

REVIEW ARTICLE

The *in vitro* diagnosis of drug allergy: status and perspectives

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Abstract

Adverse drug reactions (ADR) can result from immune-mediated (drug allergy) and nonimmune-mediated mechanisms. In both types of reaction, conclusive diagnosis and appropriate management remain major problems in daily clinical practice. This review summarizes the potentials and shortcomings of the currently available *in vitro* tests in the diagnosis of immediate (mostly IgE mediated) and nonimmediate (mostly T-cell mediated) drug allergy, particularly quantification of specific IgE, flow-assisted analysis of *in vitro* activated lymphocytes and basophils and the enzyme-linked immunosorbent spot.

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Drug hypersensitivity reactions (DHR) can be immune mediated (drug allergy) or nonimmune mediated with potentially serious consequences of diagnostic error (1). Unfortunately, for several reasons, correct diagnosis is not always straightforward or (too) easily overlooked. First, different drugs can elicit various DHR with distinct pathophysiology, generally classified according to the modified Coomb's classification. Second, the exact epitope(s) causing the DHR is (are) frequently unknown. Third, problems are compounded when several drugs are administered simultaneously. Fourth, the gold standard for diagnosis, the drug challenge, possibly double-blind placebo-controlled (DBPCDC), is hampered by obvious ethical and practical limitations especially in those with severe clinical symptoms. Finally, with novel drugs such as biological agents, currently entering clinical practice, novel patterns of DHR might be observed and further challenge the diagnostic process (2). Therefore, the availability of validated diagnostic tests that renders DBPCDC superfluous would be more than welcome. Ideally, these tests should

correctly identify the offending compound(s), minimize the number of erroneously contra-indicated agents, and identify cross-reactivity as well as safe alternatives. This review focuses on the potential pitfalls and shortcomings of the currently available *in vitro* tests in the diagnosis of immediate (mostly IgE mediated) and nonimmediate (mostly T-cell mediated) drug allergy.

Specific IgE-immunoassays

Background

Quantification of drug-specific IgE (sIgE) with IgE immunoassays relies upon the detection of a drug-(hapten)-carrier-antibody complex. Basically, the drug-(hapten)-carrier conjugate is coupled with a solid phase, which is incubated with the patients' serum. The amount of sIgE bound is subsequently detected with a secondary antihuman IgE antibody labeled with a radioisotope in the older radioimmunoassay

(RIA) or with an enzyme with colorimetric reading in the more recent enzyme-linked immunosorbent assay (ELISA) or with fluorescence reading in the fluorescent enzyme immunoassay (FEIA).

In an attempt to further optimize coupling of the drug, a new technique using polyamidoamine (PAMAM) dendrimers has been developed for the detection of β -lactam-specific IgE antibodies. These dendrimers can be densely penicilloylated on the surface of the molecule forming precisely defined chemical structures that specifically bind β -lactam IgE antibodies (3). Whether this PAMAM-based technique offers an advantage over the traditional cyanogen-bromide or epoxy-activated cellulose and sepharose assays remains to be established.

Results of sIgE assays have been expressed in many ways. Today, results of most (commercial) assays are expressed as arbitrary units in kUA/L. For years, the detection limit was established at 0.35 kUA/L. However, recently a new heterologous calibration scheme has been introduced, where quantification is based on the use of IgE antibody curves with a range of 0.00–100 kUA/L, with a detection limit set on 0.1 kUA/L and a cutoff value of 0.35 kUA/L for positive results.

Clinical use

Neuromuscular blocking agents (NMBA)

Allergy to curarizing NMBA constitutes a life-threatening condition that is generally documented by skin testing. However, to minimize the potentially dramatic consequences of missed diagnosis, quantification of sIgE has been advocated as an additional instrument to document allergy to NMBA (4–6). Although some research groups have used drug-specific assays (7, 8), allergy to NMBA is currently generally serologically assessed indirectly through various methods that measure IgE reactivity to quaternary ammonium structures (NH_4^+) that are considered to be the major epitopes of NMBA (9, 10). Most frequently employed methods are a choline chloride (5, 11–14), a p-aminophenyl phosphoryl choline (11, 14–17), and/or morphine-based assays (6, 7, 15, 18).

Table 1 displays the diagnostic value of immunoassays used for the diagnosis of allergy to NMBA. From this table, it appears that specificity of sIgE for various NMBA generally exceeds 90% with a sensitivity varying between 40% and 90%. Note, however, that sIgE antibodies against NH_4^+ are prevalent in the general population (6, 19) and can therefore not be used as a screening technique to identify patients at risk. Moreover, in a recent comparison between skin testing, sIgE, and basophil activation tests (BAT), it was clearly demonstrated that an isolated positive sIgE result in patients with suspected rocuronium allergy but with a negative skin test cannot be considered as diagnostic but need additional testing, e.g., BAT (20). Most important explanations for these false-positive sIgE results are an elevated total IgE (21) and intake of pholcodine (22).

