

In Vitro Tests in Drug Hypersensitivity Diagnosis

Priska Lochmatter, MSc, Anna Zawodniak, MD, Werner J. Pichler, MD*

KEYWORDS

- Drug hypersensitivity • In vitro tests
- Lymphocyte transformation test • CD69 upregulation
- Cytokines • Cytotoxicity

Drug hypersensitivity reactions (DHRs) account for approximately 15% of adverse drug reactions.¹ The clinical symptoms of DHRs are various and of variable severity. The exact pathophysiology of DHRs is mostly unknown; nevertheless, DHRs can be classified in a scheme proposed by Gell and Coombs,² which correlates the clinical phenotype to the immunologic mechanism involved. In this scheme, drug-induced IgE-mediated type I reactions (urticaria, bronchoconstriction, angioedema, and anaphylaxis), IgG-mediated type II reactions (hemolytic anemia and thrombocytopenia), immune-complex-mediated type III reactions (vasculitis and purpura) and T-cell-mediated type IV reactions (allergic contact dermatitis; fixed drug eruptions; and maculopapular exanthema [MPE], bullous exanthema, or pustular exanthema) have been differentiated.

The delayed-appearing T-cell-mediated (type IV) reactions have recently been further subclassified to correlate the heterogeneous T-cell functions to the clinically distinct pictures.³ Hence, type IV reactions can be subclassified into type IVa, IVb, IVc, and IVd according to the effector phenotype of the involved T cells. Type IVa and IVb correspond to T helper (Th) 1 and Th2 cytokine-driven reactions, respectively. Type IVc refers to cytotoxic reactions, whereas type IVd represents T-cell-induced neutrophilic inflammations, whereby interleukin (IL)-8 and possibly IL-17 are involved.

A diagnosis of delayed-type drug hypersensitivity has two aims: first, to confirm that the symptoms correspond to a DHR and, second, to identify the causative drug. It is still an area of much uncertainty, but recent progress in the understanding of these reactions opens new possibilities.

In vivo tests include skin tests and drug provocation tests. Patch, prick, and intracutaneous tests have an overall low sensitivity, even in patients with a well-documented history of DHR.^{4–8} Drug provocation tests may help to rule out immediate

Division of Allergology, Clinic of Rheumatology and Clinical Immunology/Allergology, Inselspital, Bern University Hospital and University of Bern, CH-3010 Bern, Switzerland

* Corresponding author.

E-mail address: werner.pichler@insel.ch (W.J. Pichler).

Immunol Allergy Clin N Am 29 (2009) 537–554

doi:10.1016/j.iac.2009.04.009

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reactions but are not well standardized in delayed reactions regarding dose, duration of symptoms, and definition of positivity, and are therefore difficult to perform. In some cases, an entire treatment period would be necessary because some reactions clearly depend on dose and may start after 4 to 5 days of treatment only. Moreover, they do not reveal the pathomechanism and may still bear the risk for severe reactions. Thus, they are not well accepted by physicians and patients.

In vitro tests would be safe for patients and can provide deeper insight into the pathomechanisms involved in a certain type of DHR. The nonphysiologic environment is one of the main disadvantages of in vitro tests, however. The clinical symptoms are influenced by cofactors that are not present in vitro, such as certain hepatic enzymes necessary for metabolizing inert drugs into immunogenic compounds or infections that were enhancing immune reactions at the time of the DHR. In addition, most in vitro tests are not yet standardized and still represent mainly a research tool.

In spite of these disadvantages, in vitro tests are of great interest because they guarantee absolute safety for patients and allow simultaneous assessment of T-cell responses to multiple drugs. At present, even a test with relatively low sensitivity but still good specificity would be considered helpful in many clinical situations. In this article, the best investigated in vitro tests are discussed, which aim to identify the causative drug by detecting drug-reacting cells in the peripheral blood of patients with delayed-type drug hypersensitivities (Fig. 1). Nevertheless, one has to be aware that such tests, like other immunologic tests, can only reflect a sensitization, which is a relevant risk factor for a DHR, but can never prove that a complete immune reaction with inflammation would take place on re-exposure.

PATHOMECHANISMS OF T-CELL-MEDIATED DRUG HYPERSENSITIVITY

T cells have been shown to play a central role in mediating DHRs and are practically involved in all types of immune reactions to drugs. On antigen-specific stimulation, T cells secrete various cytokines and can orchestrate different effector mechanisms of immune response. Immediate IgE-mediated reactions are stimulated by secretion of IL-4 and IL-13; monocyte- or macrophage-rich inflammations are stimulated by secretion of interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α); eosinophilic inflammation is stimulated by secretion of IL-5; and neutrophilic inflammation is stimulated by secretion of IL-8, granulocyte-macrophage colony-stimulating factor, and possibly IL-17.^{3,9,10} Moreover, drug-induced cytotoxic effector T cells were shown to infiltrate and kill tissue cells in the skin and liver of patients with severe forms of drug hypersensitivity.^{11–13} The number of in vitro (eg, lymphocyte transformation test [LTT], ELISA,

