The general trends of laboratory diagnostic of drug hypersensitivity reactions

Ogólne algorytmy diagnostyki laboratoryjnej w reakcji nadwrażliwości na leki

Havrylyuk Anna, Zubchenko Svitlana, Lomikovska Marta, Kril Iryna, Lishchuk-Yakymovych Khrystyna, Pukalyak Roman, Chopyak Valentyna

Departament of Clinical Immunology and Allergology, National Medical University named Danylo Halytskyy, Lviv, Ukraine
Katedra alergologii i immunologii klinicznej, Lwowski Narodowy Uniwersytet Medyczny imienia Daniela Halickiego, Lwów, Ukraina

Summary

BACKGROUND: Drug hypersensitivity reactions (DHRs) are estimated to account for 3% to 6% of all hospital admissions and to occur in 10% to 15% of hospitalized patients resulting in morbidity, prolonged hospitalization, and increased risk of mortality.

AIM: This review aims: 1) to cast a look on DHRs drug hypersensitivity reactions as allergic and non-allergic; 2) to establish of immunopathogenesis of various DHRs; 3) to create of new laboratory trends for diagnostic of allergic and non-alergic DHRs.

MATERIALS AND METHODS: Clinically patients with DHRs can be classified as a) immediate (urticaria, angioedema, anaphylaxis etc.); b) non-immediate (delayed maculopapular eruptions, SJS/TEN, DRESS, vasculitis and cytopenia). Laboratory diagnostic of DHRs generally starts with *in vivo* tests (skin prick tests/ skin testing or intradermal testing) and continue with *in vitro* tests (radio immunoassays, fluor immunoassays, flow cytometry).

RESULT: We propose the new trends to differential diagnostic of DHRs. *In vitro* testing for immediate IgE-dependent allergies 1) tryptase; 2) histamine release test; 3) specific IgE; 4) cellular *in vitro* tests - BAT, CAST-ELISA; for drug-specific T cell-mediated reactions 1) classical LTT with detecting of stimulation index; b) modern flow cytometry analysis with measuring the expression of activation surface markers on basophils; 2) ELISpot, which determines the number of cells that release relevant cytokines and cytotoxicity markers; 3) ELISA to measure released cytokines. *In vitro* testing for non-allergic reactions: 1) biochemical investigation of hepatic and renal metabolism; 2) detecting of the level of arachidonic acid metabolites - leukotrienes, prostaglandins; 3) components of complement C3a, C5a, and C5b-9; bradykinin; factor XII of coagulation system; 4) IgG; 5) infections; 6) active products of nitrosative and oxidative stress etc.

CONCLUSION: We may conclude, that the various endotypes of DHRs identifying characteristics defined by specific mechanisms with each phenotype, diagnostic *in vitro* algorithm should be based on the new laboratory technologies. The results of various laboratory tests in DHRs-diagnostic will be taken into consideration to assign modern treatment.

Keywords: *drug hypersensitivity reactions (DHRs), IgE-dependent DHRs, drug-specific T cell-mediated DHRs, non-allergic drug reactions*

Streszczenie

INFORMACJE OGÓLNE: Zgodnie z oceną, reakcje nadwrażliwości na leki (RNL) są powodem 3-6% wszystkich przypadków hospitalizacji i powstają u 10-15% hospitalizowanych pacjentów, co powoduje rozwój ciężkiego przebiegu choroby, długotrwałą hospitalizację oraz wzrost ryzyka zgonu.

CEL: 1) rozpatrzenie reakcji nadwrażliwości na leki w zależności od mechanizmu reakcji alergicznych i niealergicznych; 2) ustalenie immunopatogenezy różnych RNL; 3) zaproponowanie nowych algorytmów laboratoryjnych diagnostyki alergicznych i niealergicznych RNL.

MATERIAŁY I METODY: Z klinicznego punktu widzenia RNL można klasyfikować jako: a) reakcje natychmiastowe (pokrzywka, obrzęk naczynioruchowy, anafilaksja etc.); b) reakcje opóźnione: wysypka plamkowo-grudkowa, zespół Stevensa-Johnsona/zespół Lyella, reakcje na leki z eozynofilią i objawami systemowymi, układowe zapalenia naczyń i cytopenia. Diagnostyka laboratoryjna RNL zaczyna się, z reguły od testów *in vivo* (punktowe alergiczne testy skórne typu prick/punktowe testy skórne albo testy podskórne lub prowokacje lekowe) i dalej kontynuowana jest poprzez wykonanie testów *in vitro* (metody radioimmunologiczne, test immunofluorescencyjny, cytometria przepływowa).

WYNIKI: Proponujemy nowe trendy i układy diagnostyki zróżnicowanej RNL. Testy *in vitro* na natychmiastowe reakcje alergiczne IgE-zależne 1) tryptaza; 2) histamina; 3) specyficzne IgE; 4) testy komórkowe *in vitro* – test aktywacji bazofilow, CAST-ELISA; dla specyficznych reakcji na leki poprzez pośrednie limfocyty T 1) klasyczny test transformacji limfocytów z określeniem indeksu stymulacji; b) współczesna analiza metodą cytometrii przepływowej z pomiarem ekspresji markerów powierzchniowych aktywacji bazofili; 2) ELISpot, określający liczbę komórek, uwalniających odpowiednie cytokiny oraz cytotoksyczne markery; 3) ELISA do pomiaru cytokin. Testy *in vitro* na reakcje niealergiczne: 1) biochemia metabolizmu wątrobowego i nerkowego; 2) określenie poziomu metabolitów kwasu arachidonowego – leukotrienów, prostaglandyn; 3) składowe dopełniacza C3a, C5a, C5b-9; bradykinina; czynnik XII krzepnięcia krwi; 4) IgG; 5) infekcje; 6) aktywne produkty stresu oksydacyjnego i nitrozacyjnego etc.