Beta-lactam antibiotics

Table 1 also summarizes the data of sIgE to β -lactam antibiotics. Again, it appears that quantification of sIgE constitutes

a specific but not highly sensitive instrument to diagnose IgE-mediated allergy from β -lactams. Differences in sensitivity and specificity shown in Table 1 have been reported to relate to the clinical presentation, with the sensitivity generally being higher in anaphylaxis and anaphylactic shock for CAP-FEIA and RAST, respectively (23). Differences in sensitivity can also be explained by the evolution of drug consumption. This has been demonstrated for β -lactams (23, 24) and cephalosporins (25, 26).

Nevertheless, quantification of sIgE deserves a place in the diagnostic algorithm of immediate β -lactam allergy, particularly in severe anaphylactic cases when skin tests yield equivocal or negative results (24). Actually, immunoassays can be diagnostic in such cases (4) and render unnecessary additional potentially dangerous drug challenges.

A study with a large number of cases having immediate reactions to cephalosporin, using a radioimmunoassay with epoxy-activated sepharose as the solid phase, showed a sensitivity of 74.3%, demonstrating that immunoassays are a good approach to evaluate these reactions (27).

Quinolones

In fluoroquinolone-induced hypersensitivity, the radioimmunoassay with epoxy-activated sepharose has been shown to be a potential alternative to skin testing, which is unreliable because of the high number of nonspecific skin responses in control individuals (28). Unfortunately, the sensitivity of sepharose-RIA in quinolone allergy is rather low and ranges from 31.6% to 54.5% (29, 30) (Table 1). These differences can be attributed to the type of quinolones involved in the reaction and the clinical manifestations in the patients, with the reactions being more severe in the latter study. *In vitro* cross-reactivity of IgE among different quinolones is frequent and suggests that a common avoidance of quinolones should still be recommended in all patients.

Nonsteroidal anti-inflammatory drugs (NSAID)

Hypersensitivity reactions to NSAIDs are generally not IgE mediated and predominantly result from a pharmacologic mechanism with inhibition of cyclo-oxygenase (COX)-1, which induces a depletion of prostaglandin E2 with continuous synthesis of cysteinyl leukotrienes and mediator release from mast cells and eosinophils. Obviously, in these cases, IgE-binding studies are of no value. However, a minority of the adverse reactions to NSAID such as pyrazolones can be IgE-mediated-type reactions. These patients generally react exclusively to only one NSAID family and can demonstrate positive sIgE and skin tests (31).

Basophil activation test

Background and principles

Basophilic granulocytes are histamine containing leukocytes bearing functional high-affinity receptors for IgE ($\text{Fc}\epsilon\text{RI}$) on their plasma membrane. Basophils share many characteristics with tissue-dwelling mast cells; especially the histamine content and the $\text{Fc}\epsilon\text{RI}$ consisting of α , β , and γ -chains. However,

Table 1 Overview of the diagnostic value of specific IgE in the diagnosis of drug allergy to antibiotics, Nonsteroidal anti-inflammatory drugs, and NMBA. (not exhaustive)

Drug	Type of reaction	Technique	N (pt)	N (ctrl)	Ref. test	Sens. (%)	Spec. (%)	References
NMBA		RIA/RAST	27	20	H	88 (OAS) 67 (succinylcholine) 41 (alcuronium)	ND	5
NMBA		RIA (PAPCC)	26	34	H+ST	97	97	16
NMBA		RAST	530		H+ST	85%	91%	70
ROC	HT+Br	RIA (PAPCC)	25	30	H+ST	Roc 92	Roc 93	21
		ImmunoCAP-FEIA				Morph 88	Morph 100	
		Roc, morph, sux, phol				Sux 72	Sux 100	
						Phol 86	Phol 100	
Pen	IR	RAST	30	30	H+ST	BPO-PLL: 86.66	BPO-PLL: 90	71
		BPO-PLL				BPO-HSA: 66.33	BPO-HSA: 83.33	
		BPO-HSA				BPO-SP: 86.66	BPO-SP: 96.66	
		BPO-SP						
AX, PenG, CU, CEP	AN $\pm$ UR-AE	CAP-FEIA	67	30	H+ST	47.7	83.3	72
Pen	AN $\pm$ UR	CAP-FEIA (c1, c6)	290	120	H	C1: 10–68 C6: 41–53 Depending on ST-positivity	>95	73
AX $\pm$ clav, PenG	AN $\pm$ UR	ImmunoCAP FEIA	74	55	H $\pm$ ST	BPO:32	BPO:98	4
		BPO				AXO:43	AXO:98	
		AXO				BPO+AXO:50	BPO+AXO:96	
AX, PenG, CEP, CU	AN $\pm$ UR	BPO+AXO	58	30	H + ST	37.9	86.7	74
		ImmunoCAP FEIA						
AX $\pm$ clav, Co, Ceftri, CEP,	UR, AN, AS	CAP-FEIA (c1, c5, c6, c7)	30	15	H $\pm$ ST $\pm$ PrT	0–25	83.3–100	23
Pen, cefaclor, AMP		RAST (BPO, AX)	76	0	H	42.9–75 74.3	66.7–83.3 ND	27
Ceftri, co, ceftazi, Pen	AS, UR-AE, AE, syncope	Sepharose-RIA (c1, c2, c5, c6)	55	32	H	54.5	100%	29
CIP, CIN, LOM, OFL, PEF,	UR-AE, glottis	Sepharose-RIA (PIP, RUF, PEF,	38	35	H $\pm$ PrT	31.57	ND	30
NOR, RUF, PIP	edema, AS	NAL, CIN, LOM, CIP, OFL)	53	0	H	58	ND	31
MOX, CIP, LOM	UR, AN, AS	Sepharose-RIA (CIP, MOX, LOM)						
PP	OAS $\pm$ prur $\pm$ tachy $\pm$ UR $\pm$ AE $\pm$ shock	ELISA (PP-HSA conjugate)						

