

UDC 616.517-07-085

¹V.D. Babacan ²L.V. Kuznetsova, ¹P.G. Kravchun, ¹N.G. Ryndina¹Kharkiv's National Medical University, Kharkiv²National Academy of Postgraduate Education named by P.L. Shupyk, Kiev

Laboratory diagnosis of drug allergy.

Part 2. Methodology for determining the activation of cells to drugs allergens

Key words: *in vitro*-diagnostics drug allergy, basophil activation test, the test antigen stimulation of cells, lymphocyte transformation test, test definition upregulation receptor CD69.

Due to the success of pharmacology and the creation of many new drugs, the use and abuse of drugs is increasing [1, 2]. Increase of allergy in the population, due to the increase in the amount of medication prescribed to patients, as well as the reactivity of the body, leading to an increase in complications of therapy, including allergic reactions to drugs [3]. Now 6 % of patients who are hospitalized, marked certain adverse reactions to medicines. Thus true allergic reactions to drugs are found in 66 % of patients, and the reaction pseudoallergy – 34 % from the indicated number of patients [3].

Most drugs – are simple organic compounds with low molecular weight (less than 1000 D) [4]. Often the primary medicament is not immunogenic because it does not bind to protein sufficiently stable covalent bond [5]. The immunogenic potential of such drugs is often defined by one or more metabolites that bind hapten in the form of various body proteins. By increasing the molecular weight and structural complexity of the molecule's ability to induce an immune response in the drug increases. Macromolecular drugs (heterologous serum, streptokinase, insulin) are full allergens. Allergic reactions to proteins and peptides are often mediated by IgE – antibodies or immune complexes. However, the mechanism of allergic reactions can also be mixed [6,7].

Besides the specific chemical structure is responsible for cross- sensitivity, which in some cases may be associated with elements of the kernel, while others can only be determined specificity of the side chains [8]. For example, consider that the frequency of cross-reactions to cephalosporins in penicillin allergy is not very high [9], since the nuclei of both different. However, cases of cross hypersensitivity to these antibiotics are described in the literature [10], which depended on the

structure of the side chains. These facts should be considered when prescribing [11].

Skin tests for the diagnosis of drug allergy immediate type can not always be used to conduct them there are many contraindications and reliability of these tests is relatively low due to the fact that the cause of the allergic reaction is often not the initial drug and its metabolites [4 12]. Therefore, for the diagnosis of drug allergy skin tests with laboratory tests are used [13].

It is absolutely reliable tests for the diagnosis of drug allergy *in vitro* does not exist, so the researchers are improving existing practices and creating new tests. In connection with this drug for the diagnosis of allergy tests are developed, which are based on activated cells allergens [14]. The advantage of that tests are that no use of specific IgE and IgG antibodies to the study medication. Restriction informativeness of these tests is the same type as the test results in allergic and in response to pseudoallergy preparatat. Earlier the same reactivity was shown in tests damaging leukocytes and stimulation of histamine release by basophils. An example of such a test is a direct test of basophil degranulation.

Direct basophil degranulation test (BDT) allows determining the antibody bound to the leukocytes. BDTs based on basophil degranulation allergy patients sensitized antibody class IgE, formed under the influence of specific allergen [15].

Basophil degranulation test is performed as follows. Fasting patient receive 10-15 ml (1 ml - 1 allergen) blood from a vein into a tube with heparin (20 IU/ml). Blood is allowed to stand for 30-45 min. Plasma was aspirated and centrifuged leukocyte 3-5 min at 500 rev/min. The supernatant was removed and leukocyte pellet resuspended in 0.5-1 ml of saline (at a

concentration of 5-10 million cells per 1 ml). In wells plate for immunological studies contribute to 0.05 ml of a suspension of leukocytes in test wells - equal volume of allergens in control - the solvent. Incubate 15 min at 37 ° C and added to each well 0.05 ml of a solution of toluidine blue. 0.025 % solution of toluidine blue stain metachromatic granules of basophils. This solution was prepared by 30 % ethanol, adjust pH to 3.2-3.4 by addition of glacial acetic acid (approx. 3-4 μ l of 10 ml of paint). Then the suspension is filled Goryaev's chamber and count the number of stained basophils. In the control group must contain at least 30 cells. The reaction is considered positive if the number of stained cells in the experiment was reduced by 30 % or more relative to control. If there are 50 or more basophils control test is positive when reducing the number of basophils in a test sample by 20 % or more as compared with the control. Drugs used in concentrations, procaine 2.5 mg/ml, analgin 0.5 mg/ml vitamin B1 1 mg/ml. Fig. 1 shows the results of direct basophil degranulation test. A - microscopic unchanged basophils. B - microscopic basophil degranulation in the state, magnification $\times 900$. Despite demonstrative test, due to its low reproducibility in different laboratories to standardize the method used flow cytometry.

Basophil activation test using flow cytometry. This method allows measuring the level of in vitro IgE- dependent response to allergens in patients' sensitized basophils by analysis using flow cytometry. Basophil activation in the presence of the allergen occurs in stages, starting with changes within the cell (signal transduction from Fc ϵ RI p38MAPK by phosphorylation and calcium influx), with subsequent changes in the cell membrane (appearance CD203c) and the development of basophil degranulation (the appearance of CD63 on the membrane of basophils occurs after merger intracellular granules to the cell membrane and release of mediators) [16, 17].

Reagents comprising a set allow us to find the differences between the activated basophils and basophils, which are dormant, by flow cytometry. For this purpose a combination tricolor antibodies, which is a mixture of monoclonal antibodies bicolor (CD203c-PE and CD3-PC5) and rat monoclonal antibodies monochrome (CRTh2-FITC). In whole blood from healthy donors cell CRTh2 receptor expressed on eosinophils, and basophils, as well as T-helper type 2, respon-

sible for the development of humoral immunity and allergic responses [18]. Cell receptor CRTh2 and prostaglandin D receptor are associated with G-proteins and receptors are both prostaglandin D2, but when linking them with different ligand-activated intracellular signaling pathways. Prostaglandin D2 is a major metabolite of arachidonic acid, which is produced by activated mast cells and allergen is a lipid mediator of inflammation in allergic responses [19]. Monoclonal antibody 97A6 recognizes a surface antigen that is expressed exclusively on basophils in peripheral blood of man and is not expressed on other blood cells [20]. It has been proven that a monoclonal antibody 97A6 directed against CD203c [21].

CD3 antigen expressed only on the surface of mature T-lymphocytes. UCHT1 monoclonal antibodies interact with ϵ -chain complex CD3 [22].

Basophil activation test is performed as follows. Basophils express the high affinity receptor IgE (Fc ϵ R1). As a positive control of IgE-mediated basophil activation antibodies to IgE, contained in the reagent, which can recognize IgE associated receptors, and induce basophil activation.

The solution is optimized to enable a buffer with a high content of calcium, which allows activating basophils in vitro blood collected into tubes with EDTA, as anticoagulant (EDTA). Solution to stop the reaction buffer serves high-EDTA, which allows you to stop the process of basophil activation in vitro.

For sample analysis by flow cytometry is necessary to prepare a monodisperse cell suspension and erythrocytes to eliminate complicating analysis. The main component of the lysis solution is a cyclic amine which, in the presence of erythrocyte carbonic anhydrase converted to a compound capable of lysing erythrocytes.

The fixing solution allows preparing a sample of whole blood by fixation of cells in suspension during lysis of erythrocytes. This solution is used for the fixation of finished samples before analysis.

