The use of basophil activation tests in clinical-laboratory investigations in patients with beta-lactam allergy (pilot study)

Zastosowanie testów aktywacji bazofilów w badaniach kliniczno-laboratoryjnych u pacjentów z alergią na beta-laktamy (badanie pilotażowe)

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Summary

BACKGROUND: Beta (β)-lactam antibiotics (BLAs) are the first-line therapy for non-nosocomial and nosocomial bacterial infections and are most commonly reported to cause allergic reactions. Approximately 50% of all allergic patients in Europe and the USA suffer from drug allergies and BLA allergies.

The AIM of the study was to assess cross-reactivity reactions between 2nd and 3rd generation cephalosporins in patients with a medical history of BLA reactions and the risk of adverse reactions to BLAs based on the results of the basophil activation test.

MATERIALS AND METHODS: we examined 48 females and 8 males (in all 56 patients) aged 26 to 61 with primary reactions to BLAs and 24 healthy volunteers (control group). 19 (34%) patients were treated with amoxicillin, 18 (32,1%) patients were receiving amoxicillin+clavulanic acid, 6 (10,7%) patients were treated with cefuroxime, and 13 (23,2%) patients with ceftriaxone. Quantitative determination of the CD63 marker of basophil degranulation upon antigen stimulation in whole blood was performed with the use of Flow CAST (FK-CCR) (Bühlmann Laboratories AG, Switzerland). Based on the obtained BAT results, the patients were divided into two subgroups: the first group included 33 patients with positive stimulation index but lower CD63 expression (<10%), and the second group included 15 patients with a significantly higher level of CD63 expression (>10 %).

THE RESULT: We showed that patients from the second subgroup had the highest level of CD63 expression and stimulation index when amoxicillin, whereas the level of CD63 expression and stimulation index were lower whith ceftriaxone; at the same time, the level of CD63 expression and stimulation index were the lowest with cefuroxime. The patients who treated with and reacted to amoxicillin, as shown by high BAT, also had high CD63 expresiion after ceftriaxone and cefuroxime stimulation. In the first subgroup, urticarial and bronchospasm disappeared within 3 hours of the onset of symptoms in 51.5% of patients, the symptoms persisted for 2-3 days in 42.4% of patients with urticaria and angioedema, whereas maculopapular exanthema persisted for more than a week in 6.1% of the patients. Patients from the first subgroup (with low CD63 expression) had a weak reaction to the culprit antibiotic. Patients from the second subgroup had the strongest reaction to culprit antibiotics: anaphylaxis – 60.0%; Stevens-Johnson syndrome – 6.7%. We established that in patients with hypersensitivity to antibiotics the higher the baseline test scores after *in vitro* stimulation, the more severe clinical symptoms.

CONCLUSION: for patients with clinical manifestations of BLA in case of conflicting anamnesis data, it is recommended to establish true sensitization to antibiotics and to predict the occurrence of cross-reactions

Streszczenie

WPROWADZENIE: Antybiotyki beta(β)-laktamowe (BLA) są terapią pierwszego rzutu w pozaszpitalnych i szpitalnych zakażeniach bakteryjnych i są najczęściej zgłaszane jako wywołujące reakcje alergiczne. Około 50% wszystkich alergików w Europie i USA cierpi na alergie na leki, w tym na alergie na BLA.

Celem badania była ocena reakcji krzyżowych między cefalosporyną II i III generacji u pacjentów z klinicznym wywiadem reakcji na BLA i ryzyka wystąpienia niepożądanych reakcji BLA na podstawie wyników testu aktywacji bazofilów.

MATERIAŁ I METODY: przebadaliśmy 48 kobiet i 8 mężczyzn (razem 56) z pierwotnymi reakcjami BLA w wieku od 26 do 61 lat oraz 24 zdrowych ochotników (grupa kontrolna). Pacjentów 19 (34%) bylo leczonych amoksycyliną, 18 (32,1%) – amoksycylina + kwasem klawulanowym, 6 (10,7%) – cefuroksymem i 13 (23,2%) – ceftriaksonem. W celu oceny markera degranulacji bazofili CD 63 po stymulacji antygenem w pełnej krwi wykorzystano oznaczenie Flow CAST (FK-CCR) (Bühlmann Laboratories AG, Szwajcaria). Na podstawie wyników BAT pacjentów podzielono na dwie podgrupy: w pierwszej grupie było 33 pacjentów z dodatnim nizszym wynikiem indeksu stymulacji (<10%), a w drugiej grupie było 15 pacjentów z istotnie wyższym poziomem CD63 ekspresja CD63 (>10%).

WYNIK: Nasze wyniki wykazały, że pacjenci z drugiej podgrupy mieli najwyższe wyniki ekspresji CD63 i wskaźnika stymulacji dla amoksycyliny, następnie dla ceftriaksonu, a ostatni dla cefuroksymu. Byli leczeni amoksycyliną i odpowiadali na nią, jak wykazały wysokie wartosci ekspresji CD63 w BAT, ci pacjenci mieli również wysoką ekspresję CD63 po stymulacji ceftriaksonem i cefuroksymem. W pierwszej podgrupie u 51,5% pacjentów pokrzywka i skurcz oskrzeli ustąpiły w ciągu 3 godzin od wystąpienia objawów, u 42,4% pacjentów z pokrzywką i obrzękiem naczynioruchowym objawy utrzymywały się przez 2-3 dni, a u 6,1% osutka plamkowo-grudkowa - przez ponad tydzień. Pacjenci z pierwszej podgrupy (z niską ekspresją CD63) wykazywali klinicznie słabe objawy reakcji. Po leczeniu antybiotykami pacjenci z drugiej podgrupy wykazywali silniejsze objawy: u 60,0% - anafilaksja; 6,7% - zespół Stevensa-Johnsona. Wykazalismy, że u pacjentów z reakcja nadwrazliwosci na leczenie antybiotykami im wyższe wyjściowe wyniki testu po stymulacji *in vitro*, tym bardziej nasilone są ich objawy kliniczne.

