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Role of Histamine Release Test for the Evaluation of Patients with Immediate Hypersensitivity Reactions to Clavulanic Acid

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Abstract

Background: Immediate hypersensitivity reactions to clavulanic acid (CLV) seem to be on the increase. Diagnosis is mainly based on skin testing and the drug provocation test (DPT), procedures that are not risk free. The aim of this study was to evaluate whether the histamine release test (HRT) could help evaluate patients with selective hypersensitivity to CLV. ***Methods:*** Eighteen patients with immediate selective hypersensitivity reactions to CLV (positive skin tests to CLV but negative to the major and minor determinants of benzylpenicillin and amoxicillin; negative DPT to benzylpenicillin and amoxicillin) and 21 controls with tolerance to CLV were included. Direct and passive HRT, using patient whole blood or 'IgE-stripped' donor blood sensitized by patient serum, respectively, were performed by stimulating the blood with CLV, and basophil histamine release was detected by fluorometric determination. ***Results:*** The clinical symptoms were anaphylaxis (n = 6), urticaria (n = 9) and urticaria-angioedema (n = 3). The median time interval between the reaction and the study was 225 days (interquartile range, IQR: 120–387.5) and between drug intake and the development of symptoms 30 min (IQR: 6.25–30). We obtained similar data for both the direct and passive HRT, with a sensitivity and specificity of 55 and 85%, respectively, a positive predictive value of 76% and a negative predictive value of 69%. ***Conclusions:*** The sensitivity of both the direct and passive HRT for diagnosing patients with immediate allergy to CLV is less than 60%. However, the passive HRT has the advantage that it is based on the testing of serum samples that can be handled more easily than fresh blood samples.

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Introduction

β-Lactam (BL) antibiotics are still the most frequently used drugs causing IgE-mediated allergic reactions [1–4]. Although all BLs can be involved in these reactions, currently amoxicillin (AX), and to a lesser extent cephalo-

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sporins, are the most frequently involved [1–4]. AX can induce selective reactions in a large proportion of cases, implying that patients can react to AX but tolerate benzylpenicillin (BP) [5–7]. Immunochemical studies performed in these patients have shown that although side-chain chemical structures are the most important for immunological recognition and clinical response, part of the common BL structure is also required for optimal recognition [8].

Today, AX is often used in combination with clavulanic acid (CLV), a β-lactamase inhibitor. Although CLV was initially considered a non-immunogenic compound due to its chemical instability [9], it has now been reported that CLV can generate stable products from an intermediate structure [10] that may be involved in IgE recognition [9] and induce hypersensitivity reactions [7, 11–14]. Moreover, this tendency is thought to be on the increase as it has been shown that patients with selective reactions to CLV are younger than those with selective reactions to AX, probably reflecting changes in consumption patterns [13]. Therefore, the evaluation of immediate hypersensitivity reactions to AX-CLV is more complex since either AX or CLV can cause allergic reactions.

The diagnosis of selective reactions to CLV is mainly based on skin testing with this compound or by the drug provocation test (DPT), both of which are time-consuming and not free of risk. Moreover, in order to demonstrate a selective reaction to CLV, tolerance to BP and AX has to be confirmed by DPT. Among the in vitro tests used to confirm selective reactions to CLV, only studies using the basophil activation test (BAT) have shown promising results [12–14].