Basophils were stained with monoclonal antibodies in the presence of an allergen, or control. Then are performed lysis of erythrocytes and analysis of samples on a flow cytometer. After exclusion of the analysis of T-lymphocytes (as CD3-positive

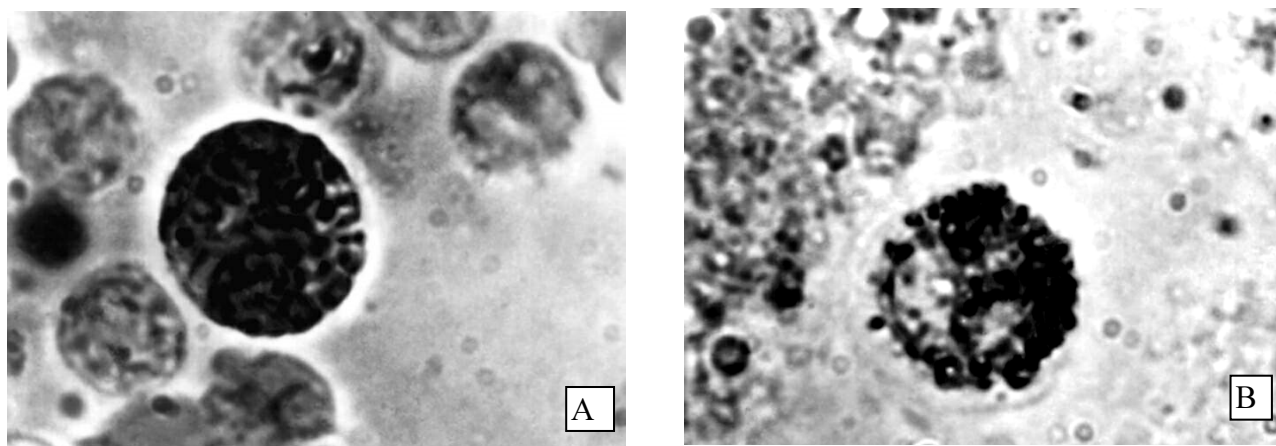


Figure 1. Direct basophil degranulation test. A - microscopic unchanged basophils, paint sample toluidine blue. B - microscopic basophil degranulation in the state, paint sample toluidine blue. Magnification $\times 900$.

cells) is being analyzed by the expression of basophils CRTh2 and CD203c. Members basophils (basophils, which are dormant) show the phenotype CRTh2posCD203c dimCD3neg, whereas in vitro activated basophils have phenotype CRTh2posCD203c brightCD3neg.

When another modification of the method determines the number of activated basophils in the level of expression of CD63 (Fig. 2, 3, 4). Upon stimulation with allergen level of CD63 expression increased 2-3 fold [23].

Basophil activation by specific allergens

Upon contact with the allergen molecules of IgE on basophils and mast cells occurs a cascade of enzymatic reactions resulting in degranulation and the release of mediators from granules (heparin, histamine). These reactions also lead to the synthesis and secretion of leukotrienes (LT), cytokines, inflammatory mediators that cause allergy symptoms. Leukotrienes are synthesized and also in the case pseudoallergy, i.e. reactions occurring without IgE. When pseudoallergy occurs basophil activation and nonspecific release of mediators (histamine, LT, prostaglandins) by trigger mechanisms: complement activation, IgG-mediated hypersensitivity, auto-IgE-antibody or through non-immune reaction (direct activation of basophils).

Test antigen stimulation of cells (Cellular Antigen Stimulation Test - CAST) is a modern method of diagnosis of immediate hypersensitivity. CAST urgency associated with the need to confirm the results of skin tests obscure independent of the use of specific IgE method. In addition, some types of allergies (to medicines, nutritional supplements) revealed very bad skin tests or serology (detection of specific IgE – sIgE), for them now only valid dangerous provocative tests [24]. The

technology is based on determining the CAST sulfidoleukotrienov (sulfidoleukotrienov – LTC₄, LTD₄, LTE₄), secreted IL -3 primed basophil under the action of the allergen in vitro. It is also called a provocative test in vitro. Thanks synthesis sulfidoleukotrienov (sLT) de novo analysis of CAST has a high specificity compared with the classic test of histamine release.

Monoclonal antibodies as a positive control are used to high affinity receptor for IgE (Fc RI), mimicking the binding IgE - antibodies to the receptor on basophils membrane. In order to obtain reliable and reproducible results, it is important to choose the correct response to allergens and their dosages, since some of them are able to interact with basophils nonspecifically in vitro (medicine, food additives, etc.).

CAST is as follows. 1. Isolation of leukocytes to a) EDTA stabilized blood are added to the dextran sedimentation of erythrocytes, b) the supernatant was centrifuged to precipitate the leukocytes (removal of dextran and platelets), and c) the precipitate of white blood cells resuspended in buffer. 2. Stimulation of leukocytes carried out: a) anti - FcεRI - antibody (positive control), b) a physiological solution (negative control)) allergen (allergens) in various concentrations. 3. The supernatants were collected and immediately determine the content of sulfide - leukotrienes (LTC₄) isolated sensitized leukocytes under the influence of anti - FcεRI - antibodies and allergens using ELISA. For the quantitative determination requires multiple dilutions of standards for the calibration curve. 4. Interpretation of the test. For medicinal allergens lower (threshold) level (cut off) about 40 pg / ml.

CAST is not recommended as the primary prick tests for the survey (they are more complex, more expensive and inferior to the standard tests detection of specific IgE (UniCAP, etc.)).

