

In Vitro Diagnosis of Delayed-type Drug Hypersensitivity

Mechanistic Aspects and Unmet Needs



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KEYWORDS

- Delayed-type drug hypersensitivity • T lymphocytes • Hapten hypothesis
- PI hypothesis • Altered peptide repertoire hypothesis

KEY POINTS

- For the effective diagnosis of delayed-type drug hypersensitivity, our laboratory currently uses the lymphocyte transformation test (LTT) and a panel of enzyme-linked immunosorbent spot assays selected from interferon gamma, interleukin (IL)-4, IL-5, IL-13, IL-17, IL-22, Fas ligand, tumor necrosis factor alpha, granzyme B, and perforin (individual panels depend on the volume of blood available and the nature of the clinical reaction).
- The availability of multiple readouts has improved clinicians' ability to diagnose reactions.
- Most studies published to date originate from well-defined test and control patient groups.
- We are currently collecting samples longitudinally from patients with cystic fibrosis exposed to β -lactam to characterize markers of the effector and regulatory immune response in hypersensitive and tolerant patients.
- A component of this analysis is a detailed assessment of the sensitivity and specificity of the LTT for the diagnosis of β -lactam hypersensitivity.

INTRODUCTION

The term delayed-type drug hypersensitivity reaction is used loosely to describe a myriad of clinical syndromes caused by a variety of different drugs. Characterization of mechanisms is difficult because the same drug has the potential to activate different arms of the adaptive immune system via diverse chemical pathways. Layered on top

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of this is the recent discovery of human leukocyte antigen (HLA) molecules as important susceptibility factors for certain reactions.

The only common aspect in all forms of delayed-type hypersensitivity reaction is the drug-specific activation of T lymphocytes. In contrast, effector T-cell responses to drugs are not detected in tolerant controls. Researchers in specialist facilities have used these observations to develop test systems that detect the drug-specific activation of T cells and, in certain circumstances, the applicability of the test for patient diagnosis has been assessed. Despite this, diagnostic tests are not yet available for routine use in the clinic. Thus, this article (1) briefly summarizes the cellular and chemical basis of delayed-type drug hypersensitivity, highlighting, when appropriate, gaps in current knowledge (Table 1); and (2) reviews the in vitro assays that are currently available for the diagnosis of drug hypersensitivity.

DRUG HYPERSENSITIVITY REACTIONS: DEFINITION AND OVERVIEW OF CLINICAL SYNