

REVIEW

In vitro diagnosis of T cell-mediated drug allergy

G. Porebski, A. Gschwend-Zawodniak and W. J. Pichler

Department of Rheumatology, Clinical Immunology and Allergology, University Bern, Bern, Switzerland

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Summary

Diagnosis of drug hypersensitivity relies on history, skin tests, *in vitro* tests and provocation tests. *In vitro* tests are of great interest, due to possible reduction of drug provocation tests. In this review we focus on best investigated *in vitro* techniques for the diagnosis of T cell-mediated drug hypersensitivity reactions. As drug hypersensitivity relies on different pathomechanisms and as a single diagnostic test usually does not cover all possible reactions, it is advisable to combine different tests to increase the overall sensitivity. Recently, proliferation-based assays have been supplemented by a panel of novel *in vitro* tests including analysis of cytotoxic potential of effector cells (granzyme B, granulysin, CD107a), evaluation of cytokine secretion (IL-2, IL-5, IL-13, and IFN- γ) and up-regulation of cell surface activation markers (CD69). We discuss the latest findings and readout systems to identify causative drugs by detecting functional and phenotypic markers of drug-reacting cells, and their ability to enable a more conclusive diagnosis of drug allergy.

Correspondence:

Werner J. Pichler, Department of Rheumatology, Clinical Immunology and Allergology, University Bern, Inselspital, CH-3010 Bern, Switzerland.
E-mail: werner.pichler@insel.ch

Introduction

People are constantly confronted with numerous xenobiotics, including drugs. Although drugs constitute one of important interventions in modern medicine, they also cause unwanted adverse drug reactions (ADRs), very common in medical practice. ADRs are important contributors to hospitalization and mortality throughout the world [1, 2]. They can be classified as type A or type B reactions. The majority of ADRs represent type B reactions: predictable side-effects due to known pharmacological actions of the drug, such as sleepiness after treatment with some antihistamines. Type B reactions are not predictable, not related to the pharmacologic activities of the drug and occur only in susceptible patients. Type B reactions comprise idiosyncratic reactions due to some individual predisposition or hypersensitivity reactions [3]. Drug hypersensitivity reactions (DHRs) account for approximately 15% of ADRs [4] and can be subdivided into drug allergic and non-allergic hypersensitivities. Non-allergic hypersensitivity reactions may imitate an allergy by direct stimulation of effector cells, but without detectable response of the adaptive immune system. Drug allergy is defined as an immunologically mediated reaction that exhibits specificity and recurrence on re-exposure to the offending drug.

DHRs are capable of inducing extremely heterogeneous clinical symptoms of variable severity. However the most

common drug allergic reactions are skin exanthemas. Any drug may elicit DHR, but antibiotics, non-steroidal anti-inflammatory drugs, and antiepileptics are the main causes of DHR. Drug-induced reactions, occurring within the first hour after the last drug administration, are generally IgE-mediated and are classified as immediate allergic reactions. Non-immediate reactions occur > 1 h after the last drug administration and the mechanisms involved seem to be heterogeneous [5]. For better explanation of a variety of clinical symptoms and mechanisms Gell and Coombs classified drug hypersensitivity, as well as other immune reactions, into four categories, I–IV [6]. IgE-mediated type I reactions are manifested clinically by urticaria, angioedema, rhinitis, bronchospasm, and anaphylactic shock. Type II reactions, less common – haemolytic anaemia and thrombocytopenia – are based on Ig-mediated cytotoxic mechanism. Immune-complex-mediated type III reactions occur, for example, in vasculitis and purpura. Type IV reactions, such as allergic contact dermatitis or maculopapular exanthema (MPE), are mediated by T cells. This type has been recently subclassified into type IVa–IVd to correlate the heterogeneous T cell functions to the clinically distinct pictures and effector cell types involved [5]. Type IVa and IVb correspond to T-helper type 1 (Th1) cytokine-driven reactions (high IFN- γ /TNF- α secretion) and Th2 cytokine-driven reactions (high IL-4/IL-5/IL-13 secretion),

respectively. In type IVc the cytotoxic reactions rely on cytotoxic T cells themselves as effector cells. They are dominant in bullous skin reactions like Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), but they seem to be present in all drug-related delayed hypersensitivity reactions. Type IVd represents T cell-induced sterile neutrophilic inflammatory reaction, e.g. acute generalized exanthematous pustulosis (AGEP).