WNIOSKI: Dla różnych endotypów RNL istnieją swoiste cechy oraz specyficzne mechanizmy dla każdego fenotypu. Jako podstawę diagnostyki *in vitro* należy brać nowe technologie laboratoryjne. Wyniki rozmaitych badań laboratoryjnych na temat diagnostyki RNL mogą być uwzględniane przy wyborze odpowiedniego leczenia.

Słowa kluczowe: *reakcje nadwrażliwości na leki (RNL), IgE-zależne RNL, specyficzne RNL poprzez pośrednie limfocyty T, niealergiczne reakcje na leki*

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Adres do korespondencji / Address for correspondence
Prof. Anna Havrylyuk
Medical University named Danylo Halyckyy,
Departament of Clinical Immunology and Alergology
Ukraine, 79010, Lviv, Pekarska str. 69, Lviv
ahavrylyuk@meta.ua

Introduction

1. Definition and risk factors of drug hypersensitivity reactions

Drugs can induce several different types of immunologic reactions as well as nonallergic drug hypersensitivity reactions (DHRs). DHRs are the adverse effects of pharmaceutical formulations (including active drugs and excipients) that clinically resemble allergy. Drug allergy is DHRs for which a definite immunological mechanism (drug-specific antibody/T cell) is suspected. Drug hypersensitivity reactions (DHR) are classified as type B adverse drug reactions (ADRs) mainly as the dose-independent, unpredictable, noxious, and unintended response to a drug and other medical substances taken at a dose normally used in humans [1,2,3].

The most important risk factors for drug hypersensitivity may be related to the chemical property and molecular weight of drugs. Other drug-specific risk factors for drug hypersensitivity include the dose, route of administration, duration of treatment, repetitive exposure to the drug, and concurrent illnesses. Host risk factors for drug hypersensitivity include age, sex, atopy. The higher risk factors for drug hypersensitivity have 1) cyclical hormonal changes in females; 2) environmental chemicals (cosmetics and detergents); 3) patients with cancer or cystic fibrosis (CF); 4) infections (Epstein-Barr virus, human immunodeficiency virus); 5) specific genetic polymorphisms; 6) autoimmune disorders (lupus erythematous) [1,4,5,6,7].

Viral and Mycoplasma pneumonia infections can lead to DHRs if a drug (mostly an antibiotic) is taken at the same time. Viral infections can also interact with drugs, leading to mild eruptions in the case of the «ampicillin rash» linked to the EBV infection and severe reaction during DRESS. The first virus shown to be reactivated in DRESS patients was the human herpesvirus (HHV)-6, but all herpesviruses can be involved. Replication of HHV-6 can be induced in vitro by amoxicillin. HHV-6 reactivation in patients with DRESS/DIHS may increase T cell activity, induce the synthesis of proinflammatory cytokines (TNF-α and IL-6). Human immunodeficiency virus (HIV) infection/acquired immunodeficiency syndrome (AIDS) made the patients predictable to the reactions, accordingly, they should be classified as drug allergies [1]. The sequential reactivations of several viruses (HHV-7, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and coxsackievirus A6 (CVA6)) were found to be coincident with the clinical symptoms of drug hypersensitivity reactions, they may also provide exogenous peptides for presentation and participate in HLA/drug/TCR interactions [6].

Genetic predisposing factors have been reported in cases of immediate-type drug hypersensitivity resulting from the use of beta-lactams, aspirin, and other NSAIDs. The induction of drug hypersensitivity requires an HLA risk allele [1,8]. These reactions can occur at first exposure and do not require sensitization [5]. Interestingly, HLA class II genes (HLA-DRA and the HLA-DRA|HLA- DRB5 interregional) have been linked to immediate reactions to beta-lactams [1]. HLA-DRB1*1302 and HLA-DRB1*0609 are associated, meanwhile, with aspirin-induced urticaria/angioedema. In addition, HLA-B44 and HLA-Cw5 have also been reported to be associated with chronic idiopathic urticaria associated with aspirin- and/or NSAID-induced hypersensitivity [6]. Some drugs, when they bind to HLA molecules, promote an exchange of embedded peptides. Abacavir (nucleoside reverse transcriptase inhibitor) binds at the F pocket antigen-binding site of HLA-B*5701, selecting an array of novel self-peptides that induce the activation of $CD8^+$ T cells inducing: a) a severe DHR similar to graft-vs-host disease without eosinophilia [1,3,8,9]; b) autoimmune-like systemic reaction manifestations [6,9]. The best example of utilizing a pharmacogenomics approach to reduce drug allergy relates to the association of HLA-B*5701 and the development of the abacavir hypersensitivity syndrome [1,8], due to the local skin and peripheral T cell activation [5]. Genotyping is available for the screening of patients at risk for SCARs including SJS/TEN, such as HLA B-5701 for patients starting abacavir treatment and HLA-B*1502 for patients exposed to carbamazepine. Metabolic pathway analysis for cytochrome isoforms of the P450 pathway can help identify patients with opioid intolerance, due to CYP2D6 deficiencies and other metabolic defects, that may place during surgery and anesthesia [5,10]. The strong association of cytochrome P450 family 2 subfamily C member 9*3 (CYP2C9*3) with phenytoin-induced SCAR) is genetically dependent [4,6].