Drug: Amp, ampicillin; AX ($\pm$ clav), amoxicillin/clavulanic acid; co,cefotaxim; ceftazi, ceftazidim; ceftri, ceftriaxone; CIN, cinoxacin; CU, cefuroxime; CEP, cephalosporin; CIP, ciprofloxacin; LEV, levofloxacin; LOM, levofloxacin; Miv, mivacurium; MOX, moxifloxacin; NAL, nalidixic acid; NOR, norfloxacin; OFL, ofloxacin; PEF, pefloxacin; PenG, penicillin G; Phol, pholcodine; PIP, pipedimic acid; PP, propylphenazone; ROC, rocuronium; RUF, rufloxacin; NMBA, Neuromuscular blocking agents.

Type of reaction: AN, anaphylaxis; AS, anaphylactic shock; Br, bronchospasm; HT, hypotension; IR, immediate allergic reaction; OAS, oral allergy syndrome; Prur, pruritus; Tachy, tachycardia; UR/AE, Urticaria/angioedema.

Technique: AXO, amoxicillin; BPO, benzyl penicilloyl; c1, penicilloyl G; c2, penicilloyl V; c5, ampicilloyl; c6, amoxicilloyl; c7, cefaclor; CAST, cysteinyl leukotriene release test; ELISA, enzyme-linked immunosorbent assay; FEIA, fluorometric enzyme immunoassay; HSA, human serum albumine; PAPCC, P-aminophenylphosphoryl-choline; PP-HSA, propylphenazone-human

serum albumine; PPL, polylysine; RAST radioallergosorbent test; RIA, radioimmunoassay; SP, aminospacer; N (pt), number of patients; N (ctrl), number of control individuals.

Ref test, Reference test; H, history; ST, skin test; PrT, provocation test; Sens, sensitivity; Spec, specificity.

ND, not determined.

specific receptors on mast cell subtypes or basophils allow their cell-selective activation. Although the percentage of basophils in the total leukocyte population is low (<1%), activation of basophils can be examined with flow cytometric techniques using basophil-specific markers, which can be helpful in diagnosing immediate DHR, including the identification of the culprit agent.

Basophils can be *in vitro* activated by different means: (i) cross-linking of FcεRI via allergen–IgE interactions, (ii) cross-linking of the FcεRI with specific human anti-IgE or human anti-FcεRI antibodies, (iii) by lectins that can cross-link FcεRI-bound IgE in an antigen-independent manner, (iv) independently of FcεRI cross-linking via different receptors or even receptor independently. Receptor-dependent stimuli include endogenous substances, such as activated complement products C3a and C5a that can bind to specific complement receptors on basophils. Several chemokines can bind to their specific receptors on basophils and activate basophils even in low concentrations. In addition, bacterial-derived N-formylmethionyl-leucyl-phenylalanine can potentially stimulate basophils. Some physical conditions can result in basophil degranulation. These can be both hyperosmolar as well as hypo-osmolar conditions. Some plasma expanders or radiocontrast drugs can activate basophils in this manner.

Traditionally, activation of basophils has mainly been analyzed by the measurement of released cysteinyl leukotriene or histamine (32). Almost two decades ago, it was shown that CD63 antibodies can be applied to identify basophil degranulation. In resting basophil, CD63 antibodies bind to the intracellular granules and only barely expressed on the surface membrane. In contrast, as a result of fusion between the granule and the membrane during exocytosis, CD63 is expressed with a high density on degranulated basophils (33). Almost a decade later, another lineage-specific marker, i.e., CD203c was discovered (34). This marker is constitutively expressed in low amounts at the surface membrane of basophils and quickly upregulates upon activation, which does not necessarily lead to degranulation.