A conclusive diagnosis of drug allergy is expected to confirm that symptoms correspond to DHR and to identify its causative agent. The evaluation of the patient should begin with a precise and detailed medical history. Unfortunately, however, many DHRs are not well documented. Danger signs of drug allergy should be considered, and a minimal laboratory investigation (differential blood count and liver function tests) may be indicated [5]. To identify the relevant drug, *in vivo* tests like skin tests and drug provocation tests may be used. Skin tests, such as patch, prick, and intracutaneous tests are the most readily available form of allergy testing for physicians, but often do not yield positive reactions, even in patients with well-documented histories [7–9]. Provocation tests are considered to be the gold standard [10]. But they are not well accepted by patients, because of the risk of severe reactions and are therefore restricted to certain specialist centres with resuscitative equipment [11]. Moreover, for delayed reactions provocation tests are not standardized and a single dose may exclude an IgE-mediated reaction, but not a delayed reaction, which may appear after a higher dose and longer treatment.

In vitro tests would be a safe procedure for patients, avoiding possible disadvantages of provocation tests: new or recall sensitizations to the drug and risk of severe adverse reactions. Moreover, they provide deeper insight into the pathomechanisms involved in DHR and allow simultaneous assessment of immune responses to multiple drugs. Nevertheless, it is necessary to be aware of the limitations of these tests: they are partly still research tools, standardization is not done for each drug with each test, as exposed controls are missing; They are useful only for certain type of drug allergies (not class II/III reactions) and reflect a sensitization, which is just a risk factor for a symptomatic immune reaction after re-exposure to the drug. However, similar limitations apply to skin tests and provocation tests. In this article we focus on T cell-mediated drug hypersensitivity and discuss the best investigated readout systems for *in vitro* tests identifying the causative drug by detecting functional and phenotype markers of specific drug-reacting cells in the peripheral blood of drug-allergic patients.

The *in vitro* use of drugs is complicated by the need for pure substance and sometimes limited solubility. Pure substance can be obtained from providers of chemicals or directly from pharmaceutical companies. The concentrations 1, 10, and 100 µg/mL of drug are often suitable;

however, lower or higher concentrations can be used for a particular drug. Concentrations inhibiting the phytohaemagglutinin-induced proliferation by > 15% are considered to be toxic and should not be used for the assays. Some drugs are not soluble in aqueous buffer/medium and have to be solved in dimethyl sulphoxide, the others are solved after mild pre-warming or addition of 1 M NaOH, and later the stock solution is further diluted into fitting concentrations.

Pathophysiology and its consequences for *in vitro* testing

Evidence that T cells are involved in the pathogenesis of drug allergy comes from several observations. Drug-specific T cells were shown to infiltrate internal organs such as the kidney and the liver [12, 13], but can also be detected in the skin biopsies of acute lesions and peripheral blood of sensitized patients with different forms of drug hypersensitivities, both benign (MPE, fixed drug eruptions) and severe [AGEP, SJS/TEN, drug rash with eosinophilia and systemic symptoms (DRESS)] [14–18]. Numerous *in vitro* assays [e.g. lymphocyte transformation test (LTT), cytokine release tests] and *in vivo* tests (patch tests, delayed reading of intradermal tests), as well as the generation of drug-specific T cell lines and T cell clones clearly confirm the central role of T cells in pathophysiology of drug hypersensitivity (Table 1).

As *in vitro* tests rely on the presence of drug-reactive T cells in blood of drug-allergic patients, persistence and frequency of these cells has a crucial impact on *in vitro* diagnosis of drug hypersensitivity. Beeler *et al.* [15] demonstrated that 1 : 250–1 : 10 000 of T cells in the peripheral blood of patients in the remission react to the relevant drug. This study also showed that T cells can persist as memory cells in peripheral blood of drug-allergic patients for up to 12 years after disease outcome. On the contrary some patients can lose reactivity 1–3 years after the original treatment with drugs that caused hypersensitivity reaction. At presently it is impossible to predict how long the reactivity of drug-specific T cells in an individual patient will persist.