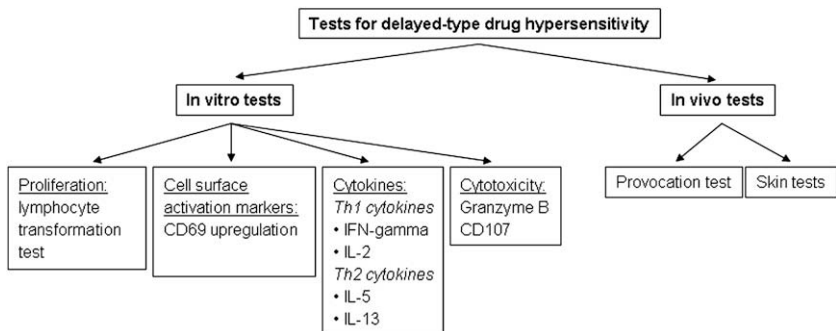


Fig. 1. Tests for the detection of drug-specific T cells in patients with delayed-type drug hypersensitivity.

enzyme-linked immunospot [ELISPOT] assay, CD69 measurement) and in vivo (delayed reading of intradermal and patch tests) tests and, in particular, the generation of drug-specific T-cell lines (TCLs) and T-cell clones (TCCs) from tissue lesions and peripheral blood of patients with different forms of drug hypersensitivity have confirmed the central role of T cells in the complex pathomechanism of drug hypersensitivity.

Two main models have been proposed to explain how T cells recognize small chemical substances, namely, the hapten or prohaptent model and the p-i concept based on pharmacologic interaction of drugs with immune receptors. According to the hapten or prohaptent model, chemically reactive drugs or their metabolites may covalently bind to certain proteins and initiate immune responses by transmitting a danger signal to the innate immune system. The modified haptenated proteins would be then processed and presented on major histocompatibility complex (MHC) molecules to hapten- or drug-specific T cells activating the specific immune system.¹⁴⁻¹⁸

In contrast, the p-i concept postulates that inert drugs unable to act as haptens may bind directly and reversibly to immune receptors, particularly to the T-cell receptor (TCR) and possibly also to the MHC. This noncovalent drug interaction with the MHC-TCR complex leads to the activation and proliferation of drug-specific T cells, as illustrated by analysis of drug-specific TCCs to various drugs (lidocaine, mepivacaine, sulfamethoxazole, lamotrigine, carbamazepine, p-phenylenediamine, and ciprofloxacin).¹⁹⁻²⁵ This concept is similar to stimulation of cells by pharmacologic activation by means of other receptors, and is hence called pharmacologic interaction with immune receptors (p-i concept).^{26,27}

Some of these drug-activated T cells can persist as memory cells in peripheral blood of drug-allergic patients, even several years after disease outcome. A recent study of Beeler and colleagues²⁸ showed that the frequency of drug-reactive T cells in peripheral blood of some allergic patients in remission is in the range of 1:250 to 1:10,000. It is thus even higher than the frequency of T cells able to react with tetanus toxoid in the same tetanus-immunized subject. It is not known how this relatively large pool of drug-specific memory T cells is maintained in spite of strict avoidance of the drug. It is possibly related to some cross-reactivity of the drug-specific T cells with other peptide antigens or even self-peptides. Indeed, approximately 30% of drug-reactive clones showed cross-reactivity with allogeneic MHC-peptide complexes.²⁹

This long-lasting persistence of drug-reactive memory T cells in peripheral blood of drug-allergic patients has a crucial impact on in vitro diagnosis of drug hypersensitivity because all available in vitro tests rely on measurement of specific effector function of drug-reactivated memory T cells. Thus, such a test is only informative if it measures the same effector mechanisms as involved in an in vivo reaction. For instance, the result of a test evaluating IgE to a drug is negative in MPE because this disease does not involve IgE formation to the drug; similarly, the result of a test for proliferation might be negative if the delayed immune reaction involved mainly cytotoxicity. These features have to be considered in interpreting test results and demonstrate why progress in diagnosis of drug allergy relies primarily on a better understanding of immunologic pathomechanisms involved.

KINETICS OF T-CELL ACTIVATION

The full activation of a T cell is assumed to require multiple sequential engagements of the TCR by the antigen presented on the MHC on antigen-presenting cells. This leads to the recruitment of costimulatory, adhesion, and signaling molecules to the antigen-TCR contact site and results in activation of the T cell. After being triggered by

a specific antigen, T cells enter a maturation process of several days, which can be divided into three phases: commitment, proliferation, and functional differentiation (Fig. 2).

Commitment follows rapidly after the triggering of a TCR by a specific antigen presented on MHC I or II; within a few minutes, the Ras-mitogen-activated protein kinase signaling pathway and the calcium- and protein kinase C-mediated signaling pathway get activated. This leads to the activation of three critical transcription factors (nuclear factor of activated T cells, activator protein-1, and nuclear factor- κ B) within half an hour after antigen recognition, which enhance the transcription of genes encoding for various cytokines, such as IL-2, IL-4, IFN γ , TNF α , and the early activation markers CD40L and CD69 within 1 to 2 hours. After 1 to 2 days, CD25 (IL-2 receptor),³⁰ CD71, human leukocyte antigen-D-related (HLA-DR), and various adhesion molecules are expressed. IL-2 has an autocrine effect on the T cells and induces the proliferation of the activated T cells, which goes along with DNA synthesis (see Fig. 2).