Currently, the determination of basophil activation by flow cytometry is called BAT. Different protocols as well as commercial kits are available. The current protocols for performing BAT are based on the analysis of whole blood. Different anticoagulants have been validated, but heparinized blood is used most frequently. Depending on the protocol, between 50 and 100 µl, whole blood per analysis is required. Diluted blood is incubated with allergen from 15 to 30 min at 37°C. During or directly following activation, the basophil-specific antibody and the activated basophil-specific antibodies (CD63 and CD203c) are added. Different strategies for basophil detection in whole blood have been developed. Previously, anti-IgE and side scatter were used to characterize basophils, but because IgE is also present on other cells such as dendritic cells, its use is less advocated. Nowadays, different basophil markers are used, such as CD123 (also present on dendritic cells) in combination with HLA-DR (negative on basophils, but positive on dendritic cells), CD203c (positive on basophils, but can be too low on resting basophils), or CCR3 (positive on basophils and eosinophils) in

combination with side scatter (eosinophils have increased side scatter compared with basophils) (35).

In the flow cytometric analysis, it is advised to analyze a minimum of 200 basophils. CD63 binding is often determined as the percentage (high) positive cells to detect the degranulated basophils. As 5–10% of the general population have basophils that do not degranulate after FcεRI cross-linking, it is important to include a positive control for the IgE-mediated releasability of basophils with anti-IgE or anti-FcεRI antibodies. In the case when this positive control is negative, the negative allergen-specific responses should be discarded to prevent false-negative results. Furthermore, the result of the test can depend on the concentration of the drugs and metabolites, and therefore, a wide concentration range is advised. It is proposed to use at least a range of 10⁴. One of the underlying problems is that drug-induced basophil activation is not *per se* IgE mediated and other receptor-dependent or receptor-independent activation routes have been suggested (35). The advantage of BAT compared with *in vivo* drug challenge is that in the case of a positive BAT cross-reactive drugs can be evaluated (36).

Although CD63 and CD203c have been used as basophil activation markers in drug allergy diagnosis, most published data on drug allergies have included CD63 and not CD203c. Therefore, this section will discuss results obtained with CD63.

Clinical use

Neuromuscular blocking agents

As emerges from Table 2, the BAT has a sensitivity varying between 36% and 92% and a specificity between 93% and 100%. As addressed elsewhere (35) and emphasized in the discussion elsewhere, there are various explanations for this generally disappointing sensitivity. Nevertheless, from these studies, it is evident the BAT can add to the diagnosis of NMBA, particularly in patients where skin tests yield equivocal or negative results. Moreover, the BAT has been proven to be complementary to skin tests in the study of cross-reactivity between NMBA and the identification of safe alternatives for future surgery (20, 36).

β-lactam antibiotics

The diagnostic approach for immediate allergic reactions to beta-lactam antibiotics generally relies on skin testing and quantification of sIgE. However, as addressed by Blanca et al. (24) in particular cases, novel instruments such as the BAT are gaining importance. There are currently five studies that have evaluated the BAT in the diagnosis of beta-lactam allergic reactions with an appropriate number of well-characterized patients. Compared with the diagnostic value of measurement of specific IgE in the diagnosis of allergy to beta-lactam antibiotics, BAT shows a higher sensitivity (about 50%) and specificity (about 90%) (Table 2).

Iodinated radiocontrast material (RCM)

Allergic reactions to iodinated radiocontrast material have been described, but their prevalence is low and has been

Table 2 Overview of diagnostic performance of the basophil activation test in the diagnosis of drug allergy

Drug	Type of reaction	N (pt)	N (ctrl)	Reference test	Sens. (%)	Spec. (%)	References
NMBA (vec, sux, roc, atra, pan, alcu)	Tachy ± HT ± UR ± AE	21	8	H + ST	64	81	75
NMBA (sux, roc, atra, vec, miv)	AN shock ± HT ± brady ± UR ± AE	39	17	H + ST	54	100	76
NMBA (Sux, atra, roc, vec, miv) midazolam, morphine, prilocaine, alfentanil,...	HT ± UR ± Br	21	10	H + ST	CD63: 79 CD203c: 36	100	77
NMBA (sux, roc, vec, atra, pan)	AN shock ± Br ± UR	47	45	H + ST	36–86	93	65
Roc	HT ± Br	14	8	H + ST	92	100	36
NMBA	Shock grade I,II,III and IV	26	23	H + ST + IgE	67	100	78
Ax, PenG, Cep, Cu	AN ± UR	58	30	H	50	93	74
β-lactam (ax, bp, cl, cu, co, peni)	UR, AN	70	40	H + ST + IgE + PrT	49	91	79
Ax	AN, UR, AE	27	14	H + ST	CD203c: 52 CD63: 22	CD203c: 100 CD63:79	80
Pen, amp, ax, cu, cip	AS ± UR ± AA ± AE ± exanthema ± dizziness ± diarrhea	24	16	H + ST + IgE	55	80	81
Metamizol	AN, UR-AE	26	30	H + PrT	42	100	40
ASA, diclofenac, ketoprofen, metamizol, piroxicam, propifenazon, lindomethacin, mefenamic acid, paracetamol, naproxen	UR ± AE ± Dyspnoe	60	30	H + PrT	66.7	93.3	39
Diclofenac	UR, AE, AA	14	12	H	No sign diff in CD63 expression between pts and ctrls		82
ASA, ibuprofen, metamizol, diclofenac, paracetamol, ketorolac	AN, UR, AE,AA	43	29	H	43	100	83
Pyrazolones	UR, AN	51	56	H + IDT + PrT	54.9	86	41
ASA	Naso-ocular symptoms, Br, AN	30	12	H + PrT	CD63:33.3 CD203c:16.7 CD63: 16.7 CD203c:22.2	CD63:79,2 CD203c:100 CD63:91.7 CD203c:100	84
Diclofenac							
ASA, NSAIDs	AERD	10	10	H + PrT	30	40	43