Also a minimal interval between the acute event and testing is debated. *In vitro* tests are normally done during remission of disease, because peripheral blood mononuclear cells (PBMC) obtained *ex vivo* from acute drug-allergic patients are strongly activated. This could lead to high background proliferation and difficulties in detecting an enhanced proliferation after drug stimulation. The time interval between acute stage and test performance allows washing out the incriminated drugs and any anti-allergic drugs, which may suppress the immune response *in vitro*. Thus, according to common opinion, *in vitro* tests should be performed after a minimal time interval of 3 weeks after the DHR. An analysis in the first 6 months or minimally first year is recommended, but later tests may

Table 1. Comparison of *in vitro* tests to detect delayed drug hypersensitivities

Test (functional/ phenotypic marker of DHR)	Method (readout systems)	Duration (day)	Advantages	Disadvantage	Remarks
LTT (proliferation of drug-specific T cells)	³ H-thymidine incorporation, harvesting and radioactivity measurement (CSFE or non-reactive methods also possible, but less sensitive)	6–7	Many drugs and drug concentrations testable; automation and many replicates possible	Long-lasting; radioactivity and expensive equipment; experience needed; not sensitive in SJS/TEN	Most widely used test; with well-documented sensitivity; best for DRESS, severe MPE
CD69 up-regulation on cell surface	Flow cytometry	2–3	Rapid; identification of reactive cell subset	Flow cytometry is difficult to standardize; automation would need improvement	Results are similar to LTT
Cytokine synthesis and secretion (e.g. IL-2, IL-5, IL-13, IFN- γ)	ELISA, ELISPOT, flow cytometry	3	Rapid; sensitive; informative regarding pathomechanism	Expensive, minimally 2 cytokines needed; specificity lower (dependent on cytokines)	May be most sensitive, but reduced specificity
Cytotoxicity (perforin, granzyme B, granulysin, CD107a)	ELISA, ELISPOT, flow cytometry	3	Rapid; may address additional mechanism	Sensitivity in severe DHRs needs more evaluation	Possibly suitable for severe reactions; CD8 and NK cells focused

DHR, drug hypersensitivity reactions; NK, natural killer; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; DRESS, drug rash with eosinophilia and systemic symptoms; LTT, lymphocyte transformation test; MPE, maculopapular exanthema.

still be positive due to the long-persisting T cells specific for the drug. There may also be differences according to the type of disease: Kano et al. [19] observed that in patients who had SJS or TEN the proliferation tests are more often positive in the acute state than during the recovery phase, while in DRESS the cells react better in remission phase.

In vitro tests aimed at detecting drug specific memory T cells, originally primed *in vivo* in the frame of the development of drug hypersensitivity. Both drugs forming hapten-carrier complexes as well as drugs which directly interact with T cell receptor (TCR) and human leucocyte antigen (HLA)-peptide complexes stimulating T cells (pharmacological interaction with immune receptors, p-i concept) can be detected by *in vitro* tests, as the result is uniformly a T cell activation, secretion of cytokines or cytotoxic mediators, and proliferation. Activation starts within a few minutes after triggering of the TCR by a specific drug antigen presented by major histocompatibility complex (MHC) class I or II, and is followed by increase of intracellular Ca²⁺, as well as the activation of early genes of antigen recognition, including critical transcription factors (nuclear factor of activated T cells, activator protein-1, and nuclear factor- κ B). Within hours a number of genes encoding for various cytokines (IL-2, 3, 4, 5, and 6, IFN- γ , TGF- β) and early activation markers (CD40L, CD69, CD25, CD71) are expressed [20]. Around 1–2 days after T cell activation, IL-2 induces the proliferation of activated T cells, which goes along with additional

gene expression (HLA-DR, CD80, adhesion molecules like VLA) [21–23] and DNA synthesis. Approximately 3–5 days after activation, T cells enter the phase of functional differentiation, which drives to production of distinct cytokine patterns (type 1, type 2, or type 17). This depends on the type of specific T cell, which has been generated during the sensitization phase. These cytokine patterns determine the effector functions of T lymphocytes. Drug-activated T cells undergo a similar differentiation to different effector cells and *in vitro* tests may grasp distinct parameters of this differentiation, ranging from surface marker up-regulation to cytokine production, proliferation, and cytotoxicity [24–26].

Cytotoxicity-based assays detect sensitization in different forms of drug hypersensitivity reactions

Cytotoxic lymphocytes [T cells and natural killer (NK) cells] monitor all cells of the body, ready to destroy any cell posing a threat to the organism. Three major pathways are used by cytotoxic lymphocytes to mediate the killing of target cells. Cell-cell contact-dependent pathways involve granule-mediated (perforin/granzymes and granulysin) (Fig. 1a) or death receptor-mediated (Fas-FasL and TRAIL-DR4/5) (Fig. 1b) killing of the target cells. The third one is based on INF- γ and TNF- α which act on the target cells distal to the effector cell (Fig. 1c).