Different studies have indicated strong associations of some HLA alleles with a high risk of severe T cell mediated reactions to drugs like carbamazepine. Drug hypersensitivity reactions between carbamazepine-induced SJS/TEN and HLA-B*1502 were found in Asian populations. The association seems to be phenotype-specific (SJS, but not hypersensitivity syndrome/drug reaction with eosinophilia and systemic symptoms/drug-induced hypersensitivity syndrome (HSS/DRESS/DIHS)) [11]. It was found that CBZ/aromatic antiepileptic drugs can directly interact with HLA-B*1502 protein [6]. While the HLA-B*1502 allele is predictive in Asian populations and Han Chinese, however, HLA-A*3101 allele was to be associated with carbamazepine-induced hypersensitivity reactions in subjects of Northern European descen [1,8]. HLA-A*3101 is associated with a spectrum of carbamazepine-induced reactions: maculopapular exanthemas, DRESS/DIHS, and SJS/TEN. HLA-B*5801 allele is associated with a high risk of allopurinol-induced DRESS and SJS/TEN in Asian and Caucasian populations [1].

The genetic variants of proinflammatory cytokines (IL-4, IL-13, IL-10, IL-18, TNF, and IFNGR1), the cytokine receptor (IL4R), the genes involved in the IgE/FceRI pathway (the galectin-3 gene (LGALS3)), and nucleotide-binding oligomerization domain (NOD) gene polymorphisms are also strongly associated with beta-lactam-induced immediate reactions.

Several genetic predisposing factors have been reported to be associated with immediate-type aspirin hypersensitivity, with those factors involving cytokines (TGFB1, TNF, IL-18, and IL-36Ra) and the production and release of mediators (LTC4S, TBXA2R, PTGER4, FCER1A, MS4A2, FCER1G, and HNMT). Immediate-type hypersensitivity to NSAIDs has also been reported to be associated with genes belonging to the arachidonic acid pathway (ALOX5, ALOX5AP, ALOX15, TBXAS1, PTGDR, and CYSLTR1) [6].

2. Classifications and mechanisms of drug hypersensitivity reactions

Clinically, DHRs can be classified as a) immediate (urticaria, angioedema, rhinitis, conjunctivitis, bronchospasm, gastrointestinal symptoms, anaphylaxis; b) non-immediate (delayed urticaria, maculopapular eruptions, fixed drug eruptions, vasculitis, Stevens-Johnson syndrome, and toxic epidermal necrolysis (SJS/TEN), drug reaction with eosinophilia and systemic symptoms (DRESS), acute generalized exanthematous pustulosis and symmetrical drug-related intertriginous and flexural exanthemas; internal organs can be affected either alone or with cutaneous symptoms (DRESS, vasculitis), and cytopenia. Immunopathologically, DHRs can be defined as allergic (antibody-mediated (IgE/IgG) or T cell mediated), and nonallergic [1,8,12]. Drug allergic reactions include: 1) IgE-mediated reactions; 2) T cell-mediated reactions; 3) pharmacologic interactions; 4) genetic predispositions. IgE-mediated reactions depend on sensitization to the culprit drug or a cross-reactive substance (table 1) [11,12].

Some groups of drugs, for example, beta-lactam antibiotics, induce hypersensitivity reactions via specific immunologic mechanisms in all age ranges, others, as contrast media, muscle relaxants, nonsteroidal anti-inflammatory drugs (NSAIDs), may induce reactions by different mechanisms, including non-immunologic. NSAID hypersensitivity reactions include both allergic and non-allergic hypersensitivity [2].

3. Pathogenesis and pathophysiology of drug hypersensitivity reactions

3.1. Immediate allergic DHRs

Immediate-type drug hypersensitivity can be realized by IgE-mediated or non-IgE-mediated mechanisms. IgE-mediated mechanisms are mediated by drug-specific IgE via an immune response to a hapten/carrier complex. In the primary drug sensitization, drug-specific IgE is formed when plasma cells are transformed from activated B cells interacting with Th2 cells. In an allergic reaction, drug allergens bind to mast cells or basophils with high-affinity Fc receptors, to which drug-specific IgE is bound, causing degranulation of the mast cells or basophils that results in the release of various mediators (histamine, leukotrienes, prostaglandins, and cytokines) [13,14].

Basophils together tissue-resident mast cells can be triggered in ways that are IgE-dependent and IgE-independent. Cross-linking of the surface-bound high-affinity IgE receptor (FcεRI) will generally occur via (glyco)proteins, chemical allergens, or autoantibodies mounted against the FcεRI or membrane-bound IgE antibodies. If not IgE-dependent, activation will mainly result from the coupling of receptors with endogenous (e.g., cytokines, anaphylatoxins, chemokines, IgG, and neuropeptides) or exogenous (e.g., pathogen-associated molecular patterns) substances. The degranulation can also result in the direct influence of opiates, iodinated contrast media, vancomycin, and quinolones [6,16].

Immediate-type hypersensitivity reactions (IDHRs) may range from urticaria and angioedema to severe near-fatal reactions, such as bronchospasm and anaphylaxis. NSAIDs are the main culprits followed by beta-lactam antibiotics [14]. Perioperative anaphylaxis also remains an issue due to the administration of various combinations of neuromuscular blocking agents (NMBAs) and induction agents (e.g., propofol, etomidate, midazolam, and ketamine) [6].

The non-IgE-mediated immunologic mechanisms are mediated by IgG antibodies or complement activation. The specific IgG bound to FcγRIII stimulates the release of platelet-activating factor (PAF) by basophils, macrophages, or neutrophils. PAF is an essential mediator in such anaphylaxis. A novel gain-of-function splice variant of FcγR - FcγRIIA has been identified with the presence of IgG anti-IgA antibodies in patients with common variable immunodeficiency who developed anaphylaxis after intravenous immunoglobulin infusion. Moreover, biological agents with IgA and infliximab have been shown to induce anaphylaxis in the absence of specific IgE but with high levels of specific IgG. Complement activation can be induced through the absence of agent-specific IgE or IgG antibody immunocomplexes in patients undergoing: a) hemodialysis with a new dialysis membrane; b) protamine neutralization of heparin; c) polyethyleneglycol infusion. Drugs solubilized in therapeutic liposomes and lipid-based excipients can form large micelles with serum lipids and cholesterol to stimulate the complement system, causing the release of C3a, C5a, and C5b-9, which trigger the activation of mast cells, basophils, and other cells via their specific receptors resulting in degranulation and mediator release [6].