Approximately 3 to 5 days after activation, some T cells undergo functional differentiation. The CD4⁺ T cells differentiate into two major subsets of effector cells, Th1 and Th2 cells, which secrete distinct cytokine patterns. These cytokine patterns determine the effector functions of activated cells. They do not only mediate the expansion of one subset but inhibit the expansion of the reciprocal subset. Therefore, once an immune response develops in one direction, it gets progressively polarized. Th1 cells are characterized by the secretion of proinflammatory cytokine IFN γ , which stimulates the microbicidal activity of phagocytes, and the production of complement-fixing and opsonizing IgG antibodies, which promote phagocytosis. In contrast, Th2 cells typically secrete IL-4, IL-5, IL-13, and IL-10, which inhibit macrophage activation but activate eosinophils and induce antibody isotype switch to mast cell-activating IgE. In 2005, Th17 cells were proposed as a third distinct Th cell subset. This subset is characterized by the secretion of IL-17 and most probably functions to eliminate specific pathogens that are not efficiently cleared by the Th1- or Th2-mediated immune response.^{31–33}

Naive T cells can also differentiate into regulatory T cells in peripheral lymphoid organs. These so-called “adaptive T-regulatory cells” produce high levels of IL-10 and transforming growth factor- β , which have an immunosuppressive function.³⁴

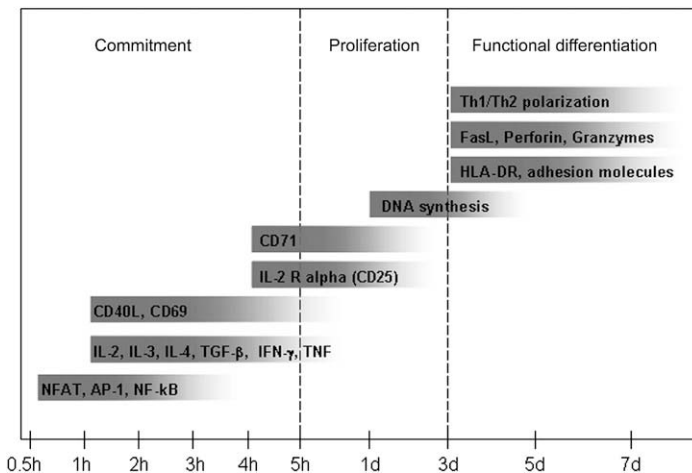


Fig. 2. Gene expression profile of antigen-activated T cells.

Antigen-activated T lymphocytes can differentiate into cytotoxic effector cells that recognize and kill target cells presenting the antigen on MHC molecules. These cells are characterized by the production of cytotoxic proteins like perforin and granzymes and proinflammatory cytokines, namely, $\text{IFN}\gamma$ and $\text{TNF}\alpha$. They can also express Fas ligand, which binds to Fas molecules on target cells, inducing apoptosis.

The measurement of these events in T-cell activation could theoretically serve to detect drug-reacting T cells *in vitro*. Because of technical limitations and the nonphysiologic *in vitro* environment, however, not all these events are suited for diagnosis of T-cell-mediated drug allergy. In the following sections of this article, *in vitro* tests that aim to detect various events in the T-cell activation induced by drugs are discussed.

LYMPHOCYTE TRANSFORMATION TEST

The LTT is the most widely used test to detect T-cell sensitization to drugs in the diagnosis of drug allergy. The principle of the LTT follows the rules of a simple proliferation test with a protein antigen. It measures proliferation and expansion of T cells after stimulation with the specific antigen (drug), which is the common effector function of different types of T-cell immune reactions (eg, Th1, Th2, Th0, Th17). Generation of drug-specific TCLs and TCCs from LTT-positive patients and the finding that peripheral blood cells from healthy drug-exposed donors do not normally show enhanced proliferation to the drug prove that the T-cell proliferation detected in the LTT is a drug-specific response found only in symptomatic patients.^{35–38} This test has been shown to be useful in patients with delayed T-cell-mediated forms of drug hypersensitivity like MPE, pustular exanthema, and bullous exanthema;^{10,23} drug rash with eosinophilia and systemic symptoms (DRESS);²² and immediate IgE-mediated reactions, including severe anaphylaxis.³⁶ Rarely, the LTT has been evaluated in other forms of drug hypersensitivity: positive reactions were reported in isolated drug fever³⁹ followed by vasculitis, in drug-induced pancreatitis (found positive with pyritinol⁴⁰), for interstitial and eosinophilic lung diseases,⁴¹ or even in some drug-induced autoimmune diseases.⁴² Often, these are case reports, and there is not enough information regarding sensitivity and specificity. In drug-induced hepatitis, the result of the LTT can occasionally be positive^{43,44} and has then been found useful in discriminating immune hepatitis from toxic reactions caused by antituberculous drugs.⁴⁵ Recently, it has also been shown useful in determination of cross-reactivity patterns in T-cell-mediated allergic reactions to iodine contrast media.⁴⁶

In the LTT, peripheral blood mononuclear cells (PBMCs) obtained from drug-sensitized patients and separated over a density gradient are cultured in the presence of suspected drug for 6 days. The drug should be available as a pure substance. If the pure substance form of the drug is not available, one can use the contents of a capsule or an injectable form of the drug. Drugs tested as tablets are mostly unsuited for an LTT. All drugs must be tested in a group of controls, and toxic concentrations have to be defined by toxicity tests (for details, see the article by Pichler and Tilch⁴⁷).