Another in vitro test is the histamine release test (HRT) using human basophils. This has been used to diagnose IgE-mediated reactions with variable results and technical difficulties mainly related to the large blood volume required, the kinetics of histamine release and the difficulty of detecting histamine levels using chromatography, fluorescence and immunoassay methods [15]. Moreover, to achieve a high sensitivity leukocyte enrichment may be required [16, 17]. To solve these issues, a simplified method based upon selective binding of histamine to glass microfibres has been developed to measure basophil histamine release in IgE-mediated allergic patients with high sensitivity and with a reduced preparation time [18]. This test provides several advantages since the isolation of cells and the extraction of histamine is not necessary, making the method suitable for routine diagnosis [19, 20]. Previously, HRT has mainly been used for experimental purposes [21, 22].
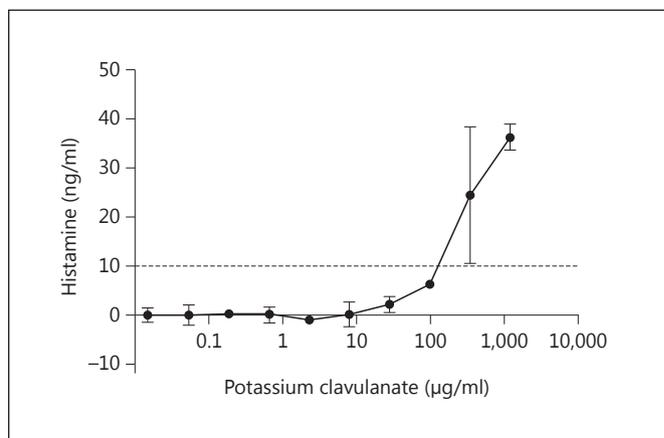

Fig. 1. Dose-response study for determining the highest concentration of CLV that did not induce unspecific histamine release (>10 ng/ml of histamine).

In this study, we used the HRT to analyse the role of histamine release for evaluating immediate allergic reactions to CLV in a well-characterized group of patients with skin test positivity to CLV.

Subjects and Methods

Patient Selection
The study group comprised 18 patients diagnosed with immediate allergic reactions (appearing less than 1 h after drug administration), selective to CLV and with skin test positivity to CLV. Patients were selected from those with a history of immediate allergic reactions to AX-CLV in the allergy units of four Spanish hospitals during 2013. For diagnosis, we followed the European Academy of Allergy and Clinical Immunology general guidelines for the evaluation of immediate reactions to BLs [23]. Selective reactors to CLV were chosen based on positive skin test results to CLV and no reaction to BP and AX in the DPT. The clinical entities presented by the patients were classified as urticaria, urticaria-angioedema or anaphylaxis, as described [2]. A control group comprising 21 healthy individuals who had previously received AX-CLV without any adverse effects and who gave negative skin test results to CLV was also included. Relevant institutional review boards approved the study and informed consent was obtained from all subjects.

Skin Testing
Skin testing was performed by prick, and if negative followed by intradermal testing as recommended [7, 23]. The maximum concentrations used were as follows: benzylpenicilloyl octa-L-lysine (PPL) 0.04 mg/ml, with a molar concentration of the benzylpenicilloyl moiety of 8.64×10^{-5} M, minor determinant (DM) 0.5 mg/ml, with a molar concentration of the sodium benzylpenilloate of 1.5×10^{-3} M, AX 20 mg/ml and CLV 20 mg/ml (DIATER

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Table 1. Clinical and demographic characteristics of the patients, and skin test and HRT results with CLV

Patient	Age, years	Sex	Reaction	Interval study, days	Interval reaction, min	Skin test concentration (wheal diameter, mm)		HRT (histamine concentration, ng/ml)	
						skin prick test	intradermal test	direct	passive
1	41	F	Anaph	150	15	20 mg/ml + (9×5)	n.d.	14	7
2	60	M	Anaph	515	5	–	20 mg/ml + (8×7)	0	10
3	61	F	Urt-Ang	485	5	–	20 mg/ml + (10×12)	3	56
4	69	F	Anaph	912	5	–	20 mg/ml + (11×10)	10	61
5	31	F	Anaph	365	30	–	5 mg/ml + (15×12)	15	56
6	67	F	Urt	180	60	–	5 mg/ml + (9×11)	9	62
7	54	F	Urt-Ang	270	10	–	5 mg/ml + (11×13)	19	24
8	34	M	Anaph	300	30	–	20 mg/ml + (10×15)	17	34
9	29	F	Anaph	425	30	–	20 mg/ml + (9×6)	33	34
10	51	M	Urt	110	30	–	0.5 mg/ml + (7×9)	7	12
11	53	M	Urt	90	60	–	5 mg/ml + (12×7)	3	4
12	58	F	Urt	170	30	–	5 mg/ml + (11×10)	9	4
13	58	M	Urt	60	30	20 mg/ml + (11×5)	n.d.	18	13
14	18	M	Urt	120	60	–	5 mg/ml + (17×13)	45	39
15	30	F	Urt-Ang	90	30	–	0.5 mg/ml + (12×10)	19	2
16	30	F	Urt	365	2	–	5 mg/ml + (20×18)	13	10
17	32	F	Urt	395	30	–	5 mg/ml + (21×16)	10	9
18	29	M	Urt	120	5	–	5 mg/ml + (28×16)	12	10