3.2. Non-IgE-mediated allergic DHRs

There are five important mechanisms of non-IgE-mediated allergic DHRs: 1) Fas-associated death domain protein (FADD) binds to the Fas–FasL complex, recruits procaspase 8, triggering the caspase cascade and resulting in intracellular DNA degradation; a suicidal interaction between Fas and FasL on keratinocytes leads to the extensive necrosis of epidermal cells in individuals with SJS/TEN; 2) drug-specific cytotoxic T lymphocytes (CTL) and NK cells produce perforin, which can promot in the entry of granzyme B into the target cells to activate the caspase cascade and the succeeding apoptosis; play more important roles in the keratinocyte death in SJS/TEN; 3) granulysin is released by CTL and NK cells, granulysin was much higher in SJS/TEN patients than the levels of other cytotoxic proteins, its depleting reduced their cytotoxicity; is strongly expressed in patients with drug-induced FDE (fixed drug eruption) and DRESS/DIHS, but not MPE (maculopapular exantema); 4) reflect the role of cytokines TNF-α, IFN-γ, TARC, IL-15, and other cytokines/chemokines in SJS/TEN, DRESS/DIHS, and AGEP (acute generalized exanthematois pustulosis); 5) syndrome-specific effector cells: a) cytotoxic $CD8^+$ T cells, NK cells and T killer cells producing the cytotoxic molecules, which causes extensive keratinocyte death and skin lesions of patients with SJS/TEN; b) regulatory T cells (Tregs) in SJS/TEN is inadequate, although present in normal frequency;

Table 1. Classification and mechanisms involved in drug allergy [11,12].

Types of reaction	Immune response	Pathomechanism	Clinical features	Chronology of the reaction
I	IgE-mediated	Mast cell and basophil degranulation	Urticaria, angioedema, bronchospasm, anaphylactic shock	minutes-6 hours after the last intake of the drug
II	IgG/IgM and complement	IgG/IgM and complement-dependent cytotoxity	Cytopenia, anaemia, thrombocytopenia	5-15 days after starting the eliciting drug
III	IgG/IgM and complement or FcR	Deposition of immune complexes	Serum sickness, urticarial, lymphadenopathy, fever, arthropathy, vasculitis	1-8 days for serum sickness/urticaria 7-21 days for vasculitis
Iva	Th1 monocyte/ macrophages via IFN-γ/TNF-α	Monocytic inflammation	Eczema, contact dermatitis, bullous exanthema	1-21 days after starting the eliciting drug
IVb	Th2 via IL-4, IL-5, IL-13, eotaxin	Eosinophilic inflammation	Maculopapular exanthema, DRESS	1-several days after starting the eliciting drug for MPE 2-6 weeks after starting the eliciting drug for DRESS
IVc	$CD4^+$/$CD8^+$cytotoxic N cells via perforin, granzyme B, FasL	Keratinocyte death	Maculopapular exanthema, SJS/TEN, pustular exanthema, fuxed drug eruption	1-2 days after starting the eliciting drug for fixed drug eruption 4-28 days after starting the eliciting drug for SJS/ TEN
IVd	T cells via IL-8/CXCL8 and GM-CSF	Neutrophilic inflammation	Acute generalized exanthematous pustulosis	1-2 days after starting the eliciting drug

c) increase in number of atypical lymphocytes or eosinophils in patients with DRESS/DIHS; d) increased numbers of $CD4^+$ T cells in the acute stage of DRESS patients [6,12].

Drug antigens might directly stimulate specific T cells, they migrate to target organs and, once reexposed to the antigen, they are activated to secrete (e.g., perforin, granzymes and granulysin) that produces tissue damage [1,6].

Antiepileptic agents and allopurinol are the most commonly reported offending medications, the symptoms often begin 2 to 6 weeks after drug administration. Damage to multiple systemic organs may occur during DRESS/DIHS syndrome. Renal, liver, cardiac, and lung involvement also occurs frequently. Several other systemic organs can also be involved in DRESS/DIHS, including the gastrointestinal tract, pancreas, central nervous system, and thyroid, while multiple organ failure associated with disseminated intravascular coagulation or hemophagocytic syndrome may also occur. Tachycardia, leukocytosis, tachypnea, coagulopathy, gastrointestinal bleeding, and systemic inflammatory response syndrome (SIRS) have also been found to be associated with poor outcomes in DRESS/DIHS patients [6].

There are four hypotheses regarding drug presentation mechanisms that have been proposed to explain how small drug antigens might interact with HLA and TCR in drug hypersensitivity: (1) the hapten theory, (2) the pharmacological interaction with immune receptors (p-i) concept, (3) the altered peptide repertoire model, and (4) the altered TCR repertoire model. It is important for hapten-drugs: 1) the availability of the binding-proteins; 2) the biotransformation and bioactivation of pro-hapten drugs to reactive metabolites, which then bind to proteins to induce the T cell-mediated immune response [6,13]. Hapten-theory is relevant for chemical compounds, but not for proteic or carbohydrate compounds of drugs such as insulin, enzymes, monoclonal antibodies, and recombinant proteins. This is also especially relevant for oral drugs that preferentially bind to proteins, for instance, albumin in gastric stomach fluid. According to the (p-i) concept, a drug can directly bind and activate T cells (providing MHC binding as well) or bind to HLA molecules, which then activate T-cells indirectly, by altering the MHC-peptide groove [3,9,15].