WNIOSEK: u pacjentów z klinicznymi objawami BLA w przypadku sprzecznych danych z wywiadu zaleca się ustalenie rzeczywistego uczulenia na antybiotyki i przewidywanie występowania reakcji krzyżowych między penicylinami i cefalosporynami nie tylko drugiej, ale i trzeciej generacji. Wyniki BAT z antybiotykami mogą być wykorzystane do opracowania zaleceń przyszłych terapii przeciwbakteryjnych.

between penicillins and cephalosporins not only of the 2nd but also of the 3rd generation. The results of BAT for antibiotics can be used to formulate future antibacterial treatments recommendation.

Keywords: *beta-lactam allergy, clinical manifestations of allergy, CD63 expression, antibiotics cross-reactions, the prognosis of the severity of allergy to antibiotics.*

Słowa kluczowe: *reakcja alergiczna na antybiotyki beta-laktamowe, kliniczne objawy alergii, ekspresja CD63, alergia krzyżowa na antybiotyki, prognozowanie skomplikowania alergii na antybiotyki.*

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Introduction

Beta-(β)-lactam antibiotics (BLA) are the first-line therapy for non-nosocomial bacterial infections and are most commonly reported to cause allergic reactions [1]. Hypersensitivity reactions to antibiotics account for 15% of all adverse drug reactions and pose a serious health problem [2,3]. Approximately 8% of all adults in southern Europe and the USA are suffering from a drug allergy, and 4.5% of adults in these countries suffer from an allergy to BLAs [4].

BLA allergies are generally presented as type I or type IV hypersensitivity reactions [5]. Amoxicillin most often causes immediate hypersensitivity reactions (IHRs) to β-lactams (BLs), followed by cephalosporins [6]. IgE-mediated I-type reactions typically occur immediately within 1-6 h of medication administration [6,7,8] and are usually manifested as urticaria (hives), angioedema/laryngeal edema, bronchospasm, respiratory compromise, vomiting or cramping abdominal pain, flushing, dyspnea, hypotension, tachycardia or anaphylaxis [3]. Severe non-IgE-mediated reactions to penicillin (interstitial nephritis, hepatitis, hemolytic anemia, serum sickness, severe cutaneous reactions - SJS, TEN, DRESS) were documented [9]. Diagnostics of immediate and non-immediate reactions to BLA poses a dilemma in clinical practice [10].

Type I hypersensitivity reactions are caused by the interaction of allergen-specific IgE with FceRI on mast cells, basophils, and eosinophils. This interaction causes cell degranulation and the release of histamine, tryptase, chymase, and carboxypeptidase A [5]. These mediators are responsible for the pathologic reactions of an immediate hypersensitivity reaction, ranging from cutaneous symptoms and respiratory symptoms to anaphylaxis [11]. Antibiotics (e.g., penicillin, cephalosporin) cause hypersensitivity by the direct release of mast cells and basophil inflammatory mediators [5,12]. The issue of genetics and family history remains unresolved; at the same time, family history of adverse reactions to beta-lactams is likely more relevant in case of non-immediate hypersensitivity reactions rather than in case of immediate ones [4,13].

Delayed (non-immediate) reactions (type IV hypersensitivity) always occurred within a period from 60 min to several weeks, following initiation of medication [4,7,8]. They were caused by CD4 T-cells responding to the epitopes of foreign allergens, for example, BLA, which appear within a period from several days to several weeks after the last administered dose [7]. These reactions have heterogeneous clinical manifestations, but may be subdivided into those with isolated, single-organ (hepatic, pulmonary, renal, hematological), or systemic, multi-organ involvements [5]. In case of a non-immediate reaction, cytotoxic and cytokine-secreting T-cells arrange inflammatory cells (i.e., neutrophils, eosinophils) [3,12].

The risk factors of a BLA allergy are as follows: 1) history of previous allergy reactions to penicillin; 2) female gender [14]; 3) route of exposure and frequency of administration - topically applied penicillin is highly immunogenic; 4) limited evidence that the oral route is less likely to cause reactions than other routes; 5) frequent courses are more likely to cause sensitization of bacteria to frequently administered intravenous antibiotics (in patients with cystic fibrosis) [15]; 6) age (20 to 49); younger children had a lower risk than older patients [14]; the likelihood of a fatal outcome (cardiovascular or respiratory comorbidity or the use of beta-blockers) is also higher in older patients; 7) atopy and asthma can be a risk factor in case of life-threatening reactions; 8) concurrent infections – EBV; HHV-6,-7,-8; CMV; SARS-Cov-2; HIV and other viruses with immune dysregulation properties [3,7,8,13]. Up to 70 % of patients with viral infections, particularly Epstein–Barr virus and SARS-CoV-2, who were receiving amoxicillin were reported to develop a self-limiting maculopapular rash [4, 10, 16].