Interval study: time between the reaction occurrence and the study. Interval reaction: time between drug administration and appearance of the symptoms. Ang = Angioedema; Anaph = anaphylaxis; n.d. = not done; Urt = urticaria.

Laboratories, Madrid, Spain). In those cases with a history of severe anaphylaxis, we performed the test with increasing dilutions until the maximum concentration was reached, as described elsewhere [23]. 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.

Drug Provocation Test

To confirm selective reactions to CLV, a single-blind, placebo-controlled DPT was performed with BP, penicillin V (PV) and AX in patients with negative skin tests to PPL, DM and AX, as reported [23]. Briefly, a DPT with BP was performed by parenteral route in a single dose of 10^6 units, followed by a 3-day therapeutic course of PV of 500 mg/8 h, when PPL and DM in the skin test were negative. If the DPT with BP and PV was negative and the skin test was negative to AX, a DPT with AX was performed. For this, incremental doses of AX were administered, with 30-min time intervals until reaching a cumulative dose of 500 mg, followed by a 3-day therapeutic course of AX 500 mg/8 h.

Direct HRT

The direct HRT was performed according to the method described by Stahl-Skov et al. [18, 19]. Briefly, whole blood samples were collected in heparinized tubes from patients and controls. These were centrifuged and plasma was removed and replaced by the same volume of PIPES buffer: 10 mM piperazine-N,N′-bis(ethane sulfonic acid), 140 mM sodium acetate, 5 mM potassium acetate, 0.6 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1 mg/ml glucose, 0.3 mg/ml HSA and 15 IU/ml heparin. Afterwards, 25 μl of washed blood in the presence of IL-3 (2 ng/ml blood) was incubated in the glass fibre-prepared microtiter plates (RefLab, Copenhagen, Denmark), with 25 μl of potassium clavulanate (DIATER Laboratories) at 100 μg/ml for 60 min at 37°C. The CLV concentration chosen for this test (100 μg/ml) was the highest value that did not lead to unspecific histamine release (>10 ng/ml) in a dose-response curve. This level corresponded to the mean + 3 SD of the histamine release values measured in 5 non-allergic subjects without a specific stimulus (fig. 1). As a positive control, we used 25 μl of anti-IgE (KPL, Gaithersburg, Md., USA) in a concentration of 5 μg/ml as well as histamine in concentrations of 0 and 50 ng/ml. After incubation, the microtiter plate was washed with H_2O for 1 min to remove cells and interfering substances. Thereafter, 75 μl of NaOH/ortho-phthalaldehyde (OPA) mixture was added to each well allowing glass fibre-bound histamine to be released and coupled to OPA. After 10 min the coupling reaction was stopped and histamine-OPA complexes stabilized by adding 75 μl of 0.59% $HClO_4$. Histamine was determined fluorometrically in the HISTAREADER™ 501 (RefLab). Results were expressed as ng/ml of histamine released.