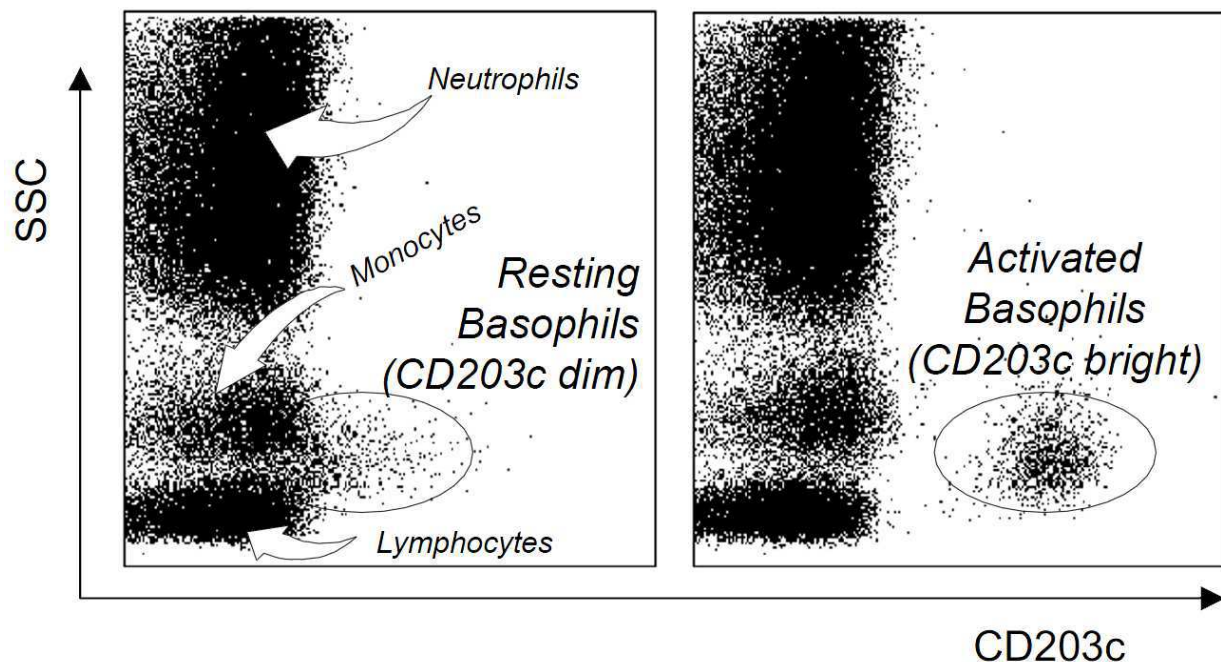


Figure 2. Test Results activate basophils, which is made using fluid cytometry. CD203c expression in whole blood before and after basophil activation. Ungated leukocytes are shown as a biparametric representation on the basis of side scatter characteristics (SSC, y-axis) and CD203c (x-axis). Left histogram depicts resting cells, basophils express low levels of CD203c (some of them are not distinguishable from lymphocytes and monocytes). Right histogram depicts cells after anti-IgE challenge, activated basophils are easily recognized on the basis of their high CD203c expression. (Bühning, H.J., "E-NPP3 (CD203c)", 2002, Leucocyte typing VII, Section New CD antigens, MC10, Oxford University Press.- P.377-378.; Boumiza R., Debard A.-L., Monneret G., The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives// Clinical and Molecular Allergy.- 2005.- № 3:9.- P. 1-8.).

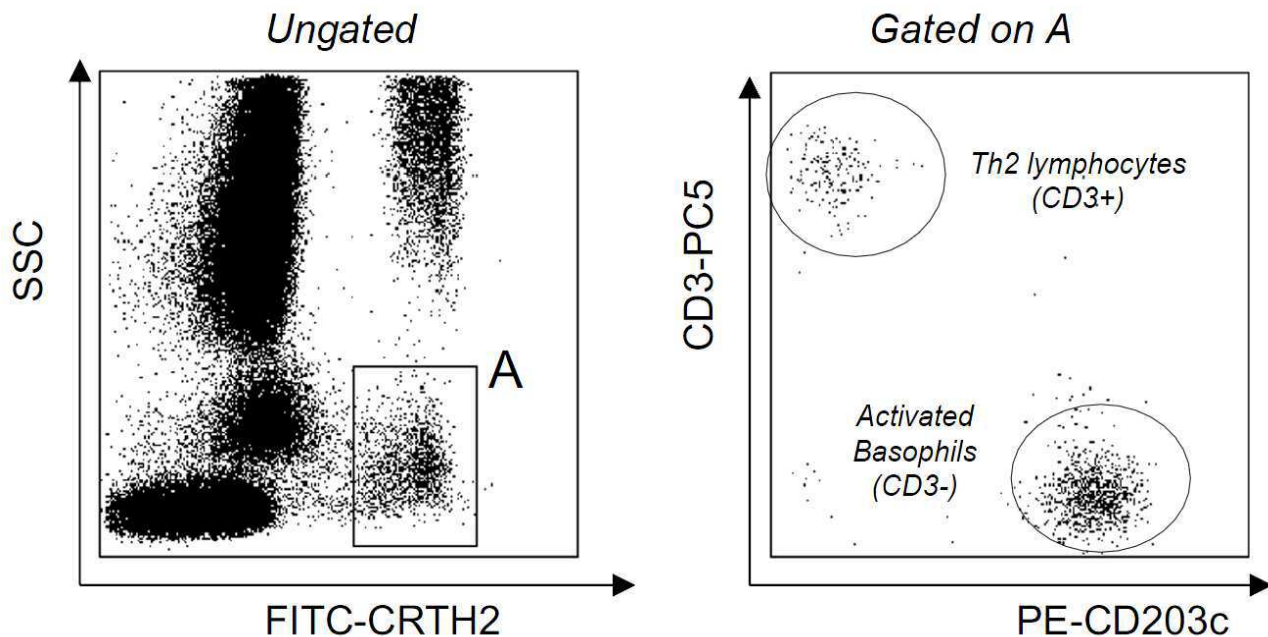


Figure 3. Identification of CRTH2 expressing cells by flow cytometry. Left histogram : ungated leukocytes biparametric representation on the basis of side scatter characteristics (SSC, Y axis) and FITC-CRTH2 (X axis). Two CRTH2 expressing cell populations are easily distinguishable: the one with high light scatterings corresponds to the eosinophil population; the second one (gating region: A) comprises Th2 lymphocytes and basophils. Right histogram: cells from the gating region (A) expressed on the basis of PE-CD203c (X axis) and PC5-CD3 (Y axis) characteristics. Th2 lymphocytes were readily separated from basophils based on their positive CD3 expression while activated basophils express high levels of CD203c without expressing CD3. These cell populations selected by using monoclonal antibodies PE-CD203c (X axis) i PC5-CD3 (Y axis). (B hring, H.J., "E-NPP3 (CD203c)", 2002, Leucocyte typing VII, Section New CD antigens, MC10, Oxford University Press.- P.377-378; Boumiza R., Debard A.-L., Monneret G., The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives// Clinical and Molecular Allergy.- 2005.-№3:9ю- P. 1-8.)

Cytometric test version of antigenic stimulation of cells - FLOW - CAST/FAST. Stages separate lymphocytes and allergen challenge for both options, and enzyme-linked immunosorbent cytometric identical. But instead of the third stage sLT FLOW - CAST determine the number of activated basophils expressing surface antigen CD63 (gp53) in response to stimulation with an allergen. The main indications for use CAST/FAST: suspicion of immediate hypersensitivity to mediator type and the absence of specific IgE, the presence of hypersensitivity to food additives and drug allergens suspicion of pseudoallergic mechanism of clinical manifestations, diagnosis of asthma and aspirinlike classes.

Beta-lactam antibiotics (penicillins, amoxicillin, ampicillin, cephalosporins) hitting a body formed hapten conjugates type transport protein that can stimulate the immune system. Using a combination of tests in vivo and in vitro can effectively and objectively diagnose clinical immediate-type hypersensitivity reactions to the beta-lactam antibiotics. Benzylpenicillin and its metabolites identified as the major antigens. It is known that the determination of allergen-specific IgE has limited value for these allergens, since they disappear for several weeks or months after the last administration of drug. Furthermore, 25% of patients with allergy to beta - lactam antibiotics have negative skin tests to determine the allergen-specific IgE. One third of these patients may be identified only by allergies CAST / FAST - one of the few tests in vitro, can detect pseudoallergic response characteristic of many medicinal compounds.

To assess the controlled stimulation of immune system cells allergens penicillin group using the CAST/FAST was a complex benzylpenicillin - polylysine (PPL), and to assess the allergenic-

ity of penicillin metabolites was selected minor determinant mixture (MDM). Thus, when included in the diagnostic algorithm CAST/FAST in many cases it is possible to avoid the provocative test in vivo. The sensitivity of this method is 70 %, a specificity of - 100 %. CAST/FAST besides allergy to benzylpenicillin can detect an increased sensitivity to amoxicillin.

Aspirin and other NSAIDs often lead to the development of allergic reactions when they are repeated administration. Determination of allergen-specific IgE is ineffective, since the sensitivity to NSAIDs has not - IgE- mediated. Found that classical cell allergotesty have negative results for the detection of hypersensitivity to aspirin. At the same time, studies have shown that aspirin / NSAIDs can induce release of basophil degranulation and sLT. CAST/FAST is developed for aspirin, diclofenac and naproxen. Sensitivity of the CAST for aspirin reaches 63 % and specificity - 93 %, for diclofenac sensitivity CAST - 53 %, for naproxen sensitivity CAST - 89 %, for metamizol (dipyrone) sensitivity CAST - 76 %, when combined with FLOW - CAST (CAST/FAST) -- 77 %.