Beta-lactams' (BL) chemical structure is formed by a 4-membered ring, but in penicillin it is fused to a 5-membered thiazolidine ring, whereas in cephalosporin it is fused to a 6-membered dihydrothiazine ring. These drugs have a side chain (R1) attached to the BL ring; in addition, cephalosporin has a second side chain (R2) attached to the dihydrothiazine ring, which is, in turn, attached to the BL nucleus, whose chemical structures distinguish one compound from the others [1,6,14]. It is currently generally assumed that cross-reactivity is primarily determined by the R1 side chain [1]. Some patients with IDHRs to amoxicillin have immunologic response and production of IgE antibodies directed at the R-group side chain (rather than the core penicillin determinants). Most hypersensitivity reactions to cephalosporin are probably directed at the R-group side chains, rather than at the core-lactam portion of the molecule [16].

Penicillins have low molecular weight, hence they must covalently bind in order to transport macromolecules and form a BLA (hapten)-carrier complex that functions as an allergen [13,17]. BLA becomes immunogenic by binding to human serum albumin (HSA). Binding to the amino acid lysine takes place via the opening of the beta-lactam ring, which results in the formation of primarily benzylpenicillin

(BPO) from benzylpenicillin. Benzylpenicilloyl-octa-L-lysine (BP-OL) and benzylpenicillinlloy l-poly-L-lysine (PPL), bound via conjugation with octa- or poly-L-lysine, are used as major determinants [4]. The penicillin central nucleus normally spontaneously opens, binds to transport proteins, and forms the major antigenic determinant, which explains all penicillin-specific immune responses. Approximately 5 determinants of penicillins are metabolized by other parts, and the resulting antigens are known as minor determinants [1]. Amoxicillin probably represents the amino group in R1 for the formation of additional minor determinants [6,17].

There are some indeterminable problems in the prognosis of the likelihood of cross-reactivity between penicillins and cephalosporins: 1) the reason why the haptenization mechanism occurs at a slower rate and is possibly more complex than in case of penicillin [8]; as for cephalosporins, the process of the hapten-protein complex formation is unknown [1]; 2) the R2 side chain acts as a "leaving group" when binding to the carrier protein, and this leads to increased beta-lactam ring reactivity [4]; 3) cephalosporin degradation does not follow the same pattern throughout the group [4]; the structure and antigenic determinants of cephalosporin-protein conjugates are difficult and are not well researched [6]; 4) other parts of the molecule (excluding R2) are necessary for the formation of the antigenic determinant; these structures serve as alternatives for the determination of *in vitro* cross-reactivity to cephalosporins in amoxicillin-allergic patients [6]; 5) a novel synthetic pyrazinone structure that serves as an antigenic determinant has been identified; this structure is formed after the reaction of the amino group in the R1 and is probably a potential antigenic determinant which mimics a cefadroxil fragment [6].

Beta-lactam allergy assessment tools can include medical history and *in vivo* tests: 1) skin tests (ST) – this represents the first level of approach for the diagnosis of type I, immediate, IgE-mediated allergy [25]; 2) drug provocation tests (DPT) (high/strong) [20]; 3) patch tests (PTs) - this is used for delayed type, cell-mediated, hypersensitivity reactions to the antibiotic [25]. *In vitro* tests (for detecting T-cell-mediated reactions) can include detecting of: drug-specific IgE, basophil activation test (BAT), inflammatory and cytotoxic mediators release of activating T cells, lymphocyte transformation test (LTT) etc. [3,4,6,7,8,10,12,18].

However, when tested with penicillin (using either skin, blood, intradermal test (IDT) or patch testing), not all of the patients will have positive results [6,14,15]. You must not conduct provocation tests with antibiotics on patients with severe reactions in their anamnesis without their consent. The utility of ST in the diagnosis of immediate and non-immediate reactions is limited, especially in case of non-immediate reactions [8, 10]. Drug-specific IgE measuring, enzyme-linked immunosorbent spot assay (for detecting cytokines, for example, IFN-γ) and the lymphocyte transformation test (LTT) can be used [12,19]. The results of T-lymphocyte reactions can only be interpreted in conjunction with all other findings and patients' medical history [20,21]. Demonstration of isolated drug-specific IgE to penicillins does not establish a BLA diagnosis [22,23]. Basophil activation test (BAT) has been an additional test for the diagnosis of drug hypersensitivity reaction (DHR) [13,24]. In the presence of the implicated drugs [4,5,24,25], especially β-lactam antibiotics, cyclosporine and quinolones [11,26], basophil activation can be detected using anti-IgE cell markers, CD63, etc. BAT has the highest significance in the cellular diagnosis of immediate reactions [4]. In general, there are no established methods for predicting the allergic potential of antibiotics [23].

The aim of this paper is to assess cross-reactivity reactions between 2nd and 3rd generation cephalosporin in patients with a medical history of BLA reactions and the risk of adverse BLA reactions based on the results of a basophil activation test.

Materials and Methods

Materials

BLA diagnostics was based on the patients' medical history, *in vivo* (skin tests, DPT), and *in vitro* diagnostics (sIgE, BAT). We examined 56 patients (48 females, 8 males) (aged 26 to 61) with primary BLA reactions and 24 healthy volunteers (control group). 8 patients were excluded from the study group because there were no changes in their CD63 expression.

The control group consisted of 24 healthy volunteers aged 30-56 – 15 females and 9 males – without systemic chronic diseases (diabetes, autoimmune diseases, allergic diseases or any accompanying decompensated diseases). According to the anamnesis and the analysis of outpatient charts, these individuals had taken β-lactams antibiotics at least 2-3 times for various bacterial infectious diseases and without any systemic or local side effects.

