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Non-immediate reactions to β -lactams: diagnostic value of skin testing and drug provocation test

A. Padial*, C. Antunez†, N. Blanca-Lopez‡, T. D. Fernandez†, J. A. Cornejo-Garcia†, C. Mayorga†, M. J. Torres§ and M. Blanca§

*Clinica Norsalud, Madrid, Spain, †Allergy Service, Hospital 12 Octubre, Madrid, Spain, ‡Research Unit for Allergic Diseases, Carlos Haya Hospital-Fundacion IMABIS, Málaga, Spain and §Allergy Service, Carlos Haya Hospital, Málaga, Spain

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Summary

Background β -Lactam (BL) antibiotics can induce non-immediate skin reactions, frequently manifested as exanthema or urticaria. The time between drug intake and the reaction appearance is generally 24–48 h. Because the mechanisms involved are not completely understood, diagnostic tests for these reactions have still to be fully validated.

Objective To evaluate the role of skin and drug provocation tests (DPTs) in the diagnosis of patients with non-immediate reactions to BL.

Methods We evaluated a group of 22 patients who developed maculopapular exanthema or urticarial exanthema after BL intake. Diagnosis was confirmed by DPT with BL. Intradermal/patch testing was performed with benzylpenicilloyl, minor determinant mixture, amoxicillin (AX), ampicillin (AMP) and the culprit drug in patients and in 22 negative controls. Immunohistochemical studies were done in the affected skin at the acute phase of the reaction and after a delayed positive skin test/DPT. IFN- γ and IL-4 were quantified in peripheral mononuclear cells, obtained during the positive response to DPT and after resolution of the symptoms.

Results From the total number of cases, 12 patients developed urticarial exanthema and 10 maculopapular exanthema after DPT. Only two of the 22 patients (9%) had a positive delayed intradermal skin test: one to AX/AMP and the other to cloxacillin. Biopsies showed a mononuclear CD4, CD8 infiltrate and activated and memory cells. The cytokine expression showed a Th1 pattern in patients, in contrast with the Th0 pattern in controls.

Conclusion In patients with non-immediate reactions to BLs (maculopapular exanthema or urticarial exanthema), the sensitivity of skin testing is low and DPT may be required to establish the diagnosis. The reproducibility of the reactions and the cytokine pattern expressed during the acute episode support a T cell-induced non-immediate response.

Keywords β -lactam, cytokine, drug provocation test, non-immediate reactions, skin tests, T cells

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Correspondence:

Miguel Blanca, Allergy Service, Hospital Civil, Málaga, Spain. E-mail: mblancag@gmail.com

Introduction

Two major mechanisms participate in allergic reactions to β -lactams (BLs): IgE-mediated responses and effector T cell-dependent reactions [1]. The classic IgE-dependent responses are immediate urticaria and/or angioedema and anaphylactic shock [2]. Reactions attributed to T cell-dependent responses are more different, varying from mild reactions, such as exanthema or delayed urticaria, to more severe reactions such as Stevens–Johnson

syndrome or toxic epidermal necrolysis [3, 4]. Organ-specific reactions, such as interstitial nephritis or hepatitis, have also been reported [5, 6]. The role of T lymphocytes has been proposed for all these reactions with *in vivo* and/or *in vitro* evidence of T cell involvement [7, 8]. *In vitro* studies show that both CD4 and CD8 T cells can be activated with highly heterogeneous functions [9–12]. Antibodies have also been suspected [2, 13, 14] but, except for haemolytic anaemia induced by IgG or IgM antibodies, no definitive role for immune complexes in

these reactions has been shown [15]. Although IgG antibodies may exist, their presence does not distinguish between reactors and exposed controls [16].

In non-immediate reactions, urticaria and exanthema are the most frequent manifestations [17]. Patch and/or intradermal tests have been proposed for their *in vivo* diagnosis [17–22]. Patients develop a typical mononuclear cell infiltrate after positive patch/intradermal testing [17–20]. In exanthematic drug reactions, the skin infiltrate has been well characterized [22, 23] but less information is available for non-immediate urticaria [18, 22, 23]. Although the lymphocyte transformation test (LTT) has also been proposed for diagnostic purposes [7, 8, 24], its role has not been definitively established [24, 25]. The LTT does not distinguish between immediate and non-immediate reactions, or between selective and non-selective responses and may also proliferate in non-exposed controls [24].