Cytotoxic mechanisms were shown to be present in nearly all drug-induced delayed hypersensitivity

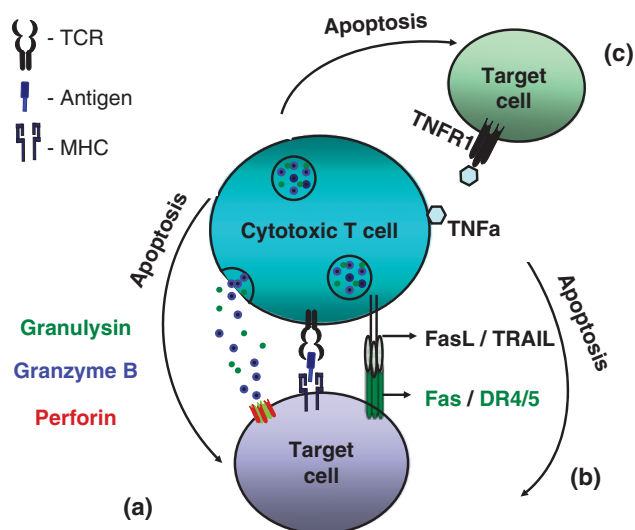


Fig. 1. Pathways of cell-mediated cytotoxicity. (a) In the granule-dependent pathway, cytotoxic molecules (perforin, granzymes, and granulysin) are realised from cytotoxic granules and induce apoptosis of the target cell. (b) In death receptor-mediated killing, FasL or TRAIL expressed on the cytotoxic cells bind Fas or DR4/5 respectively and induce target cell death. (c) TNF- α (TNF α), released from cytotoxic cells triggers death receptor (TNFR1) on the target cells. TCR, T cell receptor; MHC, major histocompatibility complex.

reactions. Histological, immunohistological and *in vitro* analyses of effector cells from acute skin lesions indicated an important role of cytotoxic mechanisms in various forms of drug-mediated hypersensitivity including maculopapular, bullous, and pustular reactions [27–31]. Studies of patients with drug-induced interstitial nephritis [12] and *ex vivo* analysis of liver cells from patient with severe DRESS syndrome [13], also indicate an important role for cytotoxic T cells in systemic drug-induced hypersensitivity reactions, even if they are clinically quite distinct. Moreover, elution of skin-infiltrating T cells and characterization of drug-specific cytotoxic T cell lines and clones from peripheral blood of drug-allergic patients confirmed that drug-dependent killing occurs [32, 33]. Perforin/granzymeB and/or the Fas/FasL-mediated drug-specific killing pathways were shown in T cell lines and clones derived from patients with maculopapular, bullous, and pustular drug eruptions. Some of these T cells had NK-like features as they expressed CD56 and CD94 and had the ability to kill the NK-sensitive cell line K562 [32]. At present, the role of NK cells or NK-T cells in drug hypersensitivity is extensively investigated. Recently, NK cell-dendritic cell interaction in hypersensitivity reactions to β -lactams was demonstrated, showing that both the innate and adaptive immune systems are involved in these drug reactions [34].

Involvement of cytotoxic lymphocytes seems to differ in different skin diseases. Hari and colleagues in a carefully conducted study evaluated the liver parameters, T

cells activation in the circulation and biopsy of acute skin lesions of patients with different forms of DHRs. She could show that a predominant activation of CD8⁺ cells lead to more severe skin symptoms or liver involvement, while predominant activation of CD4⁺ cells elicits mainly mild maculopapular reactions [18]. However, even in maculopapular drug eruptions, perforin- and granzyme B-positive, CD4⁺ and CD8⁺ T cells were found at the dermo-epidermal junction zone or in the epidermis, along with signs of keratinocyte cells damage [28]. In more severe clinical manifestations of DHRs, predominant activation of cytotoxic CD8⁺ T cells, NK cells, and NK-like T cells lead to a massive, widespread apoptosis of epidermal cells mediated by perforin/granzymeB [29] and/or FasL/Fas [35]. The arguments for a predominant role of CD8 cytotoxic T cells in most severe forms of drug hypersensitivity were also strengthened by findings concerning a striking association between particular HLA class I allotypes and certain severe DHRs [36–38]. These findings suggest that in most severe forms of drug hypersensitivity, CD8 cytotoxic cells and possibly also NK cells can be activated by MHC I-drug complexes causing massive tissue destruction. Moreover, Posadas et al. [26] showed strong associations between diseases severity and high expressions of cytotoxic markers (TNF- α , Granzyme B, perforin, and FasL), measured by quantitative-competitive PCR in mononuclear cells from drug-allergic patients in acute stage. Recently, granulysin, a cytotoxic protein secreted by T cells and NK cells, has also been implied to be involved in severe, life-threatening, bullous skin lesions of SJS/TEN [27].