Based on the obtained results, BAT patients (Fig.1) were divided into two subgroups: the first group included 33 patients with positive stimulation index but lower CD63 expression (<10%), and the second group included 15 patients with a significantly higher level of CD63 expression (>10 %).

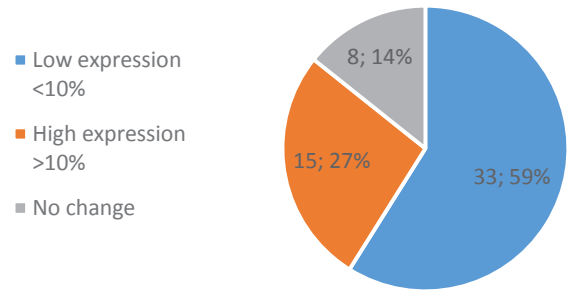


Figure 1. Groups of patients who participated in the study with different CD63 expression

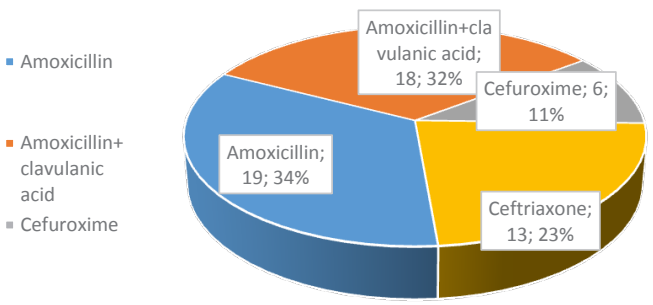


Figure 2. The number of patients who were treated with different antibiotics

19 (34%) patients were treated with amoxicillin (semisynthetic penicillin), 18 (32,1%) patients were treated with amoxicillin+clavulanic acid, 6 (10,7%) patients were receiving cefuroxime (cephalosporin of the second generation) and 13 (23,2%) patients were administered ceftriaxone (third generation cephalosporin) (see Fig. 2). Antibiotic therapy was prescribed as treatment for various diseases: 62,5% of patients had infectious and inflammatory diseases of ENT and respiratory organs, 23,2% of patients had complications after SARS-CoV-2 infection, and the remaining 14.3% of patients had other diseases caused by bacterial infections.

In 43.8% of patients the reactions occurred after the first dose; in 37.5% of patients they occurred after 2-3 doses, and in 18.8% of patients they occurred after more than 4 doses. In patients from the second subgroup, the majority of reactions occurred after the administration of the second dose of antibiotics. The amount of time that passed from initial drug administration to the first onset of symptoms (urticarial, asthma, anaphylaxis, etc.) was the same in two subgroups: in 50.0% of all patients the first symptoms appeared 20-30 min after administration; in 16.7% of patients the symptoms appeared within 5 min of administration; in 33.3% of patients the symptoms appeared more than 30 min after drug administration.

It was found that all patients from both subgroups had or have a history of these or other allergic diseases, including allergic rhinitis, atopic dermatitis, bronchial asthma, and urticarial. At the same time, in 20.8% of patients, anamnesis revealed an allergy to other groups of drugs.

According to the medical history of patients from the first subgroup, 39.4% of patients took this antibiotic for the first time. 36.3% of patients noted that they had taken the drug once or twice before. While analyzing medical history of patients from the first subgroup, it was found that the most common manifestations of drug allergy (DA) included urticaria (48.5% of patients), urticaria accompanied by angioedema (27.3% of patients), bronchospasm (18.2% of patients), exanthematous rashes (6.1% of patients). After a more detailed analysis, it was revealed that urticaria was accompanied by itching in almost all patients; at the same time, it was mainly localized in the torso and upper limbs. Angioneurotic edema was mainly localized on asymmetrical faces, namely in the areas of soft tissues (eyelids, lips); in 9.1% of patients, facial swelling was combined with swelling of the external genitalia and distal portion of the limbs. In 18.2% of patients, facial swelling was combined with swelling of the mucous membrane of the upper respiratory tract and tongue. As for patients from the second subgroup, anaphylaxis was detected in 60% of cases, urticaria accompanied by angioedema was detected in 33.3% of cases, and Stevens-Johnson syndrome (SJS) was detected in 6.7% of cases. In patients with anaphylaxis, in 86.7% of cases detailed clinical signs were characterized by a combination of skin manifestations, in 53.3% of cases they were characterized by damage to respiratory organs, and in 53.3% of cases such signs were characterized by damage to cardiovascular system. Hyperthermia, damage to the mucous membranes of the oral cavity and genitals and severe symptoms of intoxication were observed in patients with SJS manifestations.

All patients from both subgroups, who took the drug (history data) were instructed to stop taking it, and emergency care was provided. In the first subgroup, only 24.2% of patients were hospitalized, whereas all of the patients form the second subgroup required hospital treatment. The treatment was carried out according to the Unified clinical protocol of emergency, primary, secondary (specialized), and tertiary (highly specialized) medical care "Drug allergy, including anaphylaxis» of the Ministry of Health of Ukraine (Decree No. 916 of December 30, 2015) [28].

The study was conducted on the basis of the 7th revision of the principles set out in the Declaration of Helsinki Human Rights (2013), the Council of Europe Convention on Human Rights and Biomedicine, and the relevant laws of Ukraine and was approved by the Ethics Committee of Danylo Halytsky Lviv National Medical University (minutes No.6 of October 14, 2020). Informed consent was obtained from all participants before the beginning of the study.