Number of reports of anaphylactic reactions during anesthesia muscle relaxants are increasing in recent years. In this connection for relaxants CAST, sensitivity is 54-90 % and specificity - 100 %. In conjunction with skin tests sensitivity is 80 %, which reduces the risk to the patient and the need to provocative tests in vivo [23].

Reaction blastotransformation of lymphocytes (RBTL test). During RBTL test drug allergy can be identified in 60-70% of cases in the presence of immediate-type hypersensitivity [25]. When performing this technique it is shown that both drugs can induce CD4 + Th1 and / or Th2, and CD8 + T - cell response [26, 27]. However, the relatively low frequency of

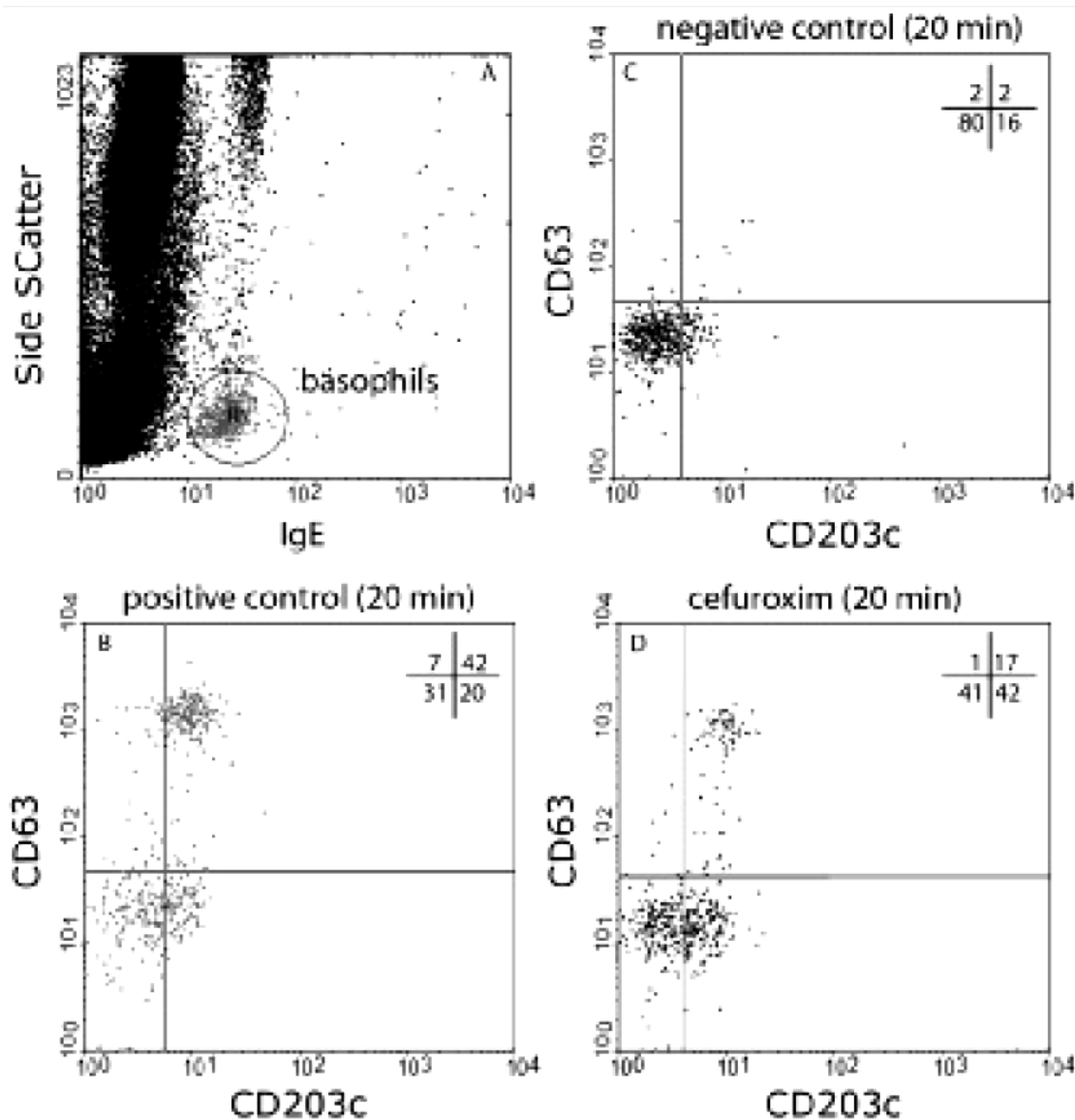


Figure 4. Submitted by increased expression of CD203c on basophils after stimulation with allergen in a patient with allergy to cefuroxime. Were identified basophils by staining CD203c: to stimulation - negative control, after stimulation with antibodies to IgE - positive control and after stimulation with allergen (cefuroxim). Activated basophils - the percentage of basophils expressed marker CD203c. Note the clear bimodal upregulation of CD63 and the more homogenous upregulation of CD203c. (D.G. Ebo, M.M. Hagendorens, C.H. Bridts, L.S. De Clerck, W.J. Stevens The basophil activation test in immediate drug allergy//Acta Clinica Belgica.- 2009.-№ 64-2.- P.129-135.).

positive reactions, high duration setting reaction (keeping in 3-4 Denya) make this method of little use for the diagnosis of drug allergy [28].

Test transformation of lymphocytes (TTL) allows to identify in vitro delayed type allergy to medicines sensitization of T-lymphocytes [29, 30]. Phenol-verografin gradient is used for isolating lymphocytes in the TTL technique. By T-lymphocytes added solutions suspected drugs in increasing concentrations. Incubation of lymphocytes with the solution of drug continue for six days. Sensitization of T-cells show increased proliferation by T-cells under the influence of the allergens according to the degree of blast transformation to increase the inclusion of 3H-thymidine incorporation into DNA (Fig. 5) [31]. The disadvantage of TTL technique is the need to use radionuclides (3H - thymidine).

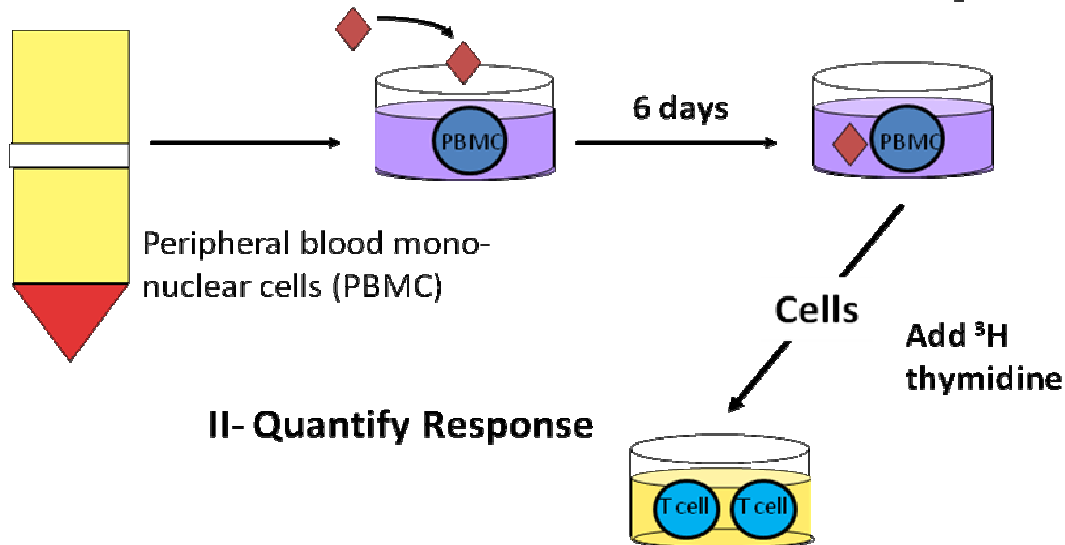
Test transformation of lymphocytes breeding succinimidyl ester carboxyfluorescein diacetate (CFSE).