Memory T lymphocytes persist in the circulation of patients with DHRs. After antigen encounter, these memory T cells undergo blastogenesis and generate cytokines, such as IL-2. The proliferative response can be measured by incorporation of ^3H -thymidine during DNA synthesis and given as the count per minute. The result is usually expressed as the stimulation index (SI), which is the ratio of cell proliferation with antigen divided by the background proliferation (without antigen). Because the spontaneous proliferation obtained with various donors differs, SI values are more comparable and easier to communicate than count-per-minute results. An SI greater than 2m

and greater than 3 for β -lactams, is considered to be a cutoff value for a positive response. An SI between 2 and 3 is considered to be weakly positive. The relevance of such a low proliferation is hard to judge without additional clinical information or other tests. Drug-specific TCCs could be also generated from cell cultures with only a moderate SI of 2.1, which suggests that this weak response in the LTT could already indicate sensitization.¹⁰ Drug-exposed but not allergic individuals do not mount a proliferative reaction to a drug.

High SI values in the LTT are not necessarily associated with the severity of clinical symptoms. Analysis of rather harmless MPE might reveal strong T-cell proliferation, whereas patients with severe forms of drug hypersensitivity, such as Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN), show only a marginally positive or even negative response.⁴⁸ This is attributable to the fact that the LTT reflects only the reactivation and proliferation of memory cells that are present in the peripheral blood of allergic patients, and the high precursor frequency of these cells is not necessarily associated with more severe clinical symptoms. In fact, the clinical severity of a DHR seems to be more closely related to the effector function of the reactive T cells. Thus, in some forms of drug hypersensitivity, cytotoxicity or a cytokine-based in vitro assay could be more useful than proliferation-based assays like the LTT.

The clinical sensitivity of a diagnostic test indicates to what extent it can detect clinically relevant sensitization. In several studies performed to date, the LTT had a general sensitivity of 60% to 70% and was often superior to skin testing for nonimmediate-type reactions.^{35,36,49–52} A lower sensitivity rate of 33% was reported by Barna and colleagues,⁵³ and a rate of 38% was reported by Berg and colleagues.⁵⁴ A prominent retrospective analysis that included 923 patients with various adverse drug reactions indicated that the sensitivity of the LTT depends on the drug tested.⁴⁹ All patients in this study were classified based on their medical history into three groups: patients with definite, highly probable, or not probable immune-mediated drug reactions. Seventy-eight of 100 patients classified as definitely drug allergic showed positive reactions in the LTT; thus, the LTT sensitivity was 78%. When only allergies to β -lactam antibiotics were analyzed, the LTT sensitivity was approximately 74%, and thus higher than that of skin tests (62%). Patients from the high-probability group and low-probability group were positive in the LTT at rates of 47% and 33%, respectively. Fifteen of 102 presumably non-drug-allergic patients had positive LTT results; thus, the authors could define the specificity of the LTT as being no higher than 85%. This relatively low specificity was attributed to the fact that all positive LTT results to nonsteroid anti-inflammatory drugs (NSAIDs) were calculated as false-positive. NSAIDs are known to cause mainly pseudoallergic (non-immune-mediated) reactions and may also slightly enhance proliferation in in vitro tests, which is explained by their ability to inhibit prostaglandin E2 synthesis.⁵⁵ This effect is not seen consistently, however, and some compounds, such as diclofenac and pyrazolones, might even cause “real” immune-mediated reactions and true-positive LTT results. Other studies indeed showed far better specificity for the LTT using various drugs.^{35–38,56} Recent analyses of LTTs to antiepileptic drugs eliciting DRESS revealed a sensitivity of 97% and a specificity of 100% for carbamazepine and lamotrigine hypersensitivity. This high specificity (93%) was recently also confirmed for β -lactam hypersensitivity.^{36–38}

Some drugs are able to elicit enhanced proliferation even in nonsensitized individuals. Examples are vancomycin, possibly paracetamol, and certain radiocontrast media, which might elicit slightly enhanced proliferation (SI of 2–4) in PBMCs of certain individuals previously not exposed. The mechanism of this is not known at the present time. In contrast, certain drugs, such as abacavir,⁵⁷ may elicit severe generalized

hypersensitivity reactions and positive delayed-type skin test results but a negative proliferation test result.

Different modifications of the LTT have been proposed. One example is the memory lymphocyte immunostimulation assay (MELISA). In this assay, PBMCs from allergic donors are depleted of monocytes before antigen stimulation. The MELISA is mainly applied to test sensitization to metals, with rather controversial results.^{58,59} Recently, Lopez and colleagues⁶⁰ postulated that a lymphocyte proliferation test with monocyte-derived dendritic cells was a more appropriate diagnostic tool in delayed hypersensitivity reactions to heparins than a classic LTT.

In conclusion, the LTT has an overall sensitivity of 60% to 70% under optimal conditions. It is thus suitable as a complement to other tests. Nevertheless, it is still a controversial method because of several disadvantages: it requires sterile cell cultures; it takes a long time and is cumbersome; the drug-specific proliferation depends on the quality of the culture medium; and it involves radioactivity, and thus expensive equipment. In the hands of an experienced technician, however, it can help to define the incriminating drug in the DHR. A positive LTT result helps to define the culprit drug, but a negative test result cannot rule out drug hypersensitivity.