We report the results of studies from a well-defined positive group of cases that were evaluated prospectively after being referred to our allergy clinic because they had previously had a maculopapular exanthema or urticarial exanthema attributed to a BL derivative. After completing the study, only a low proportion of the patients responded and skin testing was of limited value. Even though most patients with a positive drug provocation test (DPT) had a negative skin test, the cytokine expression pattern and the clinical response suggested a T cell-mediated immunological mechanism.

Material and methods

Subject selection

A positive group was selected from 146 subjects referred to our clinic over a period of 2 years for evaluation of a non-immediate reaction attributed to a BL derivative and confirmed by a positive DPT. The patients were aged 18–75 years and their reaction had occurred from 4 years to 6 months before evaluation.

Maculopapular exanthema was considered to be the presence of small confluent erythematous maculae or papules disseminated over different parts of the body. Urticarial exanthema was defined as larger erythematous areas elevated from the skin, whether or not accompanied by angioedema and persisting for several days. In both circumstances, symptoms had to have occurred at least several hours after drug intake and usually within an interval of 24–48 h as defined in the ENDA position paper [18, 19]. Moreover, an immediate reaction was ruled out by following the protocol described in the ENDA guidelines [26]. Six patients whose symptoms occurred within 1 h after drug intake were excluded: two of the subjects who reported urticaria were immediate skin test positive to benzylpenicilloyl (BPO) and four to amoxicillin (AX). In

addition, two of these six patients also had specific IgE antibodies.

After establishing the final study group, we choose an equivalent control group, composed of the same number of cases, age- and sex matched, who were taking BL for the same indications as the persons in the study group but who had good tolerance.

The institutional review boards approved the study, and informed consent for the diagnostic procedures was obtained from all patients.

Drug provocation test

This was undertaken following the ENDA recommendations, with some modifications [19, 26]. Briefly, once a positive immediate skin test or the *in vitro* presence of IgE antibodies was ruled out, 5 and 50 mg of the drugs available orally (AX, cefaclor, cefalexine and cloxacillin) were given with a 1-h interval. This was followed by giving increasing doses of 100, 250, and 500 mg at 48-h intervals. For parenteral drugs (cefazoline, cefuroxime), the same concentrations and at the same intervals as above were administered parenterally. For benzylpenicillin (BP), the doses used were 10^4 and 10^5 IU with 1-h interval and, if negative, we then administered doses of 2×10^5 , 5×10^5 and 10^6 IU at 48-h intervals, also parenterally. For the first 8 h of drug administration, the patients were monitored in the drug allergy unit, after which they were followed up with a paging system. DPT with the culprit drugs was only done when subjects were negative to penicillin determinants.

Intradermal/patch testing

These were done as described [17, 19]. The maximum concentration for BPO was 5×10^{-5} M, for minor determinant mixture (MDM) 2×10^{-2} M, for AX 20 mg/mL, for ampicillin (AMP) 20 mg/mL, for cloxacillin 20 mg/mL and for cephalosporins 2 mg/mL. Readings were made at 24, 48 and 72 h.

Patch testing was done by mixing powdered drug in petrolatum at a concentration of 5% w/w, as described [17]. In all cases, the occlusion time was 48 h and readings were made at 24, 48 and 96 h.

Specific immunoglobulin E antibodies

Testing was carried out using the Pharmacia CAP system for the haptens BPO, AX and AMP [27]. These are the more relevant haptens-inducing allergic reactions in our population. For cephalosporins, a RIA assay validated in our laboratory was used [28].

Sample collection

Serum samples were obtained at the start of the study for quantitation of specific IgE antibodies. Blood samples were obtained after a positive DPT during the occurrence of the reaction and 1 month after its resolution. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Nycomed, Oslo, Norway) from heparinized venous blood and processed for RNA extraction and cDNA synthesis for cytokine quantitation.