The technique is based on conducting flow cytometry with coloring proliferating T- lymphocytes using non-radioactive labels – succinimidyl ester carboxyfluorescein diacetate (CFSE) [32]. CFSE penetrates the T-cells dye that is able to bind the amino group of cytoplasmic proteins. During cell division CFSE labeled proteins are distributed equally between the daughter cells, thereby doubling the fluorescence intensity of normal T cells. At the same time, fluorescence antigen specific T-cell decreases.

Lymphocyte transformation test principle for breeding (CFSE). Ficoll-verografin gradient is used for isolation T-cells. After that T-cells are incubated with CFSE (at a concentration of 5 μ M) for 10 min at 37°C and washed with excess paint.

I- Activation *in vitro*

Varied concentrations of pure drug, incubate at 37°C with 5% CO₂



II- Quantify Response

**Harvest cells and count radioactivity,
results: cpm or stimulation index (SI =
drug stimulated cpm/unstimulated cpm)**

Figure 5. Test transformation of lymphocytes (explanation to figure contained in the text).

(Pichler WJ, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. //Allergy.- 2004.-№59.-P. 809-820.)

Thereafter, the labeled T cells are cultured with a potential allergen (with a drug, such as phenytoin, at a concentration of 50 µg/ml). After that T- cells are diluted with 5% autologous plasma in concentration of 1x10⁶ cells/ml in presence of humidified 5 % CO₂. After 7 days of culturing in the presence of 5 % humidified CO₂ T cells stimulate a second potential allergen (a drug, such as phenytoin concentration of 50 µg/ml) (Fig. 6). Then, T-cells are added to phorbol acetate of meristat (50 ng/ml) and ionomycin (1 µg/ml) in the presence of brefeldin (10 µg/ml), the solution was incubated for 6 hours. Activation of T-cells occurs when the cell surface receptor interaction with its specific ligand molecule, which leads to hydrolysis of inositol phospholipids, diacylglycerol and inositol phosphates by the action of phospholipase C. Diacylglycerol, which is an allosteric activator of protein kinase C, and inositol phosphate, which stimulates the release of intracellular calcium ion (Ca²⁺) leads to activation of T cell response - lymphocyte production of interleukin 2, which follows to activation of T - cells. Phorbol acetate meristat, a structural analogue of diacylglycerol and activates protein kinase C. In normal growth conditions IL -2 cells do not produce or produces in small quantities. Phorbol acetate meristat through activation of protein kinase C can activate T cells and stimulate a low level production of IL-2. When phorbol acetate meristat activates T cells in the presence of a co-stimulant such as phytohemagglutinin or allergen production of IL-2 increases significantly. T cells were then stained with anti- PG -5 CD4- labeled antibodies using FIX & PERM cell permeabilization kits (Caltag Laboratories,

Burlingame, CA, USA), and fluorescence was counted.

The test of CD69-T-cells upregulation.

After activation, lymphocytes express several molecules on their surface. One of these markers is CD69, which is manifested in the early period after cell activation [33, 34]. Beeler A. et al [35] evaluated the diagnostic utility of CD69 in 15 patients with delayed-type hypersensitivity reactions and found that in the case of a positive test activation of T- lymphocytes observed increase CD69 on T-cells 48 h after stimulation of drugs involved in allergic reaction. The same study showed that upregulation of CD69 T-cells after stimulation with the drug - an allergen is much higher than the number of proliferating T-cells, which was detected by staining of T-lymphocytes ether diacetate succinimidyl carboxyfluorescein. Further analysis showed that the drugs stimulate specific components and only T- cells are responsible for the increased secretion of IL-2 and expression of CD69 [35]. Limitation of this method is that some drugs can cause CD69 activation of T-lymphocytes even in the absence of specific recognition of the drug. Therefore, drugs used in any analysis should be evaluated in people without allergies [36]. Determination of CD69 upregulation is a measure of the immune effects, has the advantage of performing TTL before, because the test is much faster and does not require the use of radionuclides as tracers.

Execution of the method of CD69 T-lymphocytes upregulation of receptors marker (Fig. 7.). Test consists in the following. Lymphocytes were isolated by Ficoll-verografin gradient and cultured as described previously [37]. Drugs used in

non-toxic concentrations of solutions prepared immediately before use. Drugs used in the following concentrations: 100, 200, 500 µg/ml amoxicillin; 1, 10, 100 µg/ml vancomycin; 1, 10, 100 µg/ml carbamazepine; 1, 10, 100 µg/ml sulfapyridine; 1, 10, 100 µg/ml cefuroxime; 50, 100, 200 µg/ml sulmetoksazola; 10, 50, 100 µg/ml of phenytoin; 1, 10, 50 µg/ml of clavulanic acid; 1, 10, 100 µg/ml levofloxacin et al [38].

Freshly isolated T - lymphocytes (2×10^5) were cultured in three duplicate 96 well plates with a U - shaped bottomed tissue culture in the presence of the indicated concentrations of the drug as well as tetanus toxoid or interleukin 2 - a positive control. The appearance of the surface marker CD69 on CD4 +/CD8 + T-lymphocytes measured by flow cytometry in the presence of human monoclonal antibodies (PE - CD69, APC-CD3, FITC-CD-8, CD-4 PERCP-I, PE - IgG1) and in isotypic negative control. Data are expressed as cytometric normalized mean fluorescence intensity. The stimulation index was determined as the ratio of normalized average fluorescence intensity of cells cultured in the presence of antigen separated by the normalized mean fluorescence intensity of cells cultured in the absence of antigen [38].

Before painting T - peripheral blood lymphocytes stimulated with solutions of drugs or antigens for 24 h, parallel incubates unstimulated T-lymphocytes - negative control. Monensin was added to a concentration of 6 µg/ml in the last 8 h of incubation, before the determination. Cells were washed 2 times with ice-cold phosphate-buffered saline containing 0.5 % bovine serum albumin (BSA) and 0.1 % sodium azide and color within 20 min of human monoclonal antibodies (PE - CD4 mAb, PERCP - CD3 mAb, FITC - CD69 nAb). Cells were fixed with a solution of Cytofix/Cytoperm for 20 minutes, resuspended in Per/Wash solution containing a drug and incubated for 30 min in the dark. As a control, isotype FITC- and alofikotsianin - conjugated IgG1

and IgG2a. After washing twice with Perm/Wash cells were resuspended in phosphate buffered saline containing 0.5 % BSA and 0.1% sodium azide and analyzed as described above [38].

Example receptor CD69 upregulation on the surface of CD4 + T-cells in a patient with an allergy to sulfapyridine shown in Fig. 8. During exposure, sulfapyridine and tetanus toxoid 1.9 % and 1.1 % of CD4 + cells showed an increased amount of SD69 + receptor, and only 0.1 % of these cells were incubated with corresponding receptors in a medium without the addition of drugs. After 6 and 12 hours after antigen challenge upregulation of receptors on the surface of CD69⁺ CD4⁺ as T - cells, and CD8⁺ T-cells, were detected. Increasing the incubation period (36 or 72 hours) was accompanied by the appearance receptor CD69⁺, however incubation for 18 h minimum for a detection of an elevated level of receptor CD69⁺ [38]. Elevated levels of CD69⁺ were not detected in the control without the addition of the drug. For practical purposes, we can recommend 48 hours of incubation of CD4⁺ T-cells with the addition of the drug (Fig. 8.).