CD69 UP-REGULATION AS A MARKER FOR T-CELL-MEDIATED DRUG HYPERSENSITIVITY

Different surface molecules are expressed on T cells after stimulation, such as CD25, CD69, CD71, and HLA-DR. The CD69 molecule is one of the earliest markers expressed on T cells, B cells, and natural killer (NK) cells after stimulation by various mitogenic substances.^{61–63} CD69 is a membrane type II C-type lectin, which belongs to the family of NK receptors and is transiently expressed on activated lymphocytes. The surface expression of this molecule allows its flow cytometric detection on different T-cell subsets by fluorescence-conjugated antibodies. Maino and colleagues⁶⁴ reported that CD69 is a valuable marker for early 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*. CD69 was rapidly unregulated on CD3+ T cells within 4 hours after stimulation and could be measured by multicolor flow cytometry. Various other studies reported CD69 as an in vitro marker for T-cell activation by mitogenic substances or superantigens.^{65,66} Also, in T-cell-mediated DHRs, CD69 has been shown to be an in vitro marker for drug-induced T-cell activation. CD69 was up-regulated in T cells from patients who had drug-induced MPE, erythema multiforme, SJS, and DRESS when assessed by flow cytometry.^{67,68} A study by Beeler and colleagues⁶⁹ addressed the suitability of CD69 up-regulation as a tool for diagnosis of delayed-type drug hypersensitivity. It showed that CD69 measurement is a promising tool for diagnosing delayed-type drug hypersensitivity. Compared with other activation markers, such as CD71 or CD25, CD69 was the most suitable because of its rapid up-regulation and stronger increase in surface expression after drug stimulation.⁶⁹ In this study, PBMCs from 15 patients with previous DHRs were stimulated with the culprit drug. After 48 hours, substantial CD69 up-regulation on CD4+ and CD8+ T cells of all 15 patients was observed. This CD69 up-regulation was drug-specific because no drug-induced CD69 up-regulation was detected in control subjects. In 15 patients, a positive CD69 test result correlated to a positive LTT result, which was performed in parallel to the CD69 test.⁶⁹ These findings suggest that CD69 measurement is comparable to the LTT. The same study also suggested that the drug activates only few drug-specific T cells, which produce cytokines that activate many “bystander” T cells. This amplification occurred only in presence of drug-specific T cells, and therefore substantially

increases the sensitivity but does not decrease the specificity of this assay.⁶⁹ In conclusion, flow cytometric CD69 measurement seems to be suitable for routine diagnosis, similar to the LTT, but with the advantage that it does not require radioactive substances and is less time-consuming (Fig. 3). It is, however, a flow cytometry-based test, which is difficult to standardize. A large prospective study is required to evaluate this test and to determine sensitivity and specificity in drug hypersensitivity diagnosis. This prospective study is currently being performed by the authors' drug allergy research group in Bern.

MEASUREMENT OF DRUG-INDUCED CYTOKINE PRODUCTION FROM EX VIVO PERIPHERAL BLOOD MONONUCLEAR CELLS

Cytokines are secreted by the cells of the innate and adaptive immune system, and they mediate cell activation, proliferation, and differentiation during an immune response. The cytokine pattern induced by a certain antigen determines the type of immune response; therefore, cytokines play a key role in the effector phase of the immune response.

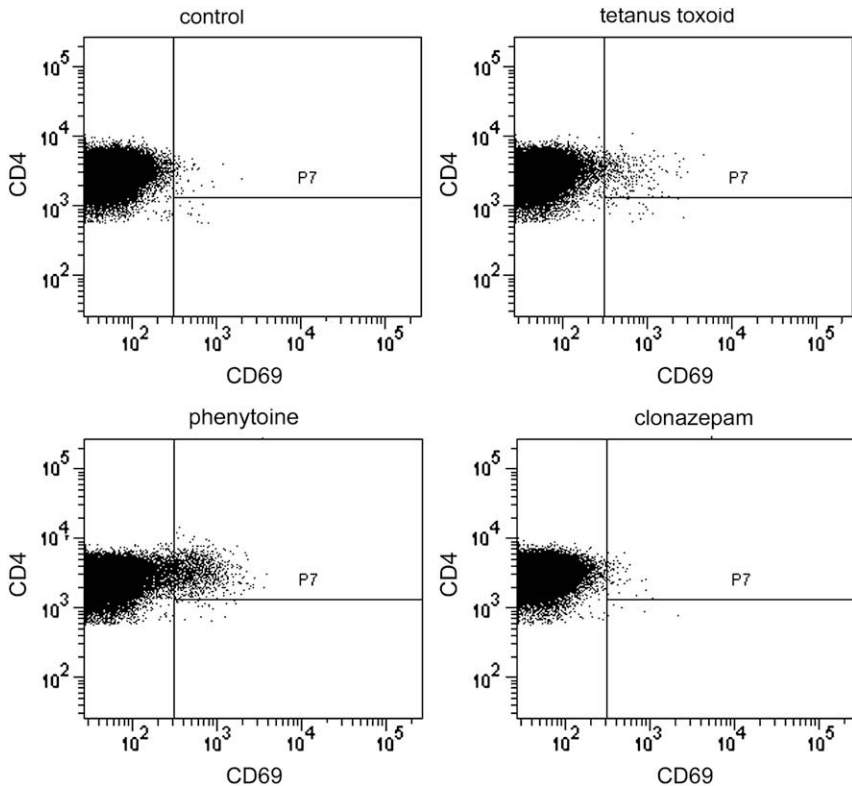


Fig. 3. CD69 expression on CD4⁺ T cells from a drug-allergic donor after drug stimulation. PBMCs from a phenytoin-allergic donor were stimulated for 48 hours with culture medium alone (negative control), tetanus toxoid (positive control), the culprit drug phenytoin, and the unrelated drug clonazepam. Flow cytometric analysis showed CD69 up-regulation on CD4⁺ T cells in response to tetanus toxoid and phenytoin but not in response to clonazepam (A. Zawodniak, P. Lochmatter, unpublished data, 2008).