For immunohistochemical studies, a 4 mm punch skin biopsy was obtained at the site of the positive cutaneous response (in the case of skin test) or of the affected skin after a DPT reaction. The biopsies were fixed in 10% formalin, routinely processed, and paraffin-embedded until the corresponding study.

Quantification of messenger ribonucleic acid expression

Total RNA was extracted in TriPure (Roche, Indianapolis, IN, USA) in accordance with the manufacturer's instructions and treated with DNase I (Sigma, Madrid, Spain). One microgram of total RNA was reverse transcribed with random hexamers and Moloney murine leukaemia virus-reverse transcriptase at a final volume of 20 μ L. Specific cDNA amplification of the human cytokines IL-4 and IFN- γ was carried out with 2 μ L of cDNA by using FastStart DNA Master SYBR Green I (Roche). The specific primers were 5'-CATCGGCATTTTGAACGAGGTCA-3'; 5'-CTTATCGATGAATCCAGGCATCG-3' for IL-4. 5'-CATTGA AAGCCTAGAAAGTCTG-3'; 5'-CTCATGAATGCATCCTTT TTCG-3' for IFN- γ . The specificity of the reaction products was confirmed by melting curves. The concentration was obtained by crossing-point extrapolation into a seven-point standard curve with known cDNA concentrations by using Light Cycler system software and results were expressed as relative units to represent the ratio of the concentration between the specific mRNA and the house-keeping (β 2-microglobulin) mRNA.

Immunohistochemical studies

Microtome sections (8 μ m) were processed for haematoxylin-eosin and immunohistochemical staining as described [17], with the following polyclonal antibodies: CD4 (Novocastra Lab, Newcastle upon Tyne, UK), CD8, CD45RO and HLA-DR (Dako, Ely, Cambridgeshire, UK) and CLA (BD Pharmingen, San Diego, CA, USA). The binding of these primary antibodies (rabbit and goat IgG) was detected using an anti-rabbit IgG conjugated to peroxidase-labelled dextran polymer and anti-goat IgG horse-radish peroxidase (HRP)-conjugated antibody (Zymed Lab, San Francisco, CA, USA). Visualisation of the signal was carried out with a 3,3'-diaminobenzidine substrate kit (Sigma, St Louis, MI, USA).

Statistical studies

Comparison of cytokines during the acute episode in patients and controls was made by a non-parametric test for non-related samples (Mann-Whitney test) and comparison between the acute phase and the resolution period within the patient group was made by the Wilcoxon test. 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

Results of drug provocation testing

A total of 22 patients who fulfilled the inclusion criteria were included in the study. Their mean age was 38.9 ± 16.52 years and the median time interval between the drug administration and the appearance of the symptoms was 24 h (interquartile range of 17.5–34.5). The drugs considered to have caused the symptoms were AX in 12 patients, AMP in 1, BP in 4, cefaclor in 2, cefuroxime in 1, cefazoline in 1, cefalexine in 1 and cloxacillin in 1. Twelve (54%) patients developed urticarial exanthema and 10 (45%) maculopapular exanthema (see Table 1).

Thirteen patients who reacted to aminopenicillins had good tolerance to BP. Four patients whose clinical records reported a reaction to AX responded to a BP and in these, no aminopenicillins were given in accordance with the ENDA guidelines [19]. Cephalosporins elicited a reaction in five patients, four had good tolerance to BP and AX but one also had a positive response to BP (see Table 1). In six patients with a positive DPT, a skin biopsy was obtained from the affected skin. Four developed a maculopapular exanthema and two urticarial exanthema, according to the criteria described above. The results showed a high T cell (CD4 and CD8 cells) infiltrate, with expression of CLA and HLA-DR at both the perivascular site and the dermal-epidermal junction. These markers were also found to a lesser extent in the epidermis, where a moderate keratinocyte death was observed with haematoxylin-eosin stain. Comparing the results of maculopapular exanthema vs. urticarial exanthema, we found a greater presence of CD4⁺ cells with a higher expression of CLA and HLA-DR in the former. Figure 1 shows a typical immunohistochemistry result for urticarial exanthema and maculopapular exanthema.