Methods

For flow cytometry, quantitative determination of basophil degranulation upon antigen stimulation in whole blood with the use of Flow CAST (FK-CCR) and CAST Allergens (Bühlmann Laboratories AG, Switzerland) was used. Each patient had their tubes labeled: 1. PB = patient background (only Stimulation Buffer); 2. PC1 = stimulation control with anti-FcεRI Ab; 3. PC2 = stimulation control with fMLP 4. A1-Ceftriaxone (Bag2-C35, concentration: 4 mg/mL); A2 – Cefuroxime (Bag2-C33, concentration: 2.5 mg/mL); A3 – Amoxicillinum (Bag2-C81, concentration: 2.5 mg/mL). 50 μl of the corresponding stimulus were added to each tube for each patient. 100 μl of stimulation buffer, 50 μl of patient whole blood specimen collected with EDTA as an anticoagulant, and 20 μl of staining reagent were added to each tube. They were then mixed and incubated for 15 minutes at 37 C in a water bath. The whole blood samples were lysed and fixed with 2 ml of pre-warmed (18-28°C) Lysing Reagent and mixed gently for 5-10 min at 18-28 C. After washing, the cell pellet was stored in 300 ml of Wash Buffer, at 2-8°C, protected from light. Flow cytometry was performed on a BD FacsCalibur flow cytometer with the use of a 488 nm argon laser diode (blue-green excitation light). The flow cytometer detects Forward Scatter (FSC), Side Scatter (SSC), two fluorochromes FITC and PE. 300 basophil cells were analyzed, which required a total of 50'000-100'000 leukocytes per sample.

Statistical calculations were performed with the help of the Student's t-test. At the same time, two independent groups checked whether differences were normally distributed (Gaussian distribution). The independent-sample t-test was performed in order to compare the results of patients and the control group. In the event of non-normal distribution, statistical calculations were performed for the compared groups with the use of the non-parametric Mann-Whitney U test. The difference between the groups was significant with the reliability is 95%.

Results

An accurate medical history is crucial for the assessment of patients who report HSRs to β-lactams. We have identified statistical differences between the obtained results on the basis of patients' history. In some cases, however, it is not possible to classify the reaction only using the data obtained during this period [8]. Based on the clinical manifestations (56 patients, 100%), the results of the diagnostics of type reactions indicated 77% of the immediate

reactions, and 23% of the reactions were delayed. However, based on the time that passed from the occurrence of a reaction, the results of the diagnostics of type reactions indicate that 61% of the reactions were immediate and 39% of the reactions were delayed (Figures 3a, 3b).

We decided that in order to determine the types of reactions, it is necessary to carry out experimental *in vitro* exposition of our patients' basophils using standard solutions of antibiotics, with subsequent performance of a basophil activation test (BAT). We performed BAT for such patients and calculated the stimulation index after the incubation of basophils (for 15 min) in standard solutions of ceftriaxone, cefuroxime and amoxicillin. Thus, based on the obtained data, we can confirm that the time of reaction occurrence and the type of clinical manifestations are insufficient to diagnose the condition of patients with reactions to beta-lactam antibiotics.

Our patients were divided into two subgroups: patients whose level of CD63 expression was slightly higher than in the control group (<10%) and patients whose level of CD63 expression was significantly higher than in the control group (>10%).

Furthermore, we examined the following parameters (Figures 4 a, b, c, d, e, f): 1) CD63 basophil activation parameters (on the left); 2) stimulation index after incubation in the solutions of ceftriaxone (4 a, b), cefuroxime (4 c, d) and amoxicillin (4 e, f).

In patients with BLAs, spontaneous CD63 expression (without stimulation) was 3.23±1.09% in comparison to 2.57±0.91% (P=0.64) in the control group. Stimulation with anti-FcεRI Ab positive control in patients with BLA was 77.65±19.22%, in comparison to 56.22±16.20% (P=0.40) in the control group, and stimulation with fMLP positive control in patients with BLA was 40.54±11.24%, in comparison to 36.42±10.44% (P=0.79) in the control group. In the first subgroup of patients with an allergy to ceftriaxone, the mean CD63 expression was 5.81±2.44%, in comparison to 3.37±1.07% (P=0.36) in the control group and 2.57±0.91% (P=0.22) in the control group without stimulation. In the second subgroup of patients with an allergy to ceftriaxone, the mean CD63 expression was 24.37±6.75% in comparison to 3.37±1.07% (P=0.005) in the control group and 2.57±0.91% (P=0.003) in the control group without stimulation. There was a significant difference (P=0.013) in expression between the first and the second subgroups of patients who were administered ceftriaxone. The stimulation index (SI, was calculated as the percentage of activated basophils after stimulation with antibiotics, divided by the number of basophils with no stimulation) in the first subgroup of patients with an allergy to ceftriaxone was 2.07, whereas in the second subgroup it was 6.11. We found that there was no statistically significant difference in the level of spontaneous CD63 expression in patients from the two subgroups, but the stimulation index after incubation in the ceftriaxone solution was significantly higher.

In the first subgroup of patients with an allergy to cefuroxime, the mean CD63 expression was 5.66±1.45%, in comparison to 4.33±1.21% (P=0.48) in the control group and 2.57±0.91% (P=0.076) in the control group without stimulation. In the second subgroup of patients with an allergy to cefuroxime, the mean CD63 expression was 20.57±5.86%, in comparison to 4.33±1.21% (P=0.011) in the control group and 2.57±0.91% (P=0.004) in the control group without stimulation. There was a significant difference (P=0.017) in expression between the first and the second subgroups of patients who were administered cefuroxime. The SI in the first subgroup of patients with an allergy to cefuroxime was 1.92, whereas in the second subgroup it was 5.63.