Abbreviations (see legend Table 1).

ALCU, alcuronium; ASA, aspirin salicyl acid; Atra, atracurium; co, cefaclor; NMBA, neuromuscular blocking agent; NSAID, nonsteroidal anti-inflammatory drug; Pan, pancuronium; Sux, suxamethonium; Vec, vecuronium; AA, asthma attack; AERD, aspirin-exacerbated respiratory disease; IDT, intradermal testing.

estimated between 0.02% for nonionic RCM and 0.4% for ionic RCM. Around 4% of these are suggestive of an IgE-mediated mechanism (positive skin test and BAT), which is therefore not the general rule in RCM allergy (37). Direct histamine release by means of nonspecific RCM binding to membrane receptors on mast cells or basophils and indirect mast cell or basophil activation by means of the complement

or kinin cascade outweigh the genuine IgE-mediated reactions (38).

Aspirin and nonsteroidal anti-inflammatory drugs

As described elsewhere, the majority of hypersensitivity reactions to NSAID are not IgE-mediated and for this reason, the BAT will not be useful to document this hypersensitivity.

Consequently, in several Spanish studies, BAT with NSAIDs demonstrated low sensitivity ranging from 15% to 55% (39–41). In a multicenter study, it was found that BAT with NSAID was positive in healthy controls that tolerated NSAID (42). Several other groups failed in producing convincing BAT results in NSAID-sensitive groups and do not recommend to include BAT in the diagnostic follow-up of patients (43) (Table 2).

However, BAT can be useful in NSAIDs hypersensitivity when the patients react to only one NSAID in an IgE-mediated way. In these cases, BAT has shown a sensitivity between 42% and 55% with a specificity of 86–100% (40, 41). Although the sensitivity is not very high, the BAT could be useful to avoid challenge tests in the evaluation of less frequent, selective patients with severe anaphylactic reactions.

Quinolones

Contradictory results regarding the sensitivity of skin testing in quinolone allergy have been reported, and skin tests have led to positive results in healthy control subjects (28). For this reason, *in vitro* detection of IgE-mediated allergy is critical. As a commercial immunoassay is not readily available, the allergist must rely on the BAT to confirm the result of the skin test and to help find alternative less harmful drugs.

A recent study using BAT showed a high sensitivity of 71% demonstrating its utility for diagnosing patients, especially those with severe reactions to quinolones where drug provocation testing may increase the risk of an adverse reaction. The sensitivity of a specific IgE sepharose-RIA was 32% (30).

Additives in vaccines and IV fluids

In smaller studies, BAT has been used to evaluate allergic reactions to additives in vaccines and intravenous fluids such as gelatines, carboxymethylcellulose, hydroxyl ethyl starch, cremophor, and bovine serum albumin (35). Information from these studies is presently too limited to confirm whether these are IgE-mediated responses in the first place and secondly to provide insight into the sensitivity and specificity of the test for these agents.

Overall, BAT can be an interesting technique in the diagnostic follow-up of drug allergies or in the evaluation of possible cross-reactive drugs. However, many uncertainties remain, and currently, the application of BAT in adverse drug reactions (ADR) is one of the major areas of the European Basophil Activation Task Force (EuroBAT).

Lymphocyte activation tests

Background

The involvement of T cells in delayed-type drug hypersensitivity reactions (DTDHR) is well established (44). *In vitro* diagnosis of DTDHR has focused on analyzing and quantifying activated drug-specific T cells. Historically, this T-cell proliferation has mostly been assayed by lymphocyte transformation tests (LTT). However, with the advent of perfor-

mant multicolor flow cytometers, newer techniques have been developed during the last decade (*vide infra*).

The LTT is an *in vitro* diagnostic test, which measures the proliferation of T cells in the presence of the specific antigen. The technical principles of the LTT have been described extensively elsewhere (45). Briefly, peripheral blood mononuclear cells are separated and cultured in the presence of the suspected drug, mitogen as a positive control and buffer as a negative control. For drugs, the cell culture generally lasts for 5–7 days to allow sufficient proliferation of the low numbers of allergen-specific cells. Subsequently, proliferation is quantified directly by measuring DNA synthesis using incorporation of radioactive nucleosides, i.e., ³H-thymidine. Results are generally expressed as a stimulation index that is calculated by proliferation (counts per minute) with drug/proliferation (cpm) without drug.