A preliminary calibration step was performed for the HRT using increasing concentrations of histamine from 0 to 50 ng/ml. The coefficients of variation of the method were 3.3 (2.1–4.2) according to 0 ng/ml of histamine and 3.1 (1.9–4.6) according to 5–50 ng/ml of histamine.
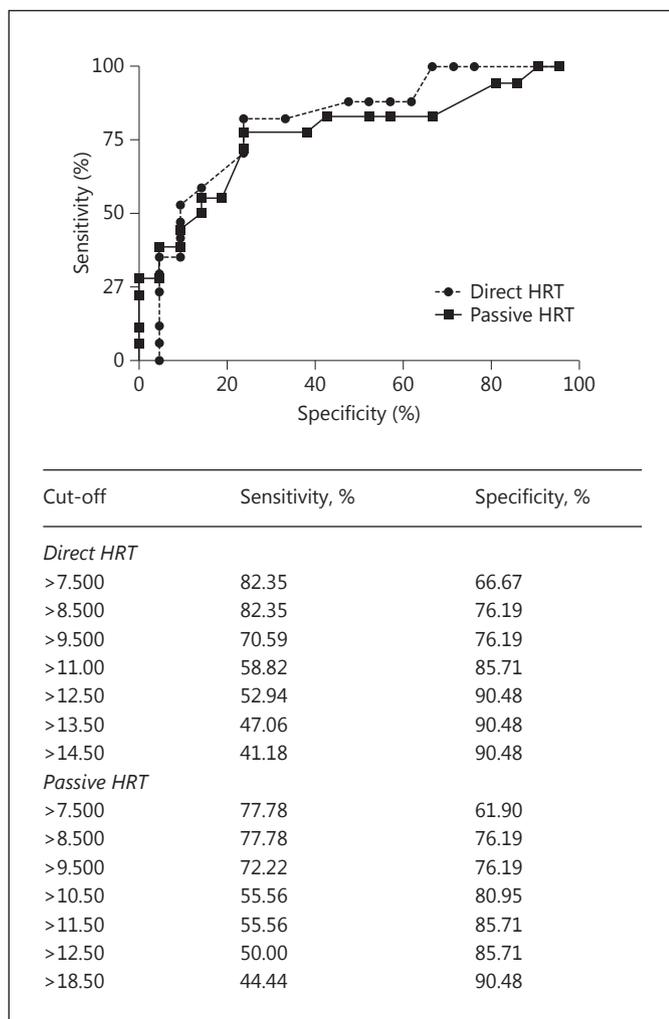
Cut-off	Sensitivity, %	Specificity, %
Direct HRT		
>7.500	82.35	66.67
>8.500	82.35	76.19
>9.500	70.59	76.19
>11.00	58.82	85.71
>12.50	52.94	90.48
>13.50	47.06	90.48
>14.50	41.18	90.48
Passive HRT		
>7.500	77.78	61.90
>8.500	77.78	76.19
>9.500	72.22	76.19
>10.50	55.56	80.95
>11.50	55.56	85.71
>12.50	50.00	85.71
>18.50	44.44	90.48

Fig. 2. ROC curve generated after comparison of direct and passive HRT to CLV of allergic vs. non-allergic subjects.

Passive HRT

Passive sensitization was performed using heparinized blood from a single non-allergic healthy donor, with the following characteristics: (1) a confirmed strong reaction to anti-IgE (histamine released: 45 ± 5 ng/ml) and (2) no response to CLV. To remove the IgE bound on the basophils surface, 3 ml of blood were treated with 10 ml of stripping buffer (0.14 M NaCl, 0.005 M KCl and 0.0134 M lactic acid) for 10 min at 4°C. Then, 0.5 ml of sera from patients or controls were incubated with 3 ml of the 'IgE-stripped' donor blood for 60 min at 37°C. Following this, the same protocol described for the direct HRT was followed. Stripping experiments were controlled by confirming no positive histamine release after stimulation of non-sensitized basophils with anti-IgE. Passive sensitization was confirmed when a strong reaction occurred (histamine released: 45 ± 5 ng/ml) once we added the serum of the patient (sensitized cells) and anti-IgE.