The diagnosis of hypersensitivity to drugs usually depends on history of the disease, and the results of skin tests performed in the laboratory confirmatory tests with drugs, such as the determination of serum specific IgE, which are available for only a few drugs. The sensitivity of these tests is not 100 %, so in some cases a need for provocation testing. New diagnostic tools, such as the BAT test antigen stimulation of cells and lymphocyte transformation test, developed a few years ago, is now thoroughly tested in leading immunological centers around the world. Their use can lead to increased performance of diagnostic tests, improving the accuracy of diagnosis of drug allergy, thereby reducing the need for provocation tests.

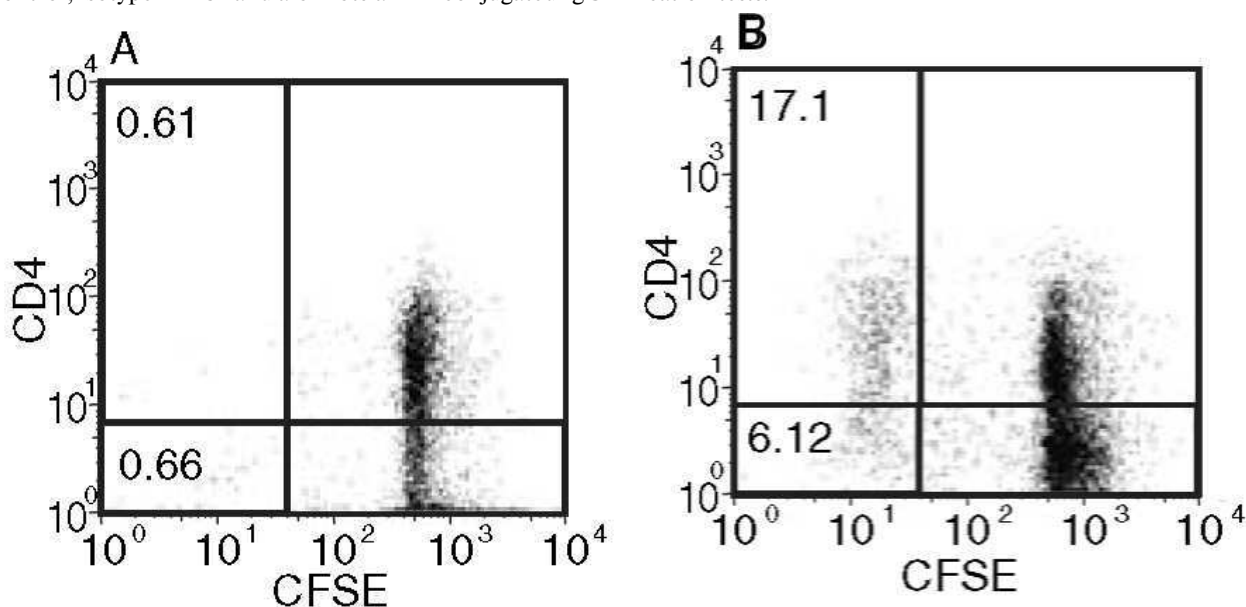
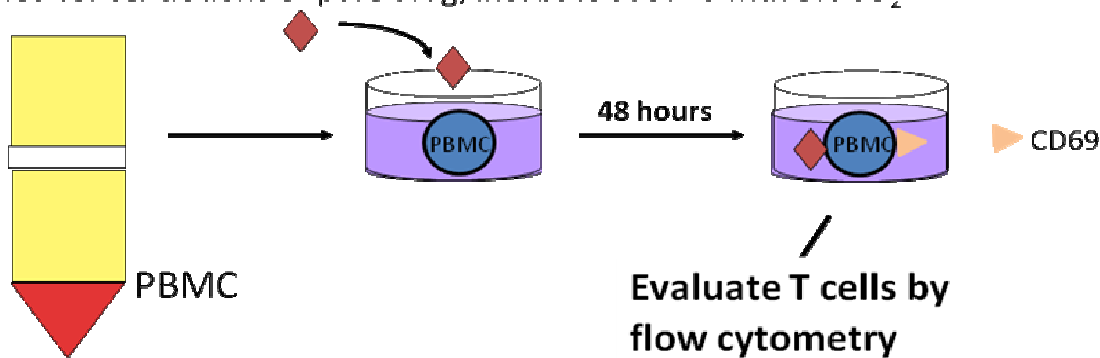


Figure 6. The results of lymphocyte transformation test a patient who has allergy to phenytoin (Tsuge I. et al., 2007).

T-cells of peripheral blood of patient labeled carboxyfluorescein succinimidyl ester diacetate (CFSE) were cultured for 7 days in the presence of 50 µg/ml phenytoin. On the 7th day of T-cells re-stimulated adding 50 µg/ml phenytoin and, then, T-cells were stimulated phorbolic meristat of acetate and ionomycin in the presence of brefeldin. A. Presented background levels of CFSE CD4+T-cells (left upper quadrant) and CD4-T-cells (left lower quadrant) in culture without phenytoin. B. Presented CFSE CD4+T-cells (left upper quadrant) and CD4-T-cells (left lower quadrant) in culture with phenytoin (Tsuge I., Okumura A, Kondo Y, Itomi S, Kakami M, Kawamura M, Nakajima Y, Komatsubara R, Urisu A. Allergen-specific T-cell response in patients with phenytoin hypersensitivity; simultaneous analysis of proliferation and cytokine production by carboxyfluorescein succinimidyl ester (CFSE) dilution assay.// Allergol Int.- 2007.-№56(2).-P.149-155.).

I- Activation *in vitro*

Varied concentrations of pure drug, incubate at 37°C with 5% CO₂



II- Quantify Response

CD69 upregulation expressed as
percent CD69 positive T cells

Figure 7. Test activation of T-lymphocytes (explanation to figure contained in the text).

(Beeler A, Engler O, Gerber BO, Pichler WJ. Long-lasting reactivity and high frequency of drug-specific T cells after severe systemic drug hypersensitivity reactions. // J. Allergy Clin. Immunol. - 2006.-№117.-P.455–462.).

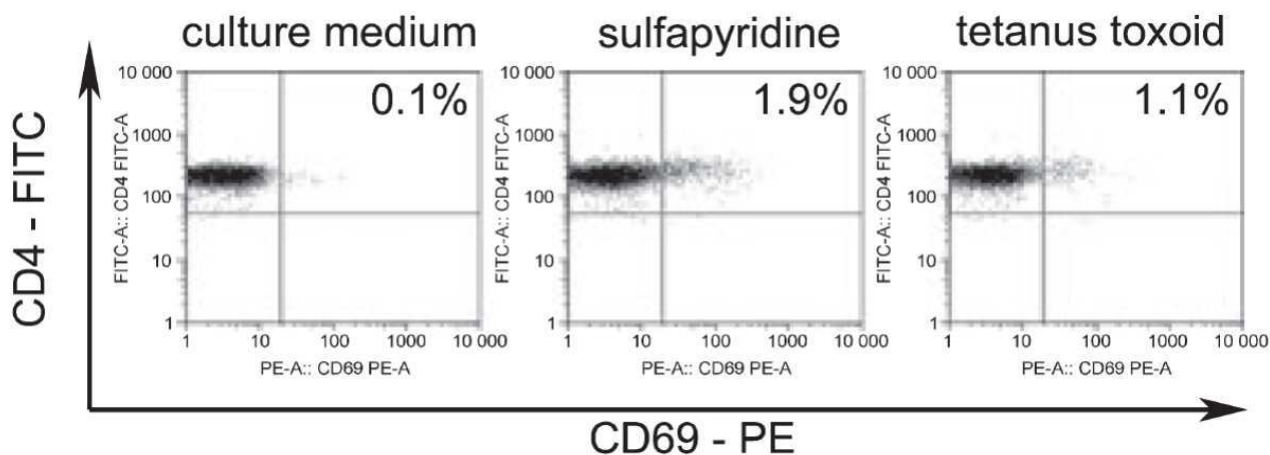


Figure 8. Flow cytometric showing increased CD69 upregulation on CD4 + T-cells.