There are nowadays alternative ways to perform LTT such as with the use of a fluorochrome, 5(6)-Carboxyfluorescein N-succinimidyl ester (CFSE), detection of nonradioactive bromodeoxyuridine (BrdU) using BrdU-specific antibodies, or newer analogues like fluorochrome labeling of 5-ethynyl-2'-deoxyuridine (EdU). To our knowledge, however, these methods have not been validated for drug hypersensitivity.

Clinical use

The clinical applications of LTT in the diagnosis of drug allergy are summarized in Table 3. From this table, it appears that LTT has predominantly been adopted to document diagnosis of different cutaneous reactions such as maculopapular eruptions (MPE), urticaria, and fixed drug eruption, the drugs mostly studied being β -lactam antibiotics and anti-epileptics, particularly carbamazepine (46–48).

The diagnostic value of the LTT depends on the drug (47) and the type of drug hypersensitivity involved. LTT is highly useful in patients with generalized exanthema of different types and drug hypersensitivity syndrome with eosinophilia and systemic symptoms (DRESS) (46) and is rather seldom positive in patients with blood cell dyscrasia, toxic epidermal necrolysis (TEN) (49), and fixed drug eruption. Overall, the LTT demonstrated a sensitivity of 60–70% and a specificity of 85–93%. The diagnostic value might significantly improve when LTT is performed at optimal timing (50). The readout can be further optimized by making use of complementary determination of cytokines produced by drug-specific stimulated T cells (such as interferon- γ or IL-5) and using optimal stimulation time/conditions. Better read out has been published with the addition of monocyte-derived dendritic cells acting as antigen-presenting cells (51).

In small groups or isolated case reports, the LTT has also been applied to identify the etiologic cause in drug fever, drug-induced pancreatitis, interstitial and eosinophilic lung diseases, and drug-induced autoimmune diseases.

As T helper cells are involved in the antibody isotype switch from IgG to IgE, LTT technology has also been applied in the diagnostic management of various immediate IgE-mediated allergies.

Table 3 Diagnostic performance of the lymphocyte transformation tests in the diagnosis of delayed drug hypersensitivity

Drug	Symptoms	N (pt)	N (ctrl)	Reference test	Sensitivity	Specificity	References
Various drugs	Different clinical manifestations	923; 100 patients with a high probability	15	H+PrT	78%	85%	47
Amoxicillin, penicillin	MPE	12 patients	6	H	83%	PPV 100% NPV 75%	85
B-lactam antibiotics	IR and NIR	IR <i>n</i> = 31 NIR <i>n</i> = 19	28	H ± RAST ± ST ± PrT	IR: 64.5% NIR: 57.9%	92.8%	48
Various drugs	Different (MPE, bullous exanthema, urticaria)	22	ND	H	67%	ND	86
Various drugs	MPE	13	13	H	75%	100%	87
Carbamazepine	Widespread rash (MPE or desquamating)	5	4	H	100%	100%	88

Abbreviations (see Table 1) IR, immediate reaction; NIR, nonimmediate reaction; MPE, maculopapular exanthema; PPV, positive predictive value; NPV, negative predictive value; ND, not determined.

Measurement of activation markers and cytokines

Nowadays, the reactivity of T cells can also be analyzed by flow cytometry assessing surface activation markers, signaling molecules, and finally intracellular cytokines, using specific monoclonal antibodies.

Background

Activation markers on the cell surface

Different activation markers are expressed and/or upregulated on T cells after stimulation, such as CD25, CD69, CD71, and HLA-DR. All these molecules are expressed on the surface of the cell and can be detected with flow cytometry using specific fluorescence-conjugated monoclonal antibodies. At present, different studies have been performed on flow cytometric detection of activation markers on T cells after activation with mitogens such as pokeweed mitogen, CD2/CD2R monoclonal antibody, superantigen staphylococcal enterotoxin B, and specific antigens such as *Candida albicans*. However, to the best of our knowledge, only one study has currently been published on the value of the measurement of activation markers in the diagnosis of DTDHR (52).

Chemokine and skin-homing receptors

As the skin is regularly affected in T cell-mediated DTDHR, T cells responsible for the immune response can be identified by their high expression of the skin-homing receptor, cutaneous lymphocyte-associated antigen (CLA). Increased levels of CLA have been detected on activated T cells during the acute phase of the allergic reactions to the drug parallel with disease severity in peripheral blood by flow cytometry (53, 54).

Apart from trafficking, some chemokine receptors can act as markers of the type of reaction (Th1/Th2). Different studies have focused on the role of CCR4, CCR7, and CCR10 in T cell-mediated skin inflammation (55–57) (Table 4).