In the first subgroup of patients with an allergy to amoxicillin, the mean CD63 expression was 6.23±1.84%, in comparison to 4.75±1.44% (P=0.53) in the control group and 2.57±0.91% (P=0.08) in the control group without stimulation. In the second subgroup of patients with an allergy to amoxicillin, the mean CD63 expression was 26.90±9.24% in comparison to 4.75±1.44% (P=0.025) in the control group and 2.57±0.91% (P=0.013) in the control group without stimulation. There was a significant difference (P=0.037) in expression of CD 63 between the first and the second subgroups of patients who were administered amoxicillin. The SI in the first subgroup of patients with an allergy to amoxicillin was 1.61, whereas in the second subgroup it was 7.21.

The results showed that patients from the second subgroup had the highest level of CD63 expression and stimulation index. Patients from the second subgroup had severe clinical manifestations, including anaphylaxis in 9 patients (60%).

Discussion

The diagnostics of penicillin allergy is complex. However, the presence of measurable anti-beta-lactam IgE is rarely, because its determination is not recommended [13,26]. Even in patients with true beta-lactam allergies, the number of IgE antibodies decreases over time [7]. Reliability of

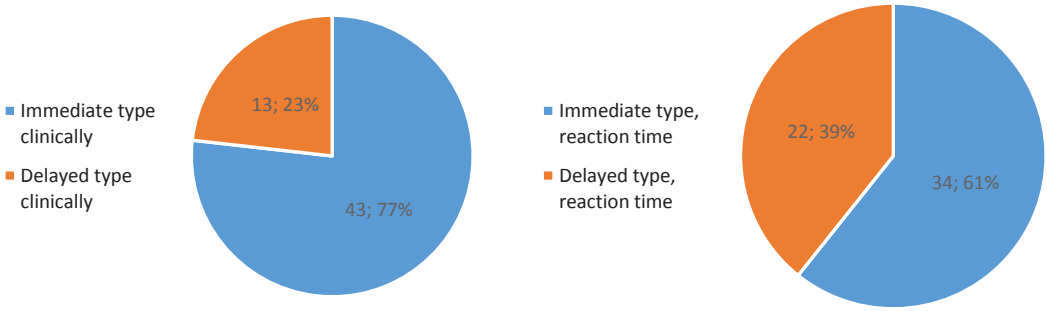


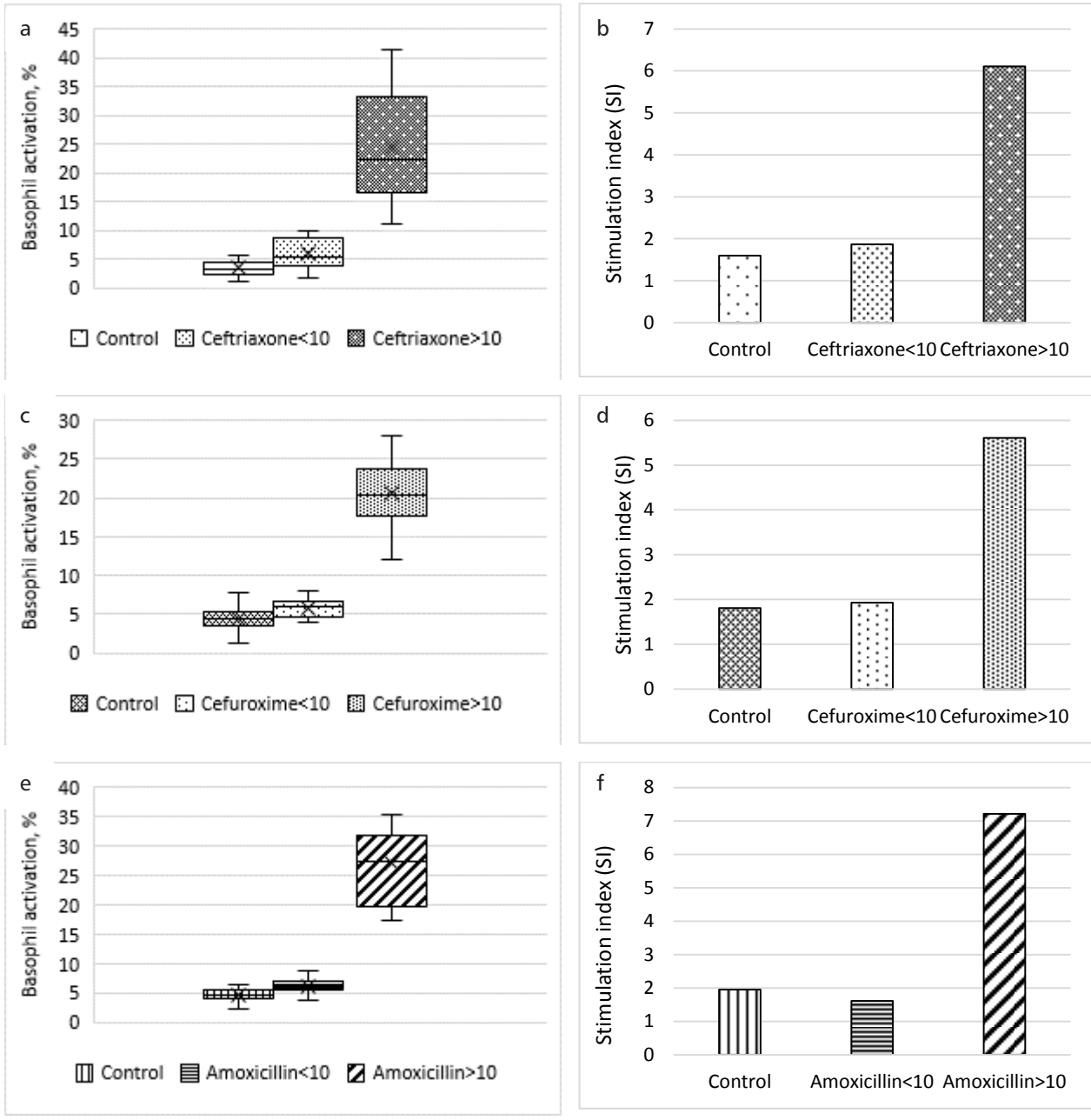
Figure 3. Comparison between the percent of patients with immediate and delayed reactions to BLAs, based on the time of reaction occurrence (1-6 hours) (a) and the first clinical symptoms (b) in patients with BLAs.