Expression of CD69 receptors on the surface of CD4 + T-cells (CD69 upregulation on CD4+ T-cells) patient 48 h after incubation in culture medium without the addition of drug or tetanus toxoid (negative control), with the addition of sulfapyridine (drug) or tetanus toxoid (positive control) stimulation. (Beeler A., Zaccaria L., Kawabata T., Gerber B.O., Pichler W. J. CD69 upregulation on T cells as an in vitro marker for delayed-type drug hypersensitivity. // Allergy. - 2008.-V.63.-P. 181–188.).

References

1. Латышева, Т. В. Тактика иммунокоррекции в интенсивной терапии аллергических и иммунопатологических состояний [Текст] : Автореф. дис... — д-ра мед. наук: 14.00.36 / Латышева Татьяна Васильевна. — М., 1996. — 46 с.
2. Новиков, Д. К. Лекарственная аллергия [Текст] / Д. К. Новиков, Ю. В. Сергеев, П. Д. Новиков. — М. : Нац. академия микологии, 2001. — 330 с.
3. Пухлик, Б. М. Лекарственная аллергия и побочные эффекты лекарственных средств в аллергологии [Текст] / Б. М. Пухлик, А. П. Виктор, С. В. Зайков. — Львів : Медицина світу, 2008. — 107 с.
4. Самойлова, Л. Н. К вопросу о диагностике лекарственной аллергии [Текст] / Л. Н. Самойлова, Т. В. Табакова // Тер. арх. — 1992. — № 10. — С. 17–24.

5. Хаитов, Р. М. Клиническая аллергология: Руководство для практических врачей [Текст] / Р. М. Хаитов. — М. : Медпресс-информ, 2002. — 623 с.
6. Adkinson, N. F. Jr. Risk factors for drug allergy [Text] / N. F. Jr. Adkinson // J. Allergy Clin. Immunol. — 1984. — Vol. 74. — P. 567–572.
7. Anderson, J. A. Allergic reactions to drugs and biologic agents [Text] / J. A. Anderson // JAMA. — 2008. — Vol. 368. — P. 2845–2857.
8. Atanaskov, M. Type-I hypersensitivity to ceftriaxone and cross-reactivity with cefalexin and ampicillin [Text] / M. Atanaskov, M. Markov // Allergy. — 2003. — Vol. 58. — P. 537.
9. Baldo, B. Immunoglobulin E binding determinants on b-lactam drugs. Current opinion [Text] / B. Baldo, N. Pham // Allergy and Clin. Immunol. — 2002. — Vol. 2. — P. 297–300.

10. *Beeler, A.* Long-lasting reactivity and high frequency of drug-specific T cells after severe systemic drug hypersensitivity reactions [Text] / A. Beeler [et al.] // *J. Allergy Clin. Immunol.* — 2006. — Vol. 117. — P. 455–462.
11. *Beeler, A.* CD69 upregulation on T cells as an in vitro marker for delayed-type drug hypersensitivity [Text] / A. Beeler [et al.] // *Allergy.* — 2008. — Vol. 63 (2). — P. 181–188.
12. *Bernard, A.* Joint report of the first international workshop on human leucocyte differentiation antigens by the investigators of the participating laboratories [Text] / A. Bernard [et al.] // *Leucocyte Typing I.* — Springer Verlag, 1984. — P. 9–135.
13. *Bernstein, I. L.* Allergy Diagnostic Testing: An Updated Practice Parameter [Text] / I. L. Bernstein [et al.] // *Annals of Allergy, Asthma & Immunology.* — 2008. — P. 1–148.
14. *Boumiza, R.* Marked improvement of the basophil activation test by detecting CD203c instead of CD63 [Text] / R. Boumiza [et al.] // *Clinical & Experimental Allergy.* — 2003. — Vol. 33, №2. — P. 259–265.
15. *Boumiza, R.* The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives [Text] / R. Boumiza, A.-L. Debard, G. Monneret // *Clinical and Molecular Allergy.* — 2005. — Vol. 3. — P. 3–9.
16. *Bühring, H. E.* NPP3 (CD203c) [Text] / H. Bühring // *Leucocyte typing VII, Section New CD antigens, MC10.* — Oxford University Press. — 2002. — P. 377–378.
17. *De Weck, A. L.* Cellular allergen stimulation test (CAST) — A new dimension in allergy diagnostics / A. L. De Weck [et al.] // *Allergy Clin. Immunol. News.* — 1993. — Vol. 5. — P. 9–14.
18. *De Weck, A. L.* Flow cytometric cellular allergen stimulation test. Technical and clinical evaluation of a new diagnostic test in allergy and pseudo allergy [Text] / A. L. De Weck, M. L. Sanz // *ACI International.* — 2002. — Vol. 14, №5. — P. 204–215.
19. *Ebo, D. G.* The in vitro diagnosis of drug allergy: status and perspectives [Text] / D. G. Ebo [et al.] // *Allergy.* — 2011. — Vol. 66. — P. 127–128.
20. *Gamboa, P. M.* Flow-cytometric cellular allergen stimulation test to assess basophil activation in allergic patients to beta-lactam antibiotics with positive and negative skin test [Text] / P. M. Gamboa, M. L. Sanz // *Allergy.* — 2000. — Vol. 56, №68. — P. 5.
21. *Grushalla, R.* Drug metabolism, danger signals and drug-induced hypersensitivity [Text] / R. Grushalla // *J. Allergy Clin. Immunol.* — 2001. — Vol. 108. — P. 475–488.
22. *Hertl, M.* Predominance of epidermal CD8+ T lymphocytes in bullous cutaneous reactions caused by beta-lactam antibiotics [Text] / M. Hertl [et al.] // *J. Invest. Dermatol.* — 1993. — Vol. 101. — P. 794–799.
23. *Hertl, M.* Lymphocyte activation in cutaneous drug reactions / M. Hertl, H. Merk // *J. Invest. Dermatol.* — 1995. — Vol. 105. — P. 955–985.
24. *Hirai, H.* Cutting Edge: Agonistic Effect of Indomethacin on a Prostaglandin D2 Receptor, CRTh2 [Text] / H. Hirai [et al.] // *J. Immunol.* — 2002. — Vol. 168. — P. 981–985.
25. *Impicciatore, P.* Incidence of adverse drug reactions in paediatric in/out-patients: a systematic review and meta-analysis of prospective studies [Text] / P. Impicciatore // *Br. Clin. Pharmacol.* — 2001. — Vol. 52. — P. 77–83.
26. *Maino, V.* Rapid flow cytometric method for measuring lymphocyte subset activation [Text] / V. Maino, M. Suni, J. Ruitenberg // *Cyto-metry.* — 1995. — Vol. 20 (2). — P. 127–133.
27. *Nagata, K.* CR-Th2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s) [Text] / K. Nagata [et al.] // *FEBS Letters.* — 1999. — Vol. 459. — P. 195–199.
28. *Padovan, E.* T-cell recognition of penicillin G: structural features determining antigenic specificity [Text] / E. Padovan // *Eur. J. Immunol.* — 1996. — Vol. 26. — P. 42–48.
29. *Park, B.* Advances in molecular toxicology-towards understanding idiosyncratic drug toxicity [Text] / B. Park [et al.] // *Toxicology.* — 2000. — Vol. 153. — P. 39–60.
30. *Pawanka, R.* WAO White Book on Allergy 2011–2012: Executive Summary [Text] / R. Pawanka, G. Canonica [et al.] — World Allergy Organization: Milwaukee, WI, USA. — 2011. — 24 p.
31. *Pichler, W. J.* Delayed drug hypersensitivity reactions [Text] / W. Pichler // *Ann. Intern. Med.* — 2003. — Vol. 139. — P. 683–693.
32. *Pichler, W. J.* The lymphocyte transformation test in the diagnosis of drug hypersensitivity [Text] / W. Pichler, J. Tilch // *Allergy.* — 2004. — Vol. 59. — P. 809–820.
33. *Pichler, W. J.* T cell reactivity to drugs [Text] / W. J. Pichler [et al.] // *Abstracts XX Internat. Congress of Allergy and Clin. Immunol. (Sweden).* — 2011. — P. 166.
34. *Platz, I.* Hymenoptera-venom-induced upregulation of the basophil activation marker ecto-nucleotide pyrophosphatase/phosphodiesterase 3 in sensitized individuals / I. Platz [et al.] // *Int. Arch. Allergy Immunol.* — 2002. — Vol. 127 (4). — P. 293.
35. *Porebski, G.* In vitro diagnosis of T cell-mediated drug allergy / G. Porebski, A. Gschwend-Zawodniak, W. Pichler // *Clin. Exp. Allergy.* — 2011. — Vol. 41 (4). — P. 461–470.
36. *Romano, A.* Diagnosis and management of drug hypersensitivity reactions / A. Romano [et al.] // *J. Allergy Clin. Immunol.* — 2011. — Vol. 127 (3 Suppl). — P. S67–73.
37. *Torres, M. J.* Differences in the immunological responses in drug- and virus-induced cutaneous reactions in children / M. J. Torres [et al.] // *Blood. Cells. Mol. Dis.* — 2003. — Vol. 30 (1). — P. 124–131.
38. *Tsuge, I.* Allergen-specific T-cell response in patients with phenytoin hypersensitivity; simultaneous analysis of proliferation and cytokine production by carboxyfluorescein succinimidyl ester (CFSE) dilution assay / I. Tsuge [et al.] // *Allergol Int.* — 2007. — Vol. 56 (2). — P. 149–155.
39. *Zanni, M. P.* T cell reactions in patients showing adverse immune reactions to drugs / M. P. Zanni, S. von Greysen, B. Schnyder // *Inflam. Res.* — 1996. — Vol. 45 (Suppl. 2). — P. 79.