Cytokines

Many researchers have focused on the cytokine production in peripheral blood mononuclear cells from patients suffering

an ADR, in an attempt to associate certain cytokines with specific clinical phenotypes. Cytokines can be measured either intracellularly or in the supernatants. As the production of most cytokines is not always confined to a single cell type, with the intracellular measurement technique, the cytokine-producing cell can be identified, without isolation of the T cells, which can be very helpful in the search of the pathophysiological mechanism. However, a disadvantage of the intracellular technique is the absence of absolute quantitative measurement of the produced cytokine. Here, we synthesize the current literature on the assessment of cytokines, mainly focusing on the enzyme-linked immunosorbent spot (ELISPOT) and the flow cytometric techniques.

ELISPOT assay

Recent studies have analyzed the value of ELISPOT assay, as a new method for the detection of cytokine release in patients allergic to drugs (58–60). The ELISPOT assay is based on the principle of an ELISA detecting antigen-induced secretion of a cytokine trapped by an immobilized specific anticytokine antibody and visualized by an enzyme-coupled secondary anti-cytokine antibody. These methods allow the detection of Th1 and Th2 cytokines (IL-2, IFN- γ , IL-4, IL-5, and IL-13) or cytotoxic protease and granzyme B. With a lower detection threshold of <25 secreting cells per million of peripheral blood mononuclear cells, the ELISPOT assay is one of the most sensitive *ex vivo* detection methods available. In the ELISPOT assay, peripheral blood mononuclear cells are obtained from a sensitized patient and cultured in the presence of the suspected drug. Sensitized lymphocytes undergo blastogenesis and secrete cytokines such as IFN- γ , IL-5, IL-13. The result can be expressed as spot-forming cells (=number of secreting cells), which is the relation between the cytokine secretion with antigen compared without antigen.

Enzyme-linked immunosorbent spot assay appears to be able to detect drug-specific T cells in blood of allergic patients. Indeed, cytokine secretion (IFN- γ , IL-5, IL-13) or cytotoxic protease (granzyme B) has been detected after *in vitro*

Table 4 Overview of activation markers, cytokines, and intracellular markers of relevance in the diagnosis of drug allergy

T cell			Technique	Application in diagnosis of drug allergy	References
Cell surface	Activation markers	CD69	Flow immunocytometry	Yes	52
		CD25, CD71, HLA-DR		To study	
	Chemokine/skin-homing receptors	CCL27–CCR10	Flow cytometry, immunohistochemistry	Yes	56
		CCR4–CCR10	Flow immunocytometry	Yes	57
		CCL27–CCR10	RT-qPCR, immunohistochemistry	Yes	55
Intracellular	Cytokines	CLA	Flow immunocytometry	Yes	53, 54
		IL-4, IFN- γ and TNF- α	PCR	Yes	63
		IL-5, IL-10, IFN- γ	Flow cytometry, ELISA	Yes	62
	Transcription factors	c-maf, GATA3 and T-bet	PCR	Yes	63
		STATs, p38MAPK		To study	
Supernatants	Cytokines	IFN- γ , IL-5, IL-13, granzyme B	Elispot	Yes	58, 59

PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; CCR, chemokine receptor; ELISPOT, enzyme-linked immunosorbent spot.

stimulation with the culprit drug but not with a tolerated drug (58, 59) in patients with DTDHR. No positive response has been detected in patients with immediate hypersensitivity to drug (59). Collectively, this preliminary data demonstrate that the ELISPOT assay appears to be a promising assay for the diagnosis of drug allergy based on the detection of drug-specific T cells. Future studies will have to confirm the usefulness of the assay in a larger number of patients.

Flow cytometry

Intracellular cytokines

Flow cytometric measurement of intracellular cytokines already has broad scientific applications in different cell types in domains such as immunology and rheumatology (61). With respect to drug allergy, the following publications were currently identified (Table 4). In the study by Martin et al. (62), IL-5/IL-10/IFN- γ with flow cytometry and IL-5/IL-2/IFN- γ with ELISA were assessed in the PBMC of 19 patients after stimulation with the culprit drug. Drug allergy was confirmed by stringent history category A according to Nyfeler and Pichler (47). Drug-specific cytokine production by peripheral blood mononuclear cells could be demonstrated in 75% of the patients using flow cytometry and in 79% using ELISA, respectively. The combination of flow cytometry and ELISA increased the sensitivity to 100%. Future comprehensive studies are mandatory to assess which cytokine or combination of cytokines is best suited for the diagnosis of drug allergy.

Measurement of transcription factors

In parallel with the study by Cornejo-Garcia et al. (63), where the expression of cytokines (IL-4, IFN- γ , and TNF- α) and transcription factors (c-maf, GATA3, and T-bet) was measured by semi-quantitative real-time PCR in peripheral blood mononuclear cells characterizing both immediate and

nonimmediate reactions to drugs, it would be interesting to do the same by means of flow cytometry. For example, similar to the flow-assisted diagnosis of immediate allergic reactions, the phosphorylation of the intracellular located signaling molecule p38MAPK (mitogen-activated protein kinase) and the different STAT (signal transducer and activation of transcription) molecules could be evaluated as marker of drug-specific T-cell activation (64).