Many researchers have focused on correlation of a clinical picture with a Th1- or Th2-type immune reaction in various DHRs. Ex vivo studies with PBMCs have revealed a controversial picture: some showed a polarized pattern with more Th1 cytokines (IL-2, IFN γ , and TNF α) in patients with delayed-type reactions and the Th2 cytokine IL-4 in patients with immediate reactions.^{70,71} In another in vitro study, immediate-type hypersensitivity reactions to penicillin or amoxicillin could not be associated with a clear Th2- or Th1-type cytokine pattern but showed a mixed Th1/Th2 pattern, whereas delayed-type reactions were restricted to a Th1 cytokine pattern.⁷² A more recent in vitro study showed no association of delayed-type DHR with sulfonamides and amoxicillin with a Th1 pattern but revealed a mixed Th1/Th2 cytokine pattern.⁷³ Other studies with TCCs and TCLs from drug-allergic patients with immediate- or delayed-type reactions have shown a heterogeneous cytokine pattern, which could not be related to a clear Th1 or Th2 phenotype and is best described as a Th0 pattern.^{19,22,74} It seems that the drug-induced cytokine pattern observed in vitro does not correlate with the ex vivo cytokine pattern observed in the acute phase of the DHR.

There have also been attempts to associate a certain cytokine with a clinical phenotype. Allergic cutaneous drug reactions, such as MPE, acute generalized exanthematous pustulosis, and DRESS, and severe forms, such as SJS or TEN, have been associated with IFN γ release in different studies.^{37,75-79} IFN γ seems to be a predominant cytokine in allergic cutaneous drug reactions and may play an important role in their pathophysiology. Different studies have shown that in vitro drug-stimulated PBMCs from patients with drug-induced cutaneous reactions secreted an increased amount of IFN γ . In a study that included 36 patients with various cutaneous adverse drug reactions tested for a wide panel of drugs, IFN γ secretion had a sensitivity of 77.8%.⁷⁵ In another study of 7 patients who had phenytoin-induced exanthema and 11 control individuals, the sensitivity of IFN γ secretion was 71.4% and the specificity was 100%.⁷⁸ In a study by Gaspard and colleagues,⁷² 6 (75%) of 8 patients with amoxicillin- or penicillin G-induced delayed-type DHRs showed induction of IFN γ expression after drug stimulation. Therefore, IFN γ might be a suitable in vitro marker for delayed-type cutaneous drug reactions and a useful tool with which to identify the responsible drug.

Another cytokine described in different forms of DHRs is the Th2 cytokine IL-5. DRESS has been shown to be associated with in vivo release of IL-5^{76,80} and, conversely, with a predominant release of IFN γ from drug-specific TCCs in vitro.³⁷ It has also been shown that IL-5 is released in vivo and ex vivo in patients who have drug-induced MPE.^{11,76,81} The measurement of IL-5 has also been proposed as a useful in vitro method for detecting drug sensitization in a study that included 10 patients who had drug-induced MPE and 10 control individuals, with a sensitivity of 92% and a specificity of 100%. Further, the combination of IL-5 measurement and the LTT was demonstrated to be a better readout for drug sensitization than the LTT alone.⁸²⁻⁸⁴

Lately, two studies have been performed that aimed to find the most suitable cytokine for the diagnosis of delayed-type DHR. In these studies, supernatants from drug-stimulated patients' PBMC cultures were screened for a panel of different cytokines. In a study by Khalil and colleagues⁷⁹ in which 18 patients who had MPE in response to penicillins and 12 control subjects were screened for drug-specific in vitro secretion of IL-2, IL-5, and IFN γ . IFN γ and IL-2 were suggested as useful methods for the assessment of penicillin allergy, with a sensitivity of 87% and a specificity of 100%. In a recent study, PBMCs of 10 patients with delayed-type DHR to amoxicillin or sulfonamides and 5 control subjects were screened for the secretion of 17 cytokines and chemokines after drug stimulation in vitro so as to identify marker cytokines for delayed-type drug

hypersensitivity. Cytokine secretion by healthy nonallergic controls after drug stimulation was used to set a cutoff point. Four cytokines, namely, IL-2, IL-5, IL-13, and IFN γ , were predominantly secreted after drug stimulation and were significantly increased compared with nonallergic controls. IL-2, IL-5, and IL-13 were secreted in 80% of patients and IL-5 was secreted in 60% of patients after a 3-day drug stimulation, whereas in the controls, cytokine secretion was lower than the cutoff for positivity.⁷³

The gene expression level of cytokines can be measured by molecular techniques, such as quantification of mRNA by reverse transcription polymerase chain reaction (RT-PCR), which detects relative changes in transcription, or by competitive quantitative RT-PCR, which allows absolute quantification of transcript concentrations. At the protein level, cytokines can be measured intracellularly by flow cytometry with fluorescent-conjugated monoclonal antibodies. This method might not be sensitive enough, however. More sensitive methods for measuring secreted cytokines in the cell culture supernatant are enzyme-linked immunoassays, such as the ELISA or ELISPOT assay, or multiplexed bead-based flow cytometric assays, such as Bioplex (Biorad Laboratories, Reinach, Switzerland) or Luminex xMAP (Luminex, Toronto, Canada) assay, which allow the simultaneous measurement of a panel of cytokines.