4. Pathomechanisms of non-immunologic drug hypersensitivity reactions

The suggested pathomechanisms of non-immunologic reactions include the following: 1) nonspecific mast cell or basophil histamine release (e.g., opiates, radiocontrast media, and vancomycin); 2) bradykinin accumulation (angiotensin-converting enzyme inhibitors); 3) complement activation (e.g., protamine); 4) possibly an alteration in arachidonate metabolism (e.g., aspirin and other nonsteroidal anti-inflammatory drugs); 5) the pharmacological action of

certain substances inducing bronchospasm (e.g., β-blockers, $S0_2$, released by formulations containing sulfites) [1, 17]. Nonsteroidal anti-inflammatory drugs (NSAIDs) (except pyrazolones) are believed to be rarely among the causes of IgE-mediated anaphylaxis, but anaphylaxis I is more commonly related to an aberrant arachidonic acid metabolism. The non-IgE-mediated immunologic mechanisms can be mediated by IgG antibodies and activation of complement-, kinines- and coagulation systems [6]. Cysteinyl leukotrienes (LTs) are major mediators generated during NSAID-induced reactions. Overproduction of leukotriene E4 (LTE4) is associated with the overexpression of 5-lipoxygenase and related enzymes. Deficient generation of prostaglandin E2 is accompanied by a downregulation of COX-2 and lower baseline production of lipoxin A4 (LXA4) in peripheral blood leukocytes [18].

The nonimmunologic-type hypersensitivity reaction directly activates mast cell degranulation without involving the activation of the immune system. Several specific agents induce different mechanisms beyond the direct immunoglobulin-mediated activation or complement activation. Oversulfated chondroitin sulfate-contaminated heparin was found to have caused various cases of anaphylaxis; the direct activation of the kinin system with increased production of bradykinin, C3a, and C5a. The triggering of factor XII-driven contact system activation-mediated bradykinin formation also plays a key role in anaphylaxis. NSAIDs, including aspirin, can result in anaphylactic reactions via the inhibition of cyclooxygenase with a decrease in the production of prostaglandins and the increased generation of cysteinyl leukotrienes. Vancomycin can directly activate mast cells and/or basophils, leading to the release of histamine. This mechanism was to be mediated via the calcium-dependent activation of phospholipase-C and phospholipase-A2 pathways. Opiates (e.g., meperidine, codeine, and morphine) also cause histamine release via direct mast cell degranulation [6]. Respiratory reactions induced by aspirin or other NSAIDs are not immunologically mediated but represent the across-reactive type of the reaction, that has been associated with inhibition of COX-1, which results in depletion of prostaglandin E2 with the unstrained synthesis of cysteinyl leukotrienes and mediator release from basophils, mast cells and eosinophils [14]. These act as chronic eosinophilic inflammation [18]. Eosinophils contain several preformed mediators and cytotoxic enzymes within cytoplasmic granules. The most abundant preformed substances are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), and eosinophil peroxidase (EPO). These mediators caused desquamation and destruction of the epithelium, and lead to airway and alveolar damage and lung dysfunction. Eosinophils also release superoxide anion, hydrogen peroxide [19], leukotrienes, and various cytokines that cause tissue injury and inflammation. Infiltrated eosinophils express the inducible type of nitric oxide (NO) synthase (iNOS), which generates higher amounts of NO relative to the constitutive type of NOS (cNOS), they also possess nicotinamide adenine dinucleotide (NADPH) oxidase complex [10]. Activated NADPH oxidase catalyzes oxygen to superoxide anion, which enters further redox pathways to generate hydrogen peroxide in the presence of superoxide dismutase, or hydroxyl and nitrogen dioxide radicals, after combining with NO. NO rapidly reacts with superoxide anion to form highly reactive nitrogen species (RNS) such as peroxynitrite, causes tissue injury and stimulates the production of proinflammatory cytokines and chemokines [20].

There is an increase in nitrosative stress (NS) in the airways, measured by a greater number of sputum cells (macrophages and neutrophils) expressing 3-nitrotyrosine (3-NT). The levels of reactive persulfides (cysteine persulfide and polysulfides), which are potent scavengers of reactive oxygen species, were also reduced in such reactions. The levels of the enzyme's cystathionine-β-synthase and cystathionine-γ-lyase, which generate persulfides, were increased and this could be due to their upregulation as a compensatory response to peroxynitrite ions [21]. Antioxidant (AO) systems exist to regulate the redox balance [22].

5. Diagnosis of DHRs

Important information to note when taking a patient history: Which medications were used before and at the time of the reaction (create a timeline if necessary)? Which diseases were already present at that time and were responsible for the use of drugs? Precise chronology: 1) The duration of medication use; 2) The time interval between the last use of the medication and the onset of symptoms; 3) Duration of the reaction; 4) Period of allergy consultation or testing; 5) Symptoms of the drug-related reaction (both subjective and objective symptoms) and which organ systems were involved in chronological order of occurrence, as well as laboratory findings and possible treatment interventions. Possible augmentation factors, such as infectious diseases and physical exertion. Known drug hypersensitivity and other known allergies. General patient history: age, sex, atopy history, other disorders, and current drug use [15,23].

5.1. *In vivo* tests

Diagnosis of IDHRs generally starts with skin prick tests (SPT)/ skin testing (ST) or intradermal testing (IDT) and/or quantification of specific immunoglobulin E (sIgE) antibodies when an IgE-mediated activation of mast cells and basophils is suspected. A very small number of drug-sIgE assays are available and clinically validated. In addition, it has to be kept in mind that IDHRs with the release of mediators by circulating basophils tissue-resident dent mast cells, do not involve IgE/FcεRI-cross-lin per se but might result from alternative mechanisms that are undetectable by sIgE antibody assays [12,16]. Drug provocation tests (DPTs) are a 'gold standard' to establish or exclude the presence of hypersensitivity to a certain drug [2,14,24]. Intradermal Test (IDT) can be used to evaluate both immediate IgE-mediated allergy and delayed-type hypersensitivity [15,24].