Although cytotoxic mechanisms appear to be present in different forms of drug-induced hypersensitivity reactions, availability of simple *in vitro* test to measure drug-induced cytotoxicity is still limited. An assay, which measures effector function of drug-induced cytotoxic cells in peripheral blood of drug-allergic patients, may supplement or represent an alternative for proliferation-based tests [32]. It could also be helpful in the diagnosis of patients with severe forms of drug allergy, where cytotoxic cells play a particularly important role and proliferation-based assays have low sensitivity. Regrettably, a standard chromium release assay [39] and more recently used methods that monitor the release of fluorescent dyes from target cells require a radioactive labelling of target cells and are rather cumbersome and semiquantitative [40, 41]. MHC multimers did not meet the practical application in identification of drug-specific cytotoxic T cells. INF- γ ELISPOT as well as intracellular cytokine staining are commonly used but none of these methods directly examines the cytotoxic effector function of T cells [41, 42].

Lately, our group [43] adapted a very sensitive ELISPOT assay based on granzyme B release [44, 45] and a flow-cytometry-based CD107a measurement [46] to detect and characterize drug-reactive cytotoxic cells in peripheral

blood of drug-allergic patients in remission. In resting cells with cytotoxic potential, lytic molecules (granzymes, perforin, granzysin) are stored in cytotoxic granules. During degranulation of activated cells, granules reach plasma membrane, release cytotoxic molecules and expose granular membranes proteins, like CD107a (LAMP-1, lined with membrane glycoprotein-1) on the cell surface. Released molecules and the expression of CD107a can be measured, providing a positive marker for degranulation of responding cytotoxic cells. Measurement of granzyme B release by an ELISPOT is highly sensitive as plate-bound antibodies directly capture the product secreted around the cell before it is diluted in the supernatant and absorbed by high affinity receptors or degraded by proteases [44]. Therefore this test detects molecules secreted by individual cells present in low frequencies and seems to be the most robust method to detect cytotoxic cells in peripheral blood of drug allergic patients. Both Granzyme B ELISPOT as well as the degranulation marker CD107a have been already used successfully to detect peptide-specific cytotoxic T lymphocytes in patients monitored in vaccine trials and in patients with chronic virus infections [45, 47–48]. Some preliminary data showed that serum granzysin levels in patients with SJS/TEN were elevated before skin detachment or mucosal lesions develop [49]. Therefore, it was suggested that monitoring of serum granzysin enables early diagnosis in SJS/TEN patients. Furthermore we observed that stimulation with culprit drug significantly increased granzysin production in NK cells from patients, both with MPE and SJS (Fig. 2., unpublished data). Cytotoxicity-based assays might be promising tools in drug hypersensitivity diagnosis and in supplementing LTT, because they measure effector cell function distinct from proliferation and do not involve radioactivity.

Lymphocyte transformation test – prevalent assay in patients allergic to drugs

The LTT is currently the most widely used test for diagnosis of T cell-mediated drug hypersensitivity. The LTT relies on the activation and proliferation of T cells after stimulation with the specific drug under *in vitro* conditions [50]. This concept of the LTT has been confirmed by the generation of drug-specific T cell clones [51]. The test is applied in patients with delayed-type DHRs like maculopapular, pustular, or bullous exanthemas [52, 53], DRESS [54], but also may be useful in diagnosis of patient with immediate reactions [14]. Occasionally, positive results of the LTT has been reported in rare forms of drug hypersensitivity: in drug-induced pancreatitis [55] and hepatitis [56, 57], drug fever [58] followed by vasculitis, interstitial and eosinophilic lung diseases [59].