Statistical Analysis

Quantitative variables were described using the median and the interquartile range (IQR). Comparisons for variables without a normal distribution were carried out using the Mann-Whitney test. ROC curve analyses were performed to calculate the optimal cut-off value corresponding to the best sensitivity and specificity. All reported p values represent two-tailed tests, with values <0.05 considered statistically significant.

Results

The study included 18 patients with immediate selective hypersensitivity reactions and positive skin tests to CLV. The clinical characteristics and the results of the allergological work-up are shown in table 1. The median age was 46 years (IQR: 30.25–58), 11 were females, AX-CLV was the drug involved in all reactions, 9 developed urticaria, 3 urticaria-angioedema and 6 anaphylaxis (1 had a severe reaction with marked hypotension). The median time interval between the reaction and the study was 225 days (IQR: 120–387.5) and between drug intake and the development of symptoms 30 min (IQR: 6.25–30).

Skin test results were negative for PPL, DM and AX and positive for CLV in all cases. Positivity was obtained by a skin prick test in 2 patients at 20 mg/ml and by an intradermal test in the remaining 16 patients (2 at 0.5 mg/ml, 9 at 5 mg/ml and 5 at 20 mg/ml). No patient developed systemic symptoms after skin testing. Skin testing (including intradermal test) with CLV was negative in all 21 control subjects.

For HRT standardization, we first performed a dose-response study for establishing the highest concentration of CLV that did not induce unspecific histamine release, corresponding to the mean + 3 SD of background histamine release values measured in 5 non-allergic subjects (fig. 1). We found 100 μg/ml of CLV to be the best concentration for the test. ROC curve analyses were performed to determine the optimal cut-off value for considering positive results. The results showed values >11 ng/ml of histamine to be the optimal cut-off for both the direct and passive HRT (fig. 2).

Considering HRT results (table 1) there were 10 (55%) positive cases in the direct HRT and 10 (55%) positive cases in the passive HRT, with 14 (77%) cases being positive to at least one test. The sensitivity and specificity of both the direct and passive HRT was 55 and 85%, respectively, with a positive predictive value of 76% and a negative predictive value of 69%.

Comparisons of the median values of released histamine for the direct and passive HRT in patients versus

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Fig. 3. Comparison of results for the HRT to CLV between allergic and non-allergic subjects for direct HRT (**a**) and passive HRT (**b**). Results are expressed as the median of histamine release (ng/ml).
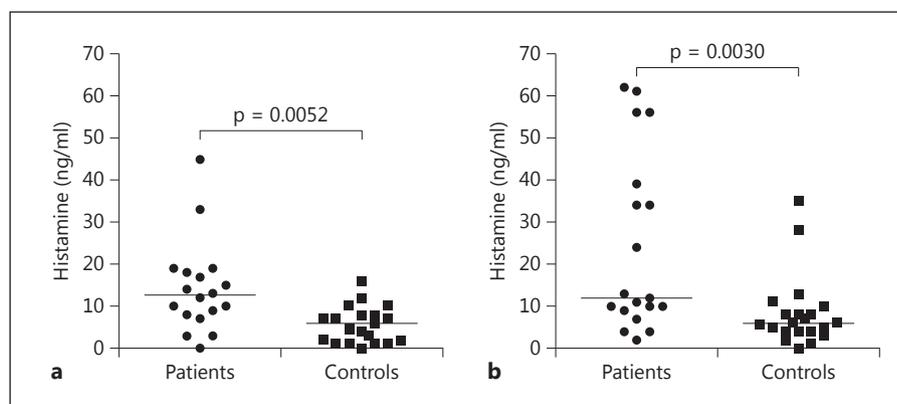
controls are shown in figure 3. Data showed significant differences between patients and controls in both the direct ($p = 0.0052$) and passive HRT ($p = 0.0030$). No differences were observed in the concentration of histamine released when comparing the direct and passive HRT (data not shown).