P - Statistical significance between groups in both comparisons (p<0,05)

specific IgE to BLAs is the subject of controversy [4]. Basophil activation test (BAT) for amoxicillin shows higher sensitivity (about 50%) and specificity (approx. 90%), compared to the quantification of sIgE antibodies [26]. BAT was used *in vitro* to provide evidence of IgE sensitization [5,29].

BAT is the main *in vitro* test for assessing patients with immediate reactions to β-lactams [12]. BAT provides significant opportunities [26]. Multiple studies on the use of BAT, which included a significant number of patients, have been performed for BLs [24,30,31,32]. *In vitro* testing is of great importance, particularly in case of severe, life-threatening reactions. It allows one to perform allergy testing even in case of high-risk patients, when *in vivo* testing is contraindicated, and in cases where skin testing is not possible, e.g. due to a skin disease. BAT can be helpful in some cases of anaphylaxis and drug-induced urticaria [4]. Patients with multiple β-lactam allergies of varying severity and antibiotic type were categorized according to the most severe reaction [33]. However, BAT sensitivity may vary depending on various factors, such as the time interval between the reaction and the test, reaction severity, and its chronology [3]. To date, no tests have been established for reliable determination of the severity of a reaction to allergens, but the use of BAT looks promising [5].

Cell activation has been confirmed by the expression of the CD63 marker in the presence of the implicated drug. BAT was used for antibiotics and may be a useful tool for the identification of sensitized patients before the occur-



*– Significant differences between the control group, the 1st, and the 2nd groups

Figure 4. CD63 basophil activation parameters (on the left); 2) stimulation index after incubation in the solutions of ceftriaxone (4 a, b), cefuroxime (4 c, d) and amoxicillin (4 e, f) in the two subgroups of patients with BLA and in the control group.

rence of reactions [5,29]. In general, assay sensitivity varies from 50% to 60%, whereas specificity easily reaches 80%. BAT/HistaFlow allows one to perform simultaneous testing of different compounds, including both active components and excipients, such as amoxicillin [26]. Despite the specificity of 93.3%, BAT sensitivity is only 50%, although it may be higher in case of cephalosporins. However, the difference was only significant when amoxicillin, rather than benzylpenicillin, was used as the hapten [8]. BAT has such advantages as the fact that testing possesses no risk to patients and that it has a significantly broader range of allergens, compared to specific IgE. At the same time, there are some disadvantages: lack of standardization, gradual negativization after the reaction, considerable technical complexity, the need for fresh blood, false-negative results (in «non-responders»), or low sensitivity [34]. Even though BATs usually have high specificity (>90%), their sensitivity rate has been highly variable, depending on the drug used. Positivity rates for BLs ranged from 44% to 63%. Moreover, BAT results can change due to the use of a particular CD63 basophil activation marker that specifically upregulates expression after drug stimulation, as it was demonstrated for BLs during clinical presentation. A negative BAT response can also be the result of alternative Mas-Related G-protein receptor-X2 (MRGPRX2-dependent) activation in skin mast cells [35,36,37,38,39]. Activation of the mast cell, connected with the MRGPRX2 receptor, does not play a role in immediate reactions to BLs [40,41]. CD63 is the most commonly used cell marker of basophil degranulation after IgE-dependent stimulation by antibiotics, but in case of BAT, there is no discrimination between IgE- and non-IgE-mediated reactions [4,11,29].

In addition to the aforementioned advantages of BAT over other *in vitro* methods, we expanded its diagnostic capabilities and suggested performing BAT for the purpose of predicting the severity of patients' reactions and identifying potential cross-reactions to other antibiotics.

We analyzed the duration of clinical manifestations in both subgroups. More than 39.4% of patients in the first subgroup took antibiotics for the first time. In 51.5% of patients from the first subgroup symptoms (urticaria, bronchospasm) disappeared within 3 hours of their appearance, in 42.4% of patients with urticaria and angioedema the symptoms persisted for 2-3 days, and in 6.1% of patients, clinical manifestations (maculopapular exanthema) persisted for more than a week. According to our data, patients from the first subgroup (with low CD63 expression) had a weak clinical response (minor urticaria, which disappeared within 2 hours, and maculopapular exanthema without systemic manifestations). Patients from the first subgroup had urticaria, angioedema, bronchospasm, and eczematous rashes.

According to the survey, 53.3% of patients from the second subgroup were taking the drug for the second time, whereas 46.7% of the patients had already taken the drug more than twice. During the analysis of the second subgroup, it was found that in 40.0% of patients, symptoms persisted during the first day, in 26.7% of patients, they persisted for up to 3 days, and in 33.3% of patients, the symptoms persisted for more than a week. After the treatment with antibiotics, 60.0% of patients from the second subgroup had anaphylaxis and 6.7% of patients had SJS. We found that the higher the baseline test scores after *in vitro* stimulation in patients with responses to antibiotic treatment, the more severe their clinical symptoms were.