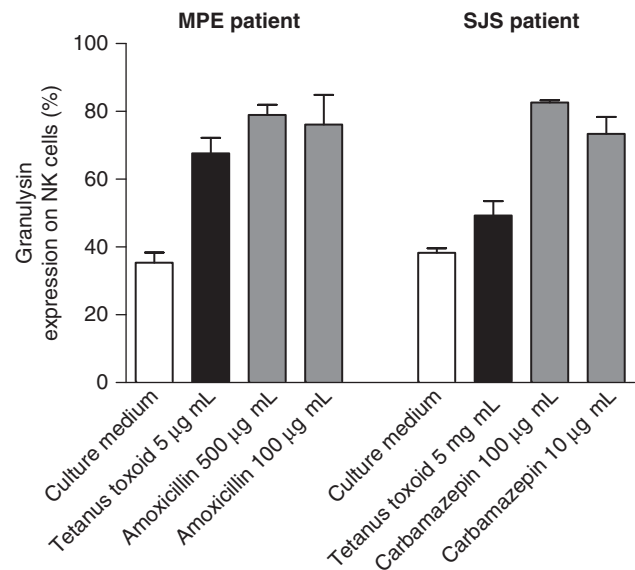


Fig. 2. Granzysin expression in NK cells after 5 days of stimulation with culprit drug, culture medium alone (negative control) or tetanus toxoid (positive control). Intracellular staining of PBMC from patients allergic to amoxicillin and carbamazepine. NK, natural killer; MPE, maculopapular exanthema; SJS, Stevens-Johnson syndrome; PBMC, peripheral blood mononuclear cells.

In the LTT, PBMC are obtained from a drug-sensitized patient and cultured in the presence of the suspected drug. Drug-specific T lymphocytes undergo blastogenesis and generate cytokines such as IL-2, followed by a proliferative response that is measured by the incorporation of ^3H -thymidine during DNA synthesis after 6 days of culture. It is possible to use a pure form of drug for the LTT. In case the pure substance is not available, an injectable form of the drug or the contents of a capsule are acceptable. We usually use following drug concentrations: 1, 10, 100 µg/mL, but occasionally lower or higher concentrations can be used as well. The appropriate concentration and solubility of the drug need to be defined previously in toxicity tests, described elsewhere [51]. Although ^3H -thymidine uptake is measured in counts per minute (c.p.m.), results of the LTT are given as stimulation index (SI), which is the ratio of cell proliferation (c.p.m.) with antigen divided by the background proliferation, without drug (c.p.m.). SI values are more comparable than c.p.m. results, because the spontaneous proliferation (expressed as c.p.m. values) obtained with different donors can differ significantly. Based on negative values in exposed but not allergic individuals, a SI > 2 and for β -lactams > 3 is interpreted as positive. The LTT to radio-contrast media should reach SI > 4 to be judged as positive. It should be taken into consideration that treatment with corticosteroids or other immunosuppressive drugs may influence the test results by suppressing the proliferation *in vitro*. On the other hand, some drugs (vancomycin, possibly paracetamol, as

well as certain radio-contrast media and non-steroidal anti-inflammatory drugs) may elicit slightly enhanced proliferation even in non-sensitized individuals [51]. SI value is not associated with the severity of clinical symptoms. The LTT in patients with MPE might reveal strong T cell proliferation and higher SI values than the LTT performed in patients with severe forms of drug hypersensitivity, such as SJS or TEN. In fact, the clinical severity of a drug hypersensitive reaction seems to be related rather to the effector function of reacting cells, than to the high frequency of these cells in the peripheral blood detected by the LTT.

Several studies performed to date indicated that the general sensitivity of the LTT in well-defined DH may lie between 60% and 70%. It depends on the drug, the type of reaction and is superior to skin testing for non-immediate type reactions [14, 18, 50, 60]. In the prominent retrospective study on the LTT accuracy, 923 patients were classified according to the imputability of DHRs [50]. In patients classified as definitely drug allergic the LTT yield a sensitivity of 78%, while patients with lower clinical likelihood of drug hypersensitivity had a respectively lower incidence of a positive LTT. Warrington and Tse [61] found the sensitivity of 51% in group of patients with clinically established allergies to several drugs. A sensitivity of 62% in a group of patients with well-documented delayed or immediate β -lactam hypersensitivity was reported by Luque et al. [14]. Lower sensitivities rates of 33% were observed by Barna et al. [62] and 38% by Berg et al. [63]. The specificity of the LTT was in the range of 85–100% in different studies [14, 16, 18, 64]. The overall specificity of the LTT is at least 85% [50], but probably higher for drugs such as lamotrigine, carbamazepine, and β -lactams. Recent analyses of the LTT to antiepileptic drugs eliciting DRESS found the specificity of 100% for carbamazepine and lamotrigine hypersensitivity [16, 65]. The specificity (93%) was recently also demonstrated for β -lactam hypersensitivity [14]. On the one hand, the LTT has some limitations: it requires experience with cellular techniques; involves radioactivity, and thus certain expensive equipment, it is rather cumbersome as it relies on a 6-day sterile culture, and, most importantly, negative results cannot exclude drug hypersensitivity. On the other hand the test is applicable with many different drugs in different DHRs and positive LTT may not only support the diagnosis of drug allergy, but can also pinpoint the responsible agent, in case patient has taken several drugs. The results of challenge tests are rarely available for *in vitro* test being under evaluation. Actually, challenge tests are contraindicated in some severe reactions for ethical reasons [11]. Therefore, diagnosis based on clinical characteristics of the patients [16, 50, 62, 63, 65] and/or skin tests [18, 61, 64], as well as collection of patients with accidental or intended re-exposure [14] are often used as the 'gold standard'.

