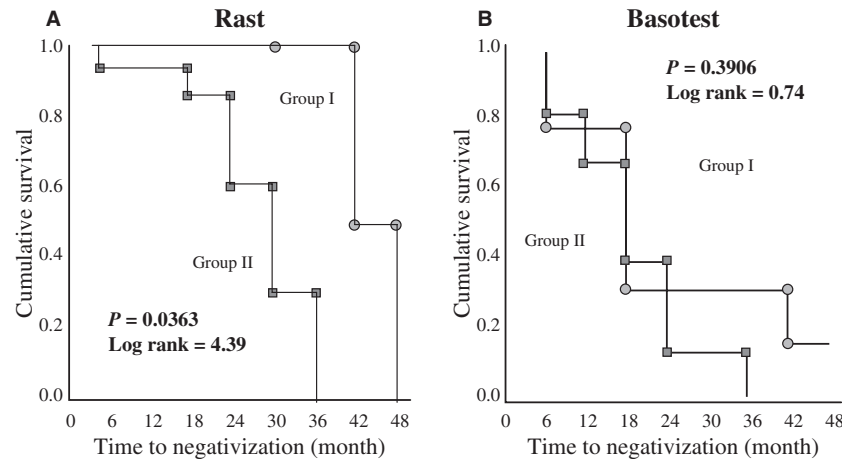


Figure 3. Comparison of the survival analysis in patients with (Group I) or without (Group II) skin tests during the study in both radioallergosorbent test (A) and basophil activation test (B).

that, after comparison of the two *in vitro* methods, the first test to become negative would be that detecting soluble IgE antibodies (RAST) followed by that detecting IgE bound to basophils (BAT). However, our results showed that the serum IgE antibodies became negative later than IgE bound to basophils. This different rate has also been observed during immunotherapy in patients allergic to wasp venom, showing that, after 3 years, 60% of the patients had a negative BAT compared with only 18% who had negative-specific IgE (25). This may be explained because, as has been reported, after a new contact with the allergen, the FcεRI aggregation not only promotes basophil survival but it can also induce apoptosis (26). Another explanation may be that the BAT assay relies on the releasability after IgE recognition, which is known to be highly variable from one patient to another (27).

When we examined whether the differences detected in the rate of negativization between the two tests could be attributed to a particular hapten, we found that this was due to AX. Similar data have been reported previously by our group in the skin testing of a group of patients followed up over 5 years, in whom the rate of negativization in those selective responders to AX was faster than in those with cross-reactions (10).

The role of skin testing, drug provocation testing or even inadvertent contact in the resensitization or maintenance of the IgE response in patients with immediate allergic reactions to penicillins has not been sufficiently addressed, although it is reported to be low, in both prospective and retrospective studies (8, 28, 29). In our study, we randomized patients into two groups, with or without periodic skin testing, in order to verify whether this had any influence on the rate of negativization of

RAST and BAT. Skin testing only influenced the RAST assay, maintaining *in vitro* positivity in the group of patients who had repeated skin tests. These results show that basophil sensitivity is lost faster, even after repeated contact with the hapten. The reasons for this could be the induction of nonreleaser basophils after stimulation with low doses of allergens, due to the post-transcriptional loss of Syk (30), similar to natural nonreleaser basophils (31).

Finally, this study raises a number of questions that need to be considered. First, this was not a study designed to analyse the natural evolution of penicillin hypersensitivity, so we do not know whether negativization of the *in vitro* testing could be accompanied by good tolerance to penicillin. Second, as there is no available skin test data from at least half the patients, we cannot estimate what percentage of skin tests remained positive throughout the time. However, this question has been answered previously by our group in a similar population, showing that in the case of selective responders to AX all skin tests were negative at 5 years and in those who cross-reacted 50% of skin tests were negative at 5 years (10). Third, in this study the BAT assay was done using BP and AX but not BPO-PLL or AXO-PLL. This was done because the addition of the latter two conjugates does not add any new information, as reported by our group and by others (14, 15). Fourth, the value of nonresponders in this study could not be well established because we used fMLP but, as has been found in several studies using anti-IgE receptor antibodies, the total number of nonresponders

was less than 10% and this, therefore, could not contribute to the decrease in BAT sensitivity (32). Furthermore, in addition to this low percentage, some patients still responded to the betalactam and they could not, therefore, be classified as nonresponders. Moreover, it happens that the nonresponder status in some patients is temporary (32).

Finally, the differential rate of negativization can be attributed to the intrinsic BAT sensitivity, although previous studies using two different methods in patients allergic to betalactams have shown similar results for both sensitivity and specificity (14, 15).

Summarizing, this study provides for the first time the rate of negativization of two *in vitro* tests that measure the presence of specific IgE antibodies to penicillins. Contrary to what was expected, BAT became negative before RAST. In addition, skin testing had a negative influence on the rate of negativization for the RAST assay, indicating that this can be a factor related to resensitization or maintaining sensitivity.

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