In conclusion, the measurement of IL-2, IL-5, IL-13, and IFN γ cytokines might be a promising tool for detecting drug sensitization in delayed-type reactions (Fig. 4). Nevertheless, studies that include a large number of patients, such as those performed for evaluation of the LTT as a diagnostic tool, are necessary to evaluate an accurate cutoff for positivity and the exact sensitivity and specificity of these assays.

CYTOTOXICITY: GRANZYME B ENZYME IMMUNOSPOT ASSAY

Analyses of drug-induced skin diseases have indicated an important role for cytotoxic mechanisms in maculopapular, bullous, and pustular reactions.^{3,11,81,85–87} In particular, histologic and immunohistologic analyses of affected skin lesions have revealed the presence of perforin- and granzyme B (GzB)-positive T-cell infiltrates.^{11,35,87} Elution of skin-infiltrating T cells and characterization of drug-specific cytotoxic TCLs and TCCs from the peripheral blood of drug-allergic patients have confirmed that drug-dependent killing occurs.^{12,88}

In maculopapular drug eruptions, perforin- and GzB-positive CD4+ and CD8+ T cells were found at the dermoepidermal junction zone or in the epidermis, along with signs of keratinocyte cell damage.¹¹ In more severe forms of DHR, predominant activation of cytotoxic CD8+ T cells, NK cells, and NK-like T cells may lead to a massive widespread apoptosis of epidermal cells mediated by perforin/GzB^{13,89} or Fas ligand/Fas.^{90,91} Recently, a role of granulysin, a cytotoxic protein secreted by T cells and NK

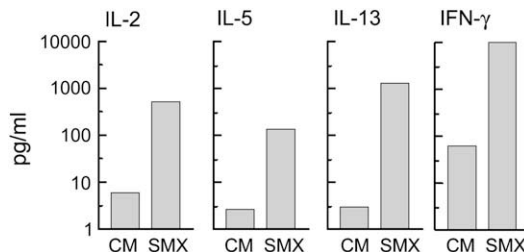


Fig. 4. Drug-induced cytokine secretion in a drug-allergic donor. Secretion of IL-2, IL-5, IL-13, and IFN γ was induced in PBMCs of a sulfamethoxazole (SMX)-allergic donor after a 72-hour stimulation by SMX compared with incubation with culture medium (CM) alone (P. Lochmatter, unpublished data, 2008).

cells, has been implied to be responsible for disseminated keratinocyte apoptosis and skin detachment in severe life-threatening bullous skin lesions (eg, SJS, TEN).⁹²

Over the past years, several approaches have been developed for the identification of antigen (peptide)-specific cytotoxic T cells on a single-cell basis.⁴⁸ A standard chromium release assay⁹³ and more recently used methods that monitor the release of fluorescent dyes from target cells are cumbersome and semiquantitative.^{94,95} MHC multimers were not of practical application in identification of drug-specific cytotoxic T cells. Intracellular cytokine staining and the IFN α ELISPOT assay are commonly used, but none of these methods directly examine the cytotoxic effector function of T cells.⁹⁶ Recently, the authors' group adapted two methods to detect drug-reactive cytotoxic cells in peripheral blood of drug-allergic patients in remission, namely, the GzB ELISPOT assay and flow cytometry-based CD107a measurement.

Cytotoxic T lymphocytes mediate the killing of target cells by means of two major pathways: a granule-dependent mechanism (perforin/granzyme) and a death receptor-mediated mechanism (Fas/Fas ligand and TNF-related apoptosis-inducing ligand [TRAIL]).^{97,98} Recently, the role of TRAIL has been described in paracetamol and virally induced liver failure.⁹⁹ However, the exact role of TRAIL in drug-induced liver disease is still unclear. Once cytotoxic T cells get activated, the lytic granules reach the plasma membrane, the content of cytotoxic granules (granzymes and perforin) is released into immunologic synapses, and the cumulative exposure of granular membrane proteins (CD107a) can be measured on the surface of responding cytotoxic cells, providing a positive marker for degranulation (Fig. 5).^{96,100} Release of GzB measured by an ELISPOT assay can be used to detect the cytotoxic function of in vivo sensitized antigen-specific T cells.¹⁰¹ The ELISPOT assay detects molecules secreted by individual cells present in low frequencies. It is highly sensitive because 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.¹⁰¹ The GzB ELISPOT assay and the degranulation marker CD107a have been used successfully to detect peptide-specific cytotoxic T lymphocytes in patients who have chronic virus infections and in patients monitored in vaccine trials.^{102–108} The GzB ELISPOT assay also seems to be the most sensitive and robust method to detect cytotoxic cells in the peripheral blood of drug-allergic patients. CD107a measurement might be useful to reveal the phenotype of reacting cytotoxic cells.