There was a positive correlation between histamine release levels obtained with the direct and passive HRT ($p = 0.0031$; Spearman's $r = 0.4670$; fig. 4). No correlation was found between the histamine release and the time interval between the reaction and the study, or between the drug intake and the development of symptoms or with the concentration of CLV required to produce a positive skin test.

Fig. 4. Scatter graph showing the positive correlation between histamine release levels obtained with the direct and passive HRT.

Discussion

BL allergy is self-reported by at least 10% of patients, although only 10% of these are truly allergic [24]. A diagnosis of BL allergy in hospitalized patients, whether confirmed or not, is a treatment disadvantage as it increases the risk of bacterial resistance, the number of days spent at hospital, the risk of opportunistic infections and the cost to the health system [25, 26]. Therefore, it has recently been claimed that BL allergy is a public health problem, particularly complicated by changes in the consumption of these antibiotics [27]. In fact, AX in combination with CLV is now the most frequently consumed BL in many countries, and therefore selective allergic reactions to CLV are becoming increasingly prevalent in children [28] and adults [11–14]. Moreover, it has recently been shown that CLV selective responses are not modified by exposure to penicillin determinants, indicating that these patients can take penicillin derivatives safely [7]. Therefore, perform-
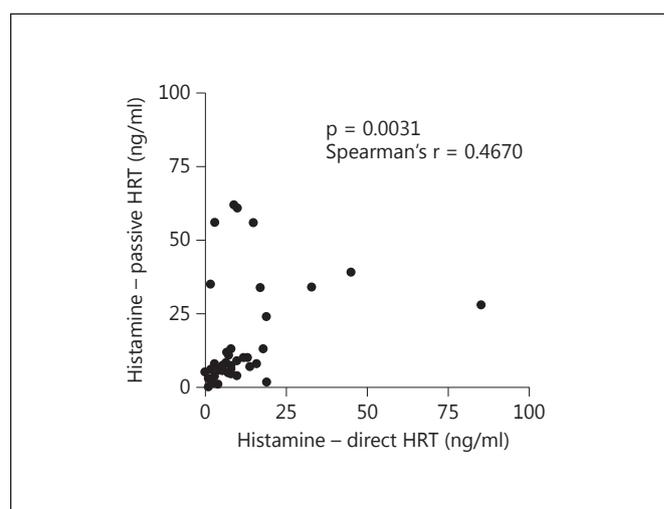
ing an accurate diagnosis of BL allergy is mandatory. However, the methods available nowadays, such as skin testing and DPT, are time-consuming, need qualified medical staff and are not risk free [23, 29]. In the recent International Consensus on Drug Allergy [30], the need for discriminating biological tests to establish the nature of culprit agents was highlighted, particularly for patients receiving several drugs simultaneously, and for severely life-threatened patients when skin tests are negative or not possible, and the DPT is contraindicated. To this end, we have performed an evaluation of HRT for the first time in patients with confirmed immediate allergic reactions to CLV and positive skin tests, and we found that more than half of these cases gave positive results.

The HRT was described in the last century. However, it has achieved limited use because it was originally time-consuming, only few patients could be tested per day and its sensitivity for diagnosing drug allergy was unknown [15]. This test has been simplified and its sensitivity has been improved using glass microfibre plates to which histamine is specifically bound [18] – this allows performing the tests in any laboratory independently of equipment availability and sending them to a reference laboratory. The advantages of the system are: (1) patient blood is tested in a number of drug concentrations from 6 to 12, (2) up to 10 drugs can be tested on the same HRT plate and (3) heparinized blood can be stored and shipped at room temperature for up to 36 h before testing. Moreover, the passive HRT allows performing the test only by sending sera.