In conclusion, flow cytometric assessment of *in vitro* drug-induced T-cell activation (cytokines, transcription factor) can be an important tool for assessing the mechanisms involved in ADR. However, nowadays, it remains mostly used as a research tool as it requires more experience in assessment and interpretation.

Discussion

Drug allergy represents an important health problem with significant morbidity and mortality with serious consequences of diagnostic error. Therefore, correct diagnosis with identification of all offending compounds and potential safe alternatives is a prerequisite to avoid future exposure.

From this review, it is clear that different *in vitro* humoral and cellular tests are valuable in the evaluation of immediate and delayed DHR, provided some important issues are kept in mind. Most importantly, it is of note that none of these diagnostic tests has an absolute predictive value. As a general rule, it appears that these tests demonstrate acceptable specificity, but sensitivity can be highly variable. False-positive results constitute an inconvenience that could result in unnecessary drug avoidance, sometimes resulting in the prescription or administration of less suitable drugs. Alternatively, false-negative results can have dramatic consequences. Several points must be taken into account regarding (false)-negative results. First, it has repeatedly been demonstrated that the outcome of the diagnostic test might be highly dependent on the time interval elapsed between the acute reaction and

actual testing, with negatvation over time (41, 50, 65–67). This indicates that further efforts are needed to establish optimal testing times, which may vary depending on the type of test used. For example, Fernandez et al. (66) demonstrated that negatvation of the BAT with amoxicillin occurred sooner than negatvation of sIgE to penicillins. Alternatively, to circumvent the potential issue of the refractory period, as is the practice with skin tests, it is recommended that diagnostic tests be postponed for 4–6 weeks after the acute event, particularly when the patient was treated with higher doses of corticosteroids. However, this recommendation is rather empiric expert based and not evidence based. Second, the diagnostic accuracy of tests significantly improves by disregarding usually predefined and rather arbitrarily chosen decision thresholds and applying drug-specific cutoffs instead (21). Such optimized drug-specific decision thresholds can be calculated through inclusion of sufficient numbers of well-characterized allergic patients and (exposed) control individuals that should allow performance of receiver operating curve analysis. Furthermore, the diagnostic accuracy of tests might benefit from optimization and standardization of the assay conditions. For example, in cellular tests, the stimulation of cells can be highly dependent on the stimulus temperature, incubation time, drug concentrations, and/or priming of the cells. Therefore, validation of such tests cannot be considered appropriate when it did not include drug-specific dose-finding experiments spanning several log scales of concentrations to establish the optimal stimulation concentrations. As some patients might exhibit a nonresponder status and not react in cellular tests, it is mandatory to perform sufficient and appropriate positive control experiments. In fact, if positive control stimulation results in nonresponsiveness, it becomes impossible to interpret negative drug stimulation. For such individuals, the test is lost as a diagnostic tool, whereas for study purposes, such results should be considered as false negatives and be included in the calculation of the predictive values.

Finally, it is also important to realize that low sensitivity of the *in vitro* test for evaluating drug allergy might also relate to the use of nonadequate drug metabolite(s) (68) or hiding the relevant epitope(s) in solid-phase techniques.

Considering the results of positive diagnostic tests, one should first keep in mind that drug allergy does not *per se*

need to relate to the active compound(s) but could also result from the applied excipients. In such cases, the excipients should be tested as this might not only improve the sensitivity of the test but also help to identify other drugs at risk.

Second, *in vitro* tests can show false-positive results as well. For example, it has been shown that *in vitro* sensitization to quaternary ammonium structures does not *per se* indicate the presence of an allergy to NMBA. Porri et al. (19) demonstrated sensitization to quaternary ammonium ions to be abundant in the general population. In a survey by Florvaag et al. (6), about 5% of the sera of unselected blood donors and 10% of the sera routinely analyzed in two allergy laboratories demonstrated IgE reactivity for morphine. In few cases, this phenomenon could relate to a deleterious effect of elevated titers of total serum IgE on the quantification of specific IgE (21) or could relate to the intake of IgE-stimulating drugs such as pholcodine (22).

In future, improvement of the diagnostic value of the tests can further be achieved by optimizing the current techniques. For example, the diagnostic performance of the flow cytometric diagnosis of drug allergy could improve by the combination of different currently available activation markers such as CD63, CD203c, and the intracellular activation marker p38MAPK (69). However, this remains to be established.

In conclusion, it is clear that the current *in vitro* techniques are helpful in assessing immediate and nonimmediate types of DHR. However, there is certainly room for improvement, and new diagnostic tools (e.g., flow-assisted quantification of activated basophils and T cells) need additional validation before entry into mainstream diagnostic use. Currently, it appears that in certain cases, *in vitro* diagnosis cannot be a reliable alternative to skin tests. However, they can be added as complementary tests to increase the overall sensitivity of the diagnostic procedure.

Conflict of interest

There are no known conflicts of interest for the authors.

Author's contribution

All authors contributed equally to this review.

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