Measurement of drug-induced cytokine production

Because their role in the effector phase of the immune response, cytokines were considered as a promising *in vitro* readout system in diagnosis of drug hypersensitivity [66]. Their early production after T cell activation would also shorten the incubation time to 48–72 h. The measurement of cytokine production can be performed in the cell culture supernatant by enzyme-linked immunoassays, such as the ELISA or the enzyme-linked immunospot technique; intracellularly by flow cytometry with fluorescent-conjugated monoclonal antibodies; as well as by molecular techniques like PCR or competitive reverse transcription PCR detecting the gene expression level of secreted cytokines.

High production of Th1-type cytokine pattern (IL-2, IFN- γ , and TNF- α) in PBMC has been reported in patients with delayed-type reactions, whereas Th2-type with preferential expression of IL-4 in patients with immediate reactions [67, 68]. Other studies have revealed a mixed Th1/Th2 cytokine pattern [69, 70]. Also studies with T cell clones from drug-allergic patients show a heterogeneous pattern of cytokines which is best described as a Th0 pattern [54, 71, 72]. This implies that it is rather difficult to correlate a certain cytokine pattern with clinical phenotype.

As IFN- γ is considered to be a predominant cytokine in drug-induced exanthemas, it might be a suitable *in vitro* diagnostic marker. Elevated IFN- γ production by drug-stimulated PBMCs has been observed in several studies, which included patients with clinical pictures as diverse as MPE, DRESS, and AGEP to carbamazepine, lamotrigine, celecoxib, and other drugs [65, 73–76]. IFN- γ secretion, as a tool to identify the responsible drug, in different studies yielded a sensitivity of 71.4–77.8% [69, 73, 75]. The measurement of IL-5, another dominant cytokine in various DHRs, has also been proposed as a useful *in vitro* method for detecting drug sensitization. It has been demonstrated that IL-5 is released *in vivo* and *ex vivo* in patients who have drug-induced MPE and DRESS [28, 77]. Also secretion of IL-5 and eotaxin are common characteristics of drug-specific T cell clones [65]. Furthermore, the combination of IL-5 measurement and the LTT was shown to be a better readout for detecting a sensitization to a drug than the LTT alone [78–80]. Recently, combining ELISA and flow cytometry were demonstrated to increase sensitivity of IL-5 and IFN- γ measurements for *in vitro* detection of drug hypersensitivities [81]. In the study by Khalil et al. [76] drug-specific secretion of IFN- γ and IL-2 from PBMC cultures could reach the sensitivity of 87% and the specificity of 100% in MPE patients allergic to penicillins. In particular clinical manifestation of DHRs like AGEP, analysis of IL-8 (CXCL8) can be useful, as this cytokine is involved in pathomechanism of the reaction [82].

Lochmatter et al. [70] presented a systematic comparison of different cytokines produced *in vitro* after drug

stimulation, and aimed to identify the most suitable cytokine or combination of cytokines for diagnosis of drug hypersensitivity. She screened the supernatants from drug-allergic patients and non-allergic controls PBMC cultures for 17 cytokines and chemokines. After a 3-day drug stimulation, the level of four cytokines, namely IL-2, IL-5, IL-13, and IFN- γ in group of patients were significantly increased compared with controls. The measurement of IL-5 as a very specific marker in combination with either IFN- γ , IL-13, or IL-2 as a more sensitive markers has been suggested as useful tool for the *in vitro* detection of T cell sensitization to drugs.