ЛАБОРАТОРНА ДІАГНОСТИКА МЕДИКАМЕНТОЗНОЇ АЛЕРГІЇ Часть 2. Методики визначення активації кліток медикаментозними алергенами

В. Д. Бабаджан, Л. В. Кузнецова,
П. Г. Кравчук, Н. Г. Рындина

Резюме

Збільшення алергопатології серед населення, пов'язане з різким підвищенням кількості лікарств, призначених хворим, а також з зміною реактивності організму, що призводить до ускладнень терапії, в тому числі алергічних реакцій на лікарства. Кожні тести для діагностики лікарської алергії невідкладного типу далеко не завжди можуть бути використані, для їх проведення існує достатньо багато протипоказань, достовірність цих тестів відносно невисока через те, що причиною виникнення алергічної реакції часто є не сам первинний лікарський препарат, а його метаболіти. Тому для діагностики лікарської алергії разом з кожними пробами застосовуються лабораторні тести.

Підтверджуючі тести з препаратами, такі як визначення специфічних сировоточних IgE, доступні тільки для декількох препаратів; крім того, чутливість цих тестів не досягає 100 %, тому в окремих випадках виникає необхідність проведення провокаційних тестів. Нові діагностичні засоби, такі як тест активації базофілів, тест антигенної стимуляції кліток, його цитометричний варіант (FLOW-CAST), з високою достовірністю дозволяють визначити наявність гіперчутливості невідкладного типу при наявності та відсутності специфічних IgE, по медіаторному типу до лікарств та інших алергенів, а також наявність псевдо-алергічного механізму клінічних проявів, здійснити діагностику аспіринової астми та подібних станів. В той же час, тест трансформації лімфоцитів дозволяє виявити алергію затриманого типу на лікарства по наявності сенсibiliзації Т-лімфоцитів.

Виконання методики тесту активації Т-лімфоцитів по маркеру підвищеної регуляції рецепторів CD69 дозволяє суттєво скоротити час реакції з тим самим прискорити отримання

результатов исследования. Таким образом, использование *in vitro* методик определения активации клеток медикаментозными аллергенами приведет к увеличению результативности диагностических обследований, повышению точности диагностики лекарственной аллергии, тем самым уменьшив необходимость проведения провокационных тестов.

Ключевые слова: *in vitro* диагностика лекарственной аллергии, тест активации базофилов, тест антигенной стимуляции клеток, тест трансформации лимфоцитов, тест определения повышенной регуляции рецепторов CD69.

Научно-практический журнал «Астма и аллергия», 2013, № 4.

В. Д. Бабаджан

Харьковский национальный медицинский университет, профессор

61001, Харьков, просп. Ленина, 4

тел.: +380(57)706-30-17, моб.: + 380(67)573-23-38

e-mail: vladdoc1@mail.ru

LABORATORY DIAGNOSIS OF DRUG ALLERGY

Part 2. Methods used for determination of drug allergens triggered cell activation

V. D. Babadzhani., L. V. Kuznetsova,

P. G. Kravchun, N. G. Ryndina

Summary

Increase of allergy in the population, associated with a significant increase in the amount of medication prescribed to patients, as well as the immune reactivity changes, which leads to increased complications of therapy, including allergic reactions to medications. Skin tests to diagnose immediate type allergy drug can not always be used. There are many contraindications to carry them out. The reliability of these tests is

relatively low due to the fact that the cause of allergic reaction is often not the initial drug and its metabolites. Therefore, skin tests with laboratory tests are usually used for diagnostic of drug allergy.

Confirmatory tests with drugs, such as the definition of specific serum IgE, only available for certain drugs. The sensitivity of these tests is not 100 %, so in some cases there is a need in provocation tests. New diagnostic agents such as basophil activation test, cellular antigen stimulation test and its flow cytometric version (FLOW-CAST) with high reliability can determine the presence of immediate hypersensitivity, the presence or absence of specific IgE, according to the type of neurotransmitter to medical and other allergens, as well as availability pseudoallergic mechanism of clinical manifestations, diagnosis of aspirin-induced asthma and similar conditions. At the same time, the lymphocyte transformation test reveals the delayed type allergies to medicines sensitization of T-lymphocytes.

Performing the test by determining of CD69 upregulation, as the T-lymphocyte activation marker, significantly reduces the response time and thereby accelerate results of the study. Thus, the use of *in vitro* methods for determination of drug allergens triggered cell activation will increase the effectiveness of diagnostic tests, improve the accuracy of diagnosis of drug allergy, thereby reducing the need in provocation tests.

Key words: *in vitro*-diagnostics drug allergy, basophil activation test, cellular antigen stimulation test, lymphocyte transformation test, CD69 receptors upregulation test.

Theoretical and practical J. «Asthma and Allergy», 2013, 4.

V. D. Babadzhani

Kharkiv National Medical University, professor

61001, Kharkiv, ave. Lenin, 4

tel.: +380(57)706-30-17, mob.: + 380(67)573-23-38

e-mail: vladdoc1@mail.ru