Diagnosis of allergy to β-lactam antibiotics and natural rubber latex generally relies upon ST [14,25]. Several types of skin tests are used in allergy diagnostics *in vivo*, for example, SPT, represents first the level of approach for the diagnosis of type I, immediate, IgE-mediated allergy, and prick-to-prick testing (PPT) with native allergens [4]. ST has been used for the diagnosis of immediate reactions to metamizole, dipyrone, and paracetamol in children [2]. Skin test procedures have not been thoroughly validated for many compounds and do not have absolute predictive value. Positive intradermal test responses independent of mast cell degranulation are not necessarily indicative of a

specific immune-mediated pathomechanistic process, and negative skin tests might not always guarantee safe reexposure to the substance being evaluated [25]. A patch test is used for delayed-type, cell-mediated, hypersensitivity reactions [4], is the most reliable technique for diagnosis non–IgE-mediated cutaneous drug reactions (maculopapular exanthems, acute generalized exanthematous pustulosis, and fixed drug eruptions), but generally is not helpful for SJS or urticarial eruptions [26].

It should be mentioned, that *in vivo* test generally has higher diagnostic value than *in vitro* test.

5.2. *In vitro* tests

5.2.1. Measurement of drug-specific IgE

Biological tests are available to establish the nature of the culprit agent. *In vitro* assay for drug-specific IgE is not available for many allergenic drugs [4]. The demonstration of isolated drug-specific IgE (to penicillins, NMBA, chymopapain, etc.) does not establish the diagnosis of a drug allergy. The absence of drug-specific circulating IgE does not rule out a diagnosis of immediate drug allergy [1].

IgE-sensitized patients present high rates of cisplatin and carboplatin sensitization without prior exposure [11]. Specific IgE (sIgE) determination is recommended within 2 weeks–6 months following a reaction [23]. However, since sIgE and especially drug sIgE are found at a very low concentration in the blood, these *in vitro* methods must be highly sensitive. The most frequent methods to evaluate specific IgE immunoassays – radioimmunoassays and fluorimmunoassays) [27]. If present sIgE in the sera recognizes the drug, it forms a drug-carrier-antibody complex which is quantified using a secondary anti-human IgE antibody labeled with a radioisotope (RIA) or a fluorescent enzyme (FEIA). RIA is generally conducted using in house techniques, such as the radioallergosorbent test (RAST); FEIA can be performed using commercial products, such as the ImmunoCAP-FEIA, ELISA although such products are only available for a few drugs (BLs, neuromuscular blocking agents (NMBAs), chlorhexidine, quinolones, and biological agents) [27]. However, these assays have low sensitivity, rarely play a critical role in patient evaluation, and are not useful in diagnosing penicillin allergy in patients with removed histories of penicillin allergy [5].

5.2.2. Basophil activation test (BAT)

BAT has been used *in vitro* to provide evidence of IgE sensitization and identification of the culprit drug. Cell activation has been demonstrated by the expression of CD63 and CD203 markers. Basophils can be detected using cell markers, such as anti-IgE, CCR3, CRTH2, CD203c, or their combination in the presence of the implicated drug [4,11, 23,27] using flow cytometry [27]. BAT has been used for multiple drugs, including iodinated radiocontrast media, β-lactam antibiotics (BLS) [7,23], antibiotics cyclosporine, quinolones (ciprofloxacin, moxifloxacin, and levofloxacin), NSAID hypersensitivity [14], general anesthetics, natural rubber latex hypersensitivity [25], performed for fluoroquinolones (FQs), pyrazolones and RCM as well as to identify chemotherapy drug reactions, and may be a useful tool to identify sensitized patients before reactions occur [11,16,27]. This test detects specific activation markers that are expressed on the surface of basophils after their incubation with the potentially responsible drug [5].

Piecemeal degranulation is mediated through the upregulation of CD203c on basophils, anaphylactic degranulation results in exposing CD63 on the surface of basophils [6,7]. Alternative methods to quantify basophil activation imply quantification of the expression of surface inhibitory receptors such as CD300a, the phosphorylation of signaling molecules such as p38 mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 5 (STAT5) (table 2) [14].

In vitro testing for immediate allergies 1) tryptase, the general mast cell protease (if possible, during the acute reaction and in the further course); 2) histamine release test (HRT), intracellular content of histamine [16,25]; 3) specific IgE antibodies; 4) cellular *in vitro* tests: a) basophile activation test (BAT), b) cellular antigen stimulation test (CAST, also referred to as CAST-ELISA) [11,23]. There are usually the other methods, for example, molecular diagnostics for detecting some exceptions (e.g., major, and minor determinants of penicillin G) [1,25].

5.2.3. Drug-specific T Cell-Mediated Reactions

The evaluation of cell-mediated DHR or NIDHR is more complex than for IDHR, mainly due to the heterogeneity of clinical symptoms. These differences in symptoms imply that in most reactions T cells are involved. The reproducing of effector reaction *in vitro* by activating T cells (inflammatory and cytotoxic mediator release) will be determined by the lymphocyte transformation test (LTT) method. The LTT should be used in high-risk patients before deciding on further investigations (moderate/strong) [28] and remain the domain of only laboratories with experience in DHRs for drug-induced type II and III allergic reactions [1].