Results of intradermal/patch testing

After intradermal testing, two patients had a delayed positive result, one to AX/AMP and one to cloxacillin (Table 1). These patients reported having had exanthematic reactions with desquamation. During the DPT, one

Table 1. Clinical characteristics of the patients included in the study

ID	Age (year)	Controlled administration	Reaction	Time interval (year)	Intradermal tests	Patch tests
1	72	BP – ; AX – ; Cefaclor+	Urticarial exa.	24	Negative	Negative
2	22	BP – ; AX +	Maculopapular exa.	48	Negative	Negative
3	60	BP – ; AX +	Desq. exa.	48	AX + ; AMP +	AX + ; AMP +
4	39	BP – ; AX +	Maculopapular exa.	24	Negative	Negative
5	33	BP – ; AX +	Maculopapular exa.	48	Negative	Negative
6	40	BP – ; AX +	Maculopapular exa.	24	Negative	Negative
7	41	BP +	Urticarial exa.	24	Negative	Negative
8	19	BP – ; AX – ; Cefaclor+	Urticarial exa.	30	Negative	Negative
9	33	BP – ; AX +	Maculopapular exa.	48	Negative	Negative
10	39	BP – ; AX +	Urticarial exa.	30	Negative	Negative
11	58	BP – ; AX +	Urticarial exa.	18	Negative	Negative
12	43	BP – ; AX – ; Cefazoline+	Urticarial exa.	12	Negative	Negative
13	62	BP +	Maculopapular exa.	24	Negative	Negative
14	30	BP +	Urticarial exa.	24	Negative	Negative
15	24	BP – ; AX +	Urticarial exa.	10	Negative	Negative
16	18	BP – ; AX +	Maculopapular exa.	48	Negative	Negative
17	34	BP + ; Cefuroxime+	Urticarial exa.	20	Negative	Negative
18	26	BP – ; AX – ; Cefalexine+	Urticarial exa.	16	Negative	Negative
19	32	BP – ; AMP +	Urticarial exa.	14	Negative	Negative
20	75	BP – ; AX – ; Cloxacillin+	Desq. exa.	24	Cloxacillin+	Cloxacillin+
21	27	BP – ; AX +	Urticarial exa.	16	Negative	Negative
22	29	BP – ; AX +	Maculopapular exa.	24	Negative	Negative

Urticarial exa, urticarial exanthema; Maculopapular exa., maculopapular exanthema; Desq. exa, desquamative exanthema; BP, benzylpenicillin; AX, amoxicillin; AMP, ampicillin; Time interval, time elapsed between drug administration and appearance of the symptoms.