Because cytotoxicity-based in vitro tests measure effector cell function distinct from proliferation, such tests might be promising tools in drug hypersensitivity diagnosis and in supplementing proliferation-based assays. In contrast to the LTT, the GzB ELISPOT test gives results as early as 48 to 72 hours after stimulation and does not involve radioactivity.

WHEN SHOULD DIAGNOSTIC TESTS BE PERFORMED?

According to common opinion, in vitro tests to identify the drug involved in a hypersensitivity reaction should not be performed during the acute stage because the immune cells are still strongly activated, causing high background proliferation and activity.³⁵ For example, in LTTs, cells obtained ex vivo from patients in the acute stage are still activated and show high spontaneous proliferation. Thus, additional in vitro drug stimulation could lead to only marginal and difficult-to-detect enhancement of proliferation or even to stimulation-induced cell death.

This concept has recently been challenged by Kano and colleagues.¹⁰⁹ They observed that the correct timing of drug hypersensitivity diagnosis with the LTT

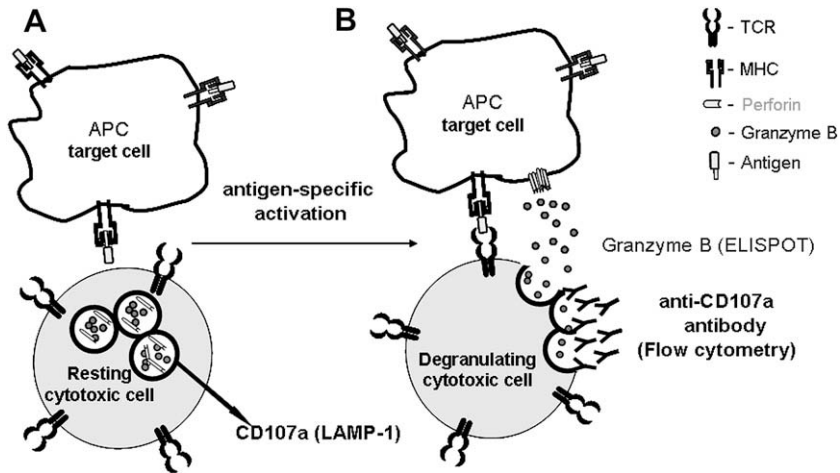


Fig. 5. Degranulation of cytotoxic cell after antigen-specific stimulation. (A) In the resting cell with cytotoxic potential, lytic molecules (granzymes and perforin) are stored in cytotoxic granules. The membrane of these granules is lined with membrane glycoproteins, including CD107a (LAMP-1). (B) Once the cell gets activated by the specific antigen, the lytic granules reach the plasma membrane, the cytotoxic molecules are released, and the cumulative exposure of granular membrane proteins (CD107a) can be measured on the surface of responding cytotoxic cells, providing a positive marker for degranulation. Granzyme B (GzB) released from the granules can be detected in the GzB ELISPOT assay. APC, antigen-presenting cell.

depends on the type of drug reaction. In patients who had DRESS, negative results were more frequently observed when the LTT was performed during the acute stage of the disease. In contrast, the culprit drug could be better identified when proliferation tests were performed during the acute stage, rather than the recovery stage, in maculopapular eruptions and SJS or TEN. Thus, the authors recommend performing the LTT within 1 week after the onset of skin rashes in patients who had maculopapular eruptions and SJS or TEN and more than 5 to 8 weeks after the event in patients who had DRESS.

In vitro diagnostic tests measure a memory T-cell response. At present, one cannot predict whether the reactivity of these drug-specific T cells in an individual patient is likely to persist for months or even years and whether these patients who lost their reactivity in in vitro tests are, in fact, likely to tolerate the drug again. The authors and others have observed a positive LTT reaction even 15 years after the original treatment with drugs that caused a delayed or even anaphylactic reaction.²⁸ Conversely, some patients lose reactivity within 1 to 3 years after the reaction. Thus, many groups carry out the tests after a minimal interval of 3 weeks but less than 3 to 6 months after the acute event. This interval is also used to wash out any immunosuppressive drugs, particularly corticosteroids, which are often used for the treatment of acute delayed reactions and may also suppress the immune response in vitro.

SUMMARY

The diagnosis of DHR is a challenging task because multiple and complex mechanisms are involved. The overall poor sensitivity of drug allergy tests has various reasons, with one being that a multitude of mechanisms are involved, not each of which might be covered by the tests applied. Therefore, at present, a practical

approach to drug hypersensitivity diagnosis is needed, combining history, various skin tests, in vitro tests, and possibly provocation tests.

With regard to in vitro tests, the “old” proliferation-based assays have been supplemented by new and more rapid tests. The latter include evaluation of cytokine secretion, up-regulation of activation markers, and analysis of cytotoxic potential. These novel in vitro tests seem promising for drug allergy diagnosis and still need further evaluation.

In vitro tests offer the advantage of safety, simultaneous assessment of T-cell responses to multiple drugs, lack of risk for resensitization, and insight into the pathomechanism. This latter aspect may improve our understanding of particular immune mechanisms in different drug allergy diseases, which could open a more pathogenesis-tailored approach to drug allergy diagnosis, and consequently result in higher sensitivity.

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