We found in this study that the sensitivity of the direct HRT was 55% and the specificity 85%. These results were similar to those found for the BAT in another study with 16 selective allergic patients to CLV with positive skin tests to CLV, in which 50% of the patients had positive results for the BAT [13]. These results indicate that the direct HRT and the BAT could be broadly equivalent in in vitro tests in terms of results, although comparative studies in the same group of patients must be performed to compare these techniques directly.

However, both the direct HRT and the BAT have the disadvantage of being cellular tests that need to be performed 24–48 h after drawing blood, meaning samples cannot be stored, so the test should be performed close to the blood collection place. These drawbacks could be avoided by using a passive sensitization of basophils from donors with serum IgE from patients. This method has been previously used to evaluate the functional characteristics of IgE in patients [31, 32]. However, the passive BAT has not shown positive results for evaluating drug hypersensitivity reactions. In the present study we have performed the direct and passive HRT with samples from the same patients and found similar results for both, with a sensitivity of 55% and a specificity of 85% for both test types. Both tests had similar ROC curves, the same cut-off point and a positive correlation, indicating that the results obtained with the two tests showed equivalent sensitivities and specificities. However, the direct and passive HRT were positive in different patients, and when the two tests were combined, the number of positive cases increased from 55 to 78%. There might be several explanations for the differences observed. In the direct HRT, false-negative results might be due to either non-responding basophils due to impaired IgE signalling or low histamine content in basophils beyond the detection limit of the HRT system.

In the passive HRT, these limitations can be overcome because donor cells are selected on the basis of high IgE responsiveness and sufficient basophil histamine content. However, false-negative results can occur if the level of specific IgE in serum is low [32].

These results are interesting because, as far as we know, this is the first study showing a positive diagnostic outcome among a large group of patients using passive sensitization and basophil stimulation in drug hypersensitivity. Although one of the main challenges of using the passive HRT is to have access to a donor with highly responding basophils, the use of a single donor, on the other hand, allows a significant reduction of the inter-individual variability due to the basophil releasability.

In fact, in the present study all the passive tests were done with basophils obtained from the same donor. It is important to take into account that the passive sensitization of stripped basophils is the only way to report that the basophil histamine release induced by a drug is mediated by specific IgE.

Finally, regarding patients included in the study, it is of note that, as reported in other studies [13], they were generally young subjects and 1/3 of them developed anaphylaxis. We did not detect any correlation in the time interval between the clinical reaction and the performance of the HRT. This contrasts with results observed in a study with selective patients to AX tested with the BAT, where the positive outcome of the BAT was inversely correlated to the time interval after the clinical reaction [33]. However, studies with larger numbers of patients are needed to confirm this possibility. Considering skin testing, 2 patients were diagnosed by prick test, and only 5 patients needed to reach the maximum concentration of the reagent in the intradermal test (20 mg/ml) to obtain a positive result. No correlation was obtained between the skin test and the HRT results. The present study does not include patients with a skin test negative to CLV but DPT positive, and therefore it is not possible to evaluate the outcome of the HRT in this group of patients.

In conclusion, we have shown in this study that the HRT can be a promising in vitro method for the evaluation of patients with immediate selective reactions to CLV, including patients where skin prick testing or provocations cannot be performed due to the risk of systemic reactions. Moreover, the passive HRT has been shown to be equivalent to the direct HRT in terms of performance, with the advantage that only patient sera are needed. Although these results should be reproduced in a larger group of patients, this study opens an area of research into the utility of the HRT for the diagnosis of allergy to other

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BLs and in patients who are skin test negative but DPT positive. Furthermore, it is important to carry out comparative studies between the HRT and other in vitro tests, such as the BAT.

Disclosure Statement

Per Stahl-Skov is a research consultant for RefLab. Fernando Pineda and Ricardo Palacios are employees of DIATER Laboratories. The remaining authors did not receive money for the present study and research is part of their daily activities.

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