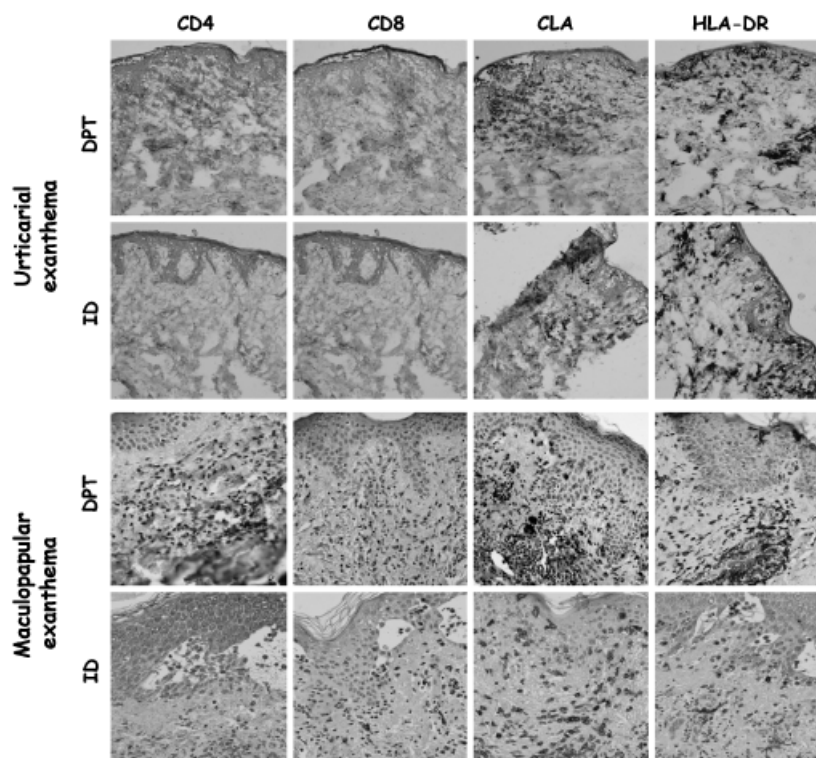


Fig. 1. Skin biopsy specimens from a typical delayed reactions: urticarial exanthema and maculopapular exanthema, both during the reaction after a DPT and after a positive cutaneous response. Samples were processed for haematoxylin–eosin and for immunohistochemical stains of the lymphocyte subpopulations CD4 and CD8, the skin homing receptor CLA and the activation marker HLA-DR. DPTs, drug provocation tests; HLA, human leucocyte antigen.

of the patients (case 3) had a mild exanthematic reaction at the dose of 100 mg that begun 8 h after drug administration and lasted for 48 h and the other (case 20) had a similar reaction but at a dose of 250 mg that started 24 h after drug intake and lasted for another 24 h. The patch tests with the same drugs were also positive in these patients. The optimal response (highest expression of the local skin lesions) was seen at 48 h.

Immunohistochemical study of the skin biopsies obtained from positive intradermal testing showed similar results and a similar distribution to that described above for positive DPT. The haematoxylin–eosin stain also showed similar results, including the keratinocyte death.

Cytokine profile in positive responders

We compared the disbalance of IL-4 and IFN- γ by analysing the changes in the ratio of IFN- γ /IL-4 expression produced between the acute phase and resolution period by the Wilcoxon test, which compares related samples. The results showed a significant increase during the acute phase of the reaction ($P=0.043$). This ratio was also significantly increased in patients during the acute episode compared with healthy controls taking BL ($P=0.0001$; Fig. 2). No differences were found when comparing the cytokine profile obtained from between the two entities.

Discussion

Although many subjects claim to be allergic to BL [29, 30], appropriate studies with diagnostic tests and DPT reveal that a very high proportion of these persons in fact have good tolerance [19]. This may be due to loss of sensitivity of the tests, well known for IgE-mediated reactions [31], though it is more likely due to falsely labelling persons as allergic, which may result in patients undergoing tests that can be costly and may involve administration of other, possibly dangerous drugs as an unnecessary alternative [19, 29].

The evaluation of immediate reactions is now well established and different studies have shown the value of the techniques used [2, 26, 32]. However, less evidence is available for non-immediate reactions [17, 33]. Although reactions compatible with a T cell response were reported from the outset, the value of intradermal testing has still not been sufficiently demonstrated [33].

Studies over the last decade have shown the value of intradermal/patch testing in patients who have allergic reactions to BL, with 60–100% of patients being confirmed positive [14, 19]. Intradermal/patch testing has therefore been recommended for the routine screening of patients with suspected non-immediate allergy to BL [17].

In a study comparing patch and intradermal testing, we showed that both tests are good alternatives, although intradermal testing seems to be more sensitive [17]. However, in that study and others, we selected the patients because they had had a previous positive intradermal test [14, 19]. The advantage of the present study is that patients were included based on the results of DPT, thus eliminating any positive bias. Surprisingly, and in contrast to previous studies [14, 19, 33], only 9% of the patients developed a positive skin test response, whereas values of 60–100% sensitivity have been reported previously [19, 33]. A possible explanation for this is that previously known positive cases already diagnosed were included in the study, giving a high number of positive cases and a possible selection bias [14, 17]. To avoid this problem, in the present study, we included subjects who were evaluated for the first time. Because delayed intradermal responses may remain after a long time, we only selected patients who had had a reaction during the 4 years before the study (up to 6 months beforehand) and who had not previously been included in any study, thus attempting to avoid this phenomenon and the presence of exanthematic reactions of another nature, including viral reactions. Another possible explanation that may have contributed to the low sensitivity of the intradermal/patch testing was that most of the cases had mild reactions, with just two cases of desquamation following the