According to results discussed above, the measurement of IL-2, IL-5, IL-13, and IFN- γ cytokines is considered as promising diagnostic tool in most DHRs. There is a need to perform studies with a larger number of drug-allergic patients and controls to provide data on the sensitivity and the specificity of these assays.

CD69 up-regulation in T cell-mediated drug hypersensitivity

After stimulation T cells express a number of surface molecules like CD25, CD69, and later CD71 [18, 54]. Such activation antigens offer the advantage of identifying specific, drug-activated lymphocytes, because they can be easily detected by flow cytometric analysis. Compared with other T cell activation markers, CD69 seems the most suitable, as it is up-regulated quickly and shows the biggest difference to baseline values [83]. This surface molecule has been already reported as a valuable marker of T cell activation after stimulation of whole blood with pokeweed mitogen, CD2/CD2R monoclonal antibodies, superantigen staphylococcal enterotoxin B, or the specific antigen *Candida albicans* 64. CD69 was also up-regulated in T cells from patients with drug-induced MPE, erythema multiforme, SJS, and DRESS [84, 85].

In the study by Beeler et al. [83] the usefulness of CD69 up-regulation in diagnosis of T cell-mediated drug allergic reactions was evaluated. The authors demonstrated substantial CD69 up-regulation on CD⁸⁺ and CD⁴⁺ in all tested patients, after 48 h of stimulation with the culprit drug. These findings correlated to the LTT result, which was positive in all patients. CD69 up-regulation was drug-specific, as it occurred neither in non-stimulated cultures nor in drug-stimulated PBMC from control subjects. The same study also demonstrated that the frequency of CD⁶⁹⁺ T cells after stimulation with drug was much higher than the real frequency of drug-specific T cells evaluated by CFSE staining or ELISPOT analysis. Further analysis revealed that drugs stimulated a few truly drug-specific T cells, which secreted cytokines (in particular IL-2). These cytokines acted on 'by-stander' T cells, which reacted with CD69 up-regulation. As this amplification only occurred if truly reactive T cells were present, it had no negative effect on the

specificity, but enhanced the sensitivity of this flow-cytometry-based test.

Therefore, CD69 measurement offers a promising alternative to the LTT, with the advantage that it does not require incorporation of radioactive substances and is less time-consuming, but it is also clear that further systematic research is required to better define the drugs and diseases where CD69 up-regulation is best suited for.

Concluding remarks

In current approach to drug hypersensitivity diagnosis detailed clinical history and skin tests are supported by a few useful *in vitro* tests. Because drug hypersensitivity relies on different and complex pathomechanisms, a single diagnostic test most likely does not cover all possible reactions. Thus, it is advisable to combine different tests to increase the overall sensitivity. With regard to *in vitro* tests, proliferation-based assays have been supplemented by novel *in vitro* assays including evaluation of different cytokine secretion, up-regulation of cell surface activation markers (e.g. CD69), and analysis of cytotoxic potential of effector cells in immune response (granzyme B, granulysin, CD107). These tests seem promising, but still need further evaluation.

At present, the tests described are limited to specialised laboratories involved in research, and are thus not available in routine medical practice. An assay that may be developed into a widely used diagnostic test has to be reproducible in different laboratories and relatively easy to perform, but also sufficiently validated in reference to the best existing diagnostic standards (e.g. skin tests and/or *in vivo* challenge tests) [7, 11]. At present the LTT is the best documented assay. Thanks to its high specificity, positive result of the LTT with a culprit drug performed at an early stage of diagnostic process allows the avoidance of *in vivo* challenges, including both skin test and drug provocative tests. The assays based on ELISPOT technique were shown to be comparable to the LTT [42, 43], but they are not so demanding in respect of equipment and avoid radioactivity and can thus be promising perspectives for the future. In some reactions, a combination of tests might be required to detect a sensitization.

Recent studies have shown the role of dendritic cells in the enhancement of drug-specific lymphocyte proliferation in patients [86, 87] and have suggested that depletion of regulatory T cells can improve the sensitivity of assays to contact allergens or if done at the right time-point after the reaction occurred [88, 89]. However, these methods need refinement and further evaluation.

Important advantages of *in vitro* tests are a reduction for drug provocation challenges, the simultaneous assessment of response to many drugs, no risk for re-sensitization due to reapplication and no severe reactions.

They are also important to have better insight into the relevant pathomechanism. This latter aspect is crucial for further progress in methods of drug allergy diagnosis.

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