The LTT improves he drug-specific activation (through the addition of IL-2, IL-7/IL-15) professional antigen-presenting cells - dendritic cells (DC) or by removal of regulatory T cells ($CD3^+CD25^+$). Likewise, modification of the LTT by addition of anti-CD3/anti- CD28 monoclonal antibodies has been also tried, which led to increased sensitivity but simultaneously reduced specificity [13]. The classical LTT is performed using PBMCs which comprise mainly lymphocytes (B, T, and NK cells), and monocytes. Upon culprit drug incubation, memory T cell activation results in cell proliferation and differentiation. However, the final classical LTT read-out parameter (stimulation index) does not differentiate exactly which cells are activated and proliferating [13,27]. The steps of the T cell activation and proliferation process are associated with changes in terms of cell numbers, transcribed genes, translated proteins, reprogrammed pathways, and thus altered cellular metabolites levels [13]. Increased glycolysis in activated T cells results in pyruvate accumulation of orofacial release from the cells. External glucose uptake and utilization play a crucial role in the T cell proliferation response. Glycolysis is a required part of the metabolic response of T cells to proliferative signals [13]. The analysis of genes, proteins, and metabolites in activated T cells would not only extend our understanding of the mechanisms of drug allergy but also identify potential biomarkers for monitoring drug-induced immune system activation in drug allergy [13]. Various surface markers are upregulated in T cells upon activation: CD69 for the early activation stage, CD25 for the later activation stage, and HLA-DR for an even later activation stage. Flow cytometry

Table 2. Characteristics of the basophil activation markers CD63, CD203c, CD300a, and p38MARK [14].

Characteristics	Cell surface activation marker CD63	Cell surface activation marker CD203c	Cell surface activation marker CD300a	Intracellular activation marker phosphorylated p38 MARK
Synonym	Gp53 (LAMP-3)	E-NPP3	IRp60	Absent
Family	tetraspanins	NPP3	Immunoglobulin superfamily	Kinases
Resting basophils	barely detectable	Constitutively expressed	Constitutively expressed	barely detectable
Lineage-specific	No	Yes	No	No
IgE-activated basophils	Upregulation – 5 min bimodal expression	Upregulation – 3 min bimodal expression	Upregulation – 5 min bimodal expression	Upregulation – 3 min bimodal expression
Relation with anaphylactic degranulation	Yes	No	Under investigation	No

analysis has been employed for measuring the expression of CD86 on activated DC and PD-1/ PD-L1, CTLA4 on T cells effectors. The biochemical studies of the AK-STAT STAT signaling pathway are very important for patients with DIHS/ DRESS [13].

Most current studies for evaluating these types of reactions have included a heterogeneous mix of patients and culprit drugs (BLs, anticonvulsants, local anesthetic, and NSAIDs [27]. Enzyme-Linked Immunosorbent Spot (ELISpot) determines the number of cells producing an inflammatory marker: relevant cytokines and cytotoxic markers, after their activation by the specific drug. The method of ELISpot arise focused on BLs and/or anticonvulsants and measures the number of cells producing IFN-γ, IL-4, IL-,5, or Granzyme B. Another approach for evaluating non-immediate-type drug hypersensitivity (NIDHR) after stimulation with the specific drug is by the determination of the cell activation and/or cytokines or cytotoxic products (granzyme B and granulysin), by flow cytometry analysis of cells in culture or ELISA using the culture supernatants [11, 23]. These methodologies have been used for determining such cytokines as IL-2, IL-5, IL-10, IFN-γ [27,32], TNF-α, IL-8/ CXCL8, serum thymus and activation-regulated chemokine (TARC), IL-4, IL-6, IL-12, IL-13, IL-15, IL-18, CCR3, CXCR3, CXCR4, and CCR10 in the skin lesions, blister fluids, PBMC, or plasma of drug hypersensitivity patients [6].

In vitro testing for IgE-non-immediate cell-mediated allergies: 1) the determination of T-lymphocyte proliferation after incubation with culprit drug: a) classical LTT with detecting of stimulation index; b) modern flow cytometry analysis with measuring the expression of activation surface markers CD69, CD25, HLA-DR, CD86, CTLA4, death markers PD-1/ PD-L1 on various immune cells; 2) ELISpot, which determines the number of cells that release relevant cytokines (IL-2, IL-5, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-18, IFN-γ, TNF-α, IL-8/CXCL8, TARC, CCR3, CXCR3, CXCR4, CCR10) and cytotoxicity markers (perforin, granzyme B, granulysin); 3) ELISA to measure released cytokines [23, 27, 28]. Additionally, there are usually the cytological and histological methods for the diagnosis of drug-induced eruptions, and a skin biopsy may not definitively exclude alternative causes [26]. These methods have been used for delayed reactions [7,30].

6. The evaluation of non-allergic DHRs

Nonimmunologic anaphylaxis, previously regarded as a pseudoallergic drug reaction, involves the direct stimulation of mast cell degranulation. These reactions are limited to certain groups of drugs, including NSAIDs, such as aspirin, as well as opiates, vancomycin, quinolones, and NMBAs. NSAIDs can also induce T cell-mediated single-NSAID-induced delayed hypersensitivity reactions (SNIDHR) [6].

The cellular allergen stimulation test, which quantitates throught leukotriene (LT) release, has been proposed for the diagnosis [2]. Diclofenac, for example, as well as several other carboxylic acid nonsteroidal anti-inflammatory drugs can cause immune-mediated liver injury, which may be explained by hepatic metabolism and selective modification of hepatic proteins [1].

The other nonallergic DHRs without an immune mechanism (NSAIDs, RCM, or opioids) produce an increase in cysteinyl leukotrienes (CysLTs), prostaglandins (PG) D2, 15-hydroxyeicosatetraenoic acid (15-HETE) with overexpression of leukotriene (LTC4) and a decrease in PGE2. Nonallergic DHRs to NSAIDs have been classified as NSAID-exacerbated respiratory disease (NERD), NSAID-exacerbated cutaneous disease (NECD,) and NSAID-induced urticaria/ angioedema (NIUA) [12,18]. Aspirin-exacerbated respiratory disease (AERD) patients are characterized by eosinophilic tissue infiltration and excessive production of CysLTs [11].