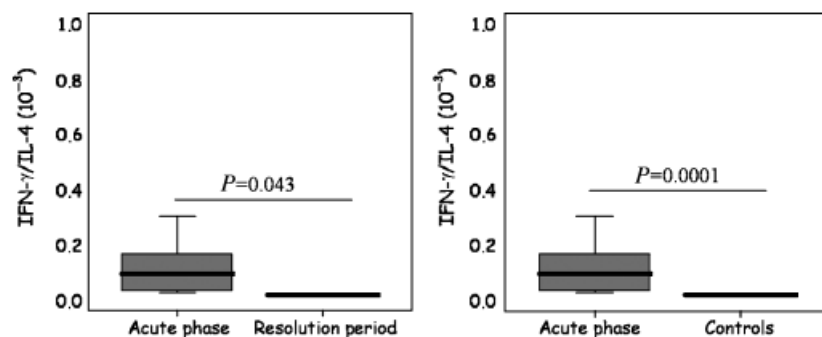


Fig. 2. (a) Comparisons of the ratio between the IFN- γ and IL-4 relative units (IFN- γ /IL-4) in PBMCs during the acute phase of the reaction and during the resolution period. (b) Comparison of the ratio between the IFN- γ and IL-4 relative units (IFN- γ /IL-4) in PBMCs between patients during the acute phase and the controls taking BL. PBMC, peripheral blood mononuclear cells.

exanthematic manifestations. Interestingly, these two cases were the only ones who were skin test positive. A clear association between positive results of the test and the severity of the reactions has been reported [6, 18]. However, whether this can be applied to more severe cases or other populations needs to be studied.

We found an important role for aminopenicillins, with almost all patients tolerating BP, as reported in other studies [14, 33]. However, in contrast to other series [14], cephalosporins are taking on an increasing role, possibly due to their increased pattern of consumption, although only one patient appeared to respond to another BL (BP or AX); whether this was due to cross-reactivity or to two non-immediate coexisting responses cannot be clarified with this type of study. Although very little evidence exists, dual immediate and non-immediate responses have been published, suggesting that patients can have independent non-cross-reactive responses to several BL [31, 34]. Although we have shown this does not exist in immediate reactions by detailed clinical and immunological studies [35, 36], in non-immediate responses, it is more difficult to verify and it was not the purpose of this study.

The induction of urticaria or exanthema by other mechanisms, e.g. IgG or another isotype-dependent response, is debatable [2, 3, 15]. Previous evidence from our group indicates that IgG antibodies play no role in allergic reactions to BL [13]. Other idiosyncratic mechanisms involved are more difficult to assess. In those cases where a biopsy was obtained, a clear T cell mechanism is supported, as seen by others for maculopapular exanthema [8, 37]. With respect to the urticarial exanthema, a role for lymphocytes has been reported although no detailed studies have been carried out [38, 39]. In these reactions, lymphocytes and other inflammatory cells are recruited to the skin and are able to secrete histamine-releasing factors that induce mast cell degranulation. This can be applied to our cases, although further studies are required to verify the hypothesis.

In our study, the cytokine pattern, determined by mRNA quantification, showed a Th1 mechanism, expressing a higher ratio of IFN- γ to IL-4, which decreased to control levels after resolution. Although this is indirect evidence, further studies are required to establish the role of these cytokines.

Summarizing, non-immediate cutaneous reactions to BL are reproducible in a certain proportion of patients. We showed that they are T cell mediated in some patients, but in others, we only have indirect evidence for this. The pattern of cross-reactivity indicates that in the event of a non-immediate reaction, consideration should be given to controlled administration because many subjects who respond to aminopenicillins have good tolerance to BP and subjects who respond to cephalosporins may have good tolerance to penicillin derivatives.

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