In case of BAT, free drugs are assumed to covalently bind proteins present in the blood through β-lactam reactivity, as a result of which a conjugate is formed, which is big enough to achieve cross-linking. This approach attempts to emulate *in vivo* conditions, but there is a lack of information about the chemical composition of the conjugate that induces basophil activation [26]. It has been found, that cross-reactivity in IgE-mediated IHRs to amoxicillin with cephadroxil – a cephalosporin with the same R1 – has a probability of 35% [6]. However, other parts of the molecule (excluding R2 substituents) are necessary for the formation of the antigenic determinant. These structures are the alternatives for determining *in vitro* cross-reactivity to cephalosporins in amoxicillin-allergic patients [6,17]. The R1 side chain remains intact and mainly accounts for cross-reactivity, while the R2 side chain makes little contribution to cephalosporin hypersensitivity [1]. The cross-reactivity rate with cephalosporins in penicillin-allergic patients with IgE-mediated reactions ranges from 0% to almost 40%, depending on the chemical structure of the BL involved, in particular on the similarity in the R1 side chain. Amoxicillin, which has the same amino R1 side chain as cefadroxil, can have high cross-reactivity. Conversely, cefuroxime, which has a different R1 side chain, has shown tolerance in patients with IHRs to penicillins. Similar results have been recently obtained for cefazolin and ceftibuten [6]. When anaphylaxis or severe reactions are not reported as adverse reactions, we suggest avoiding only cephalosporins with similar side chains [40]. Patients with a history of IHRs to first generation cephalosporins should avoid cephalosporins with similar R-group side chains [16]. Side chains are a common cause of allergic cross-reactivity [1]. Overall, scientific data about chemical differences and similarities between various generations of cephalosporins and their association with the risk of adverse reactions are contradictory.

There are a lot of scientific data about the possibility of cross-reactions between penicillin and 2nd generation cephalosporin [1,6,16,17]. Cephalosporin allergy can only be studied through the detection of IgE against the native molecule [8, 11].

As for cross-reactions between penicillin and 3rd generation cephalosporin, scientific data are also contradictory.

Clinically important cross-reactivity between penicillins and third- and second-generation cephalosporins could be between 1% and 2%. After examining 234 confirmed penicillin-allergic patients (180 immediate and 54 delayed hypersensitivity reactions), the autors found that the cross-reactivity risk was below 1%. The examination was carried out with the use of skin testing (ST) [44]. Another group of researchers evaluated 234 true penicillin-allergic patients, and there were only 1/159 positive reactions to ceftriaxone. Only 1/76 (1.3%) of those tested with 2nd generation cephalosporin (cefuroxime) had an immediate positive skin test, while having negative results of skin tests and a drug provocation test (DPT) with a 3rd generation cephalosporin cefpodoxime and ceftriaxone, in accordance with the published data on immediate drug hypersensitivity reaction (IDHR) and non-IgE-mediated drug hypersensitivity reaction (DHR). This study of 234 truly penicillin-allergic patients confirms the published data on the very low risk of cross-reactivity, namely less than 1%, with different side-chain-bearing cephalosporins like cefuroxime, ceftriaxone

and cefpodoxime. The patients were examined with the use of skin tests and drug provocation tests (DPT) [45].

Out of the 1,393 patients with confirmed BL hypersensitivity, which also had confirmed IDHR to amoxicillin, we randomly selected 54 patients (3.87%), whose cross-reactivity to cefadroxil and cefuroxime was evaluated and analyzed with the use of skin tests and drug provocation tests (DRT), as well as a radioallergosorbent test (RAST) for the detection of drug-specific IgE [46].

Based on the obtained results, we found that cross-reactions between antibiotics accounted for 6.25% (the number of all patients who had a reaction – 48, the percent of all patients with cross-reactions – 6.25%). Thus, according to our data, after performing BAT for other antibiotics (3rd generation cephalosporin), we obtained high statistically significant values of CD63 expression in case of patients with severe reactions, who were treated with amoxicillin. It turned out that patients from the second subgroup had the highest level of CD63 expression and stimulation index when amoxicillin was used, whereas the level of CD63 expression and stimulation index were lower when using ceftriaxone; at the same time, these indicators were the lowest when using cefuroxime. Based on the obtained percentage, we are confident that BAT is better than ST, DPT [45] and RAST [46] for the prognosis of cross-reactivity between cephalosporins of various generations.

The history of herpes viruses (EBV), SARS-Cov-2 virus [41], and moderate or severe course of COVID-19 (4 out of 12 (30%) patients from the second subgroup had a history of COVID-19), as well as female gender (85,3%), were additional prognostic factors for a severe reaction to antibiotics in the second subgroup of patients. The results we obtained are consistent with the published data [42], since SARS-Cov-2 and herpesviruses have important immune dysregulation properties [3], and women are more likely to report a history of drug allergies, in particular penicillin allergy [43], than men. At the same time, the number of female patients who had been referred for DHR was 2.54 times higher than the number of male patients who had been referred for this test [14].

Conclusion

The results of the Basophil activation test (BAT) for antibiotics can be used to determine true sensitization to antibiotics and predict the occurrence of cross-reactions between penicillin and cephalosporin not only in the 2nd but also in the 3rd generation.

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