Non-immunologic hypersensitivity reactions may also be mediated through the MAS-related G protein-coupled receptor-X2 (MRGPRX2) in cases involving specific drugs, such as icatibant, neuromuscular blocking drugs, and quinolone antibiotics. The interaction of certain drugs with this mast cell receptor can stimulate degranulation and the release of TNF-α and prostaglandin D2 (PGD2), among other molecules, leading to nonimmunologic anaphylactic reactions. The experimental mouse counterpart of MRGPRX2 induces pseudo-allergic reactions [6]. The Mas-related G protein-coupled receptor X2 (MRGPRX) expresses the tetrahydroisoquinoline motif (THIQ) thereby have been controlled of downstream processes between cross-linking of IgE/FcεRI (high-affinity receptor for IgE) and occupation of MRGPRX2 in basophils and mast cells (MC). Basophils develop from $CD34^{+}$ pluripotent progenitor stem cells, receptor MRGPRX2 is derived fr om $CD34^{+ve}$ progenitor

cells [16]. MC-degranulation via occupation of MRGPRX2 constitutes a novel endotype of immediate drug hypersensitivity (IDH) independent from cross-linking of IgE/FcεRI complexes. Neuromuscular blocking agents (NMBAs), fluoroquinolones (FQs), icatibant and the opiate morphine act via MRGPRX2. MRGPRX2-silencing appears on responses of activated MCs other than degranulation markers, such as the generation and release of lipid mediators, cytokines and vascular endothelial growth factor (VGEF) and to compare this approach to other means identifying human MC degranulation [29].

One of the free radicals is peroxyl radicals (ROO–•) acts in the peroxidation of fatty acids. Free radicals trigger chain reactions of lipid peroxidation, generated lipid radical reacts with oxygen, and peroxyl radicals are produced. Then, peroxyl radical transforms polyunsaturated fatty acids into lipid hydroperoxides, which are unstable and disintegrated into unsaturated aldehydes or malondialdehydes (MDAs). The lipid peroxidation counteracts cell membrane integrity itself, disrupting the membrane lipid bilayer and downregulating membrane receptors and enzymes: a) redox-sensitive transcription factor NF-κB; b) Nrf2 – another regulator of the antioxidant response. Understanding of the exact signaling pathway between the transcription factors and such markers such as cytokines, inflammatory cells warrant further research of study the effect of oxidative stress on transcriptional factors. For example, in the untreated allergic patients MDA levels were increased, and SOD level was decreased [31].

How allergens trigger ROS-generating pathways is not clear. Reactive oxygen species are partially reduced and highly reactive metabolites of O_2 that include, amongst others, superoxide (O_2–•), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). As an example, being stimulated by cytokines, growth factors, and hormones, most cell types elicit a discrete oxidative burst generating low concentrations of ROS. ROS then operate as important messengers of signal transduction through the oxidative modification of kinases and phosphatases, which are present in many signaling pathways including mitogen-activated protein kinase (MAPK) and NF-κB [31].

N-Acetylcysteine (NAC), an acetylated amino acid L-cysteine, inactivates free radicals and reactive oxygen species by directly reacting with them (direct antioxidant effect) and also supplying cysteine and endorcing the promotion of glutathione (indirect antioxidant effect). Consequently, cysteine efficiency may limit the rate of glutathione synthesis under conditions of oxidative stress. The positive effect of NAC has been demonstrated on conditions characterized by activation of lipid peroxidation (POL) and decreased production of glutathione (GSH): cardiovascular diseases, administration of acetaminophen (paracetamol) and heavy metal poisoning, HIV infection, etc. [22]. The role of allergic disorders in the skin has also not been as well studied, but in patients with physical urticarias, there are decreased blood levels of vitamin E, catalase, and glutathione peroxidase; however, increased SOD activity is also found. Furthermore, the peripheral blood monocytes from patients with severe dermatitis are primed to secrete superoxide [19].

In summary, is recommended the combining various *in vitro* tests for evaluating non-allergic DHRs. They include such investigations: 1) biochemical - hepatic enzymes; arachidonic acid metabolites - leukotrienes), prostaglandins D2, E2 [12, 14], products of nitrosative and oxidative stress, lipid peroxidation [20]; 2) immunochemical - components of complement (C3a, C5a, C5b-9), kinin- and coagulation systems, IgG; 3) PCR - viral and Mycoplasma infections [6], HLA-typing [1, 8]; 4) flow cytometry - detecting of MRGPRX2 – receptor [6, 18, 21, 31]. The mean sensitivity of *in vitro* tests is 79,6% [27].

Conclusions

1. The various endotypes of DHRs identifying characteristics defined by specific mechanisms (genetic, pharmacologic, physiologic, biologic, and/or immunologic) with each phenotype.
2. Diagnostic *in vitro* algorithm would generally be placed before or after the STs but always before DPTs, it should be a must base on the new laboratory technologies. Ideal screening tests associated with optimal sensitivity and specificity are safe to perform, should have been validated in studies with a blind comparison to a reference standard.
3. New biomarkers are described for severe delayed reactions (soluble chemokines, cytokines, granulysin, perforin, granzyme B, prostaglandins, leukotriene, and other molecular targets of inflammation), its measurement is very important to patients with acute reactions.
4. Performance of the BAT and detection of expression of receptor MRGPRX2 are recommended for diagnostic of reactions to NMBA, NSAID and b-lactam antibiotics. For testing, it is important to consider that IDHRs and NIDHRs usually correspond to different immunopathologic mechanisms.
5. HLA-typing and genotyping for prognosis of drug susceptibility can identify potential reactors and aid in protecting risk populations. Future research may be used in prospective pharmacogenomics screening, should help reduce severe, potentially fatal reactions to other her drugs.
6. In the future, the results of various laboratory tests in DHRs-diagnostic will be taken into consideration to assign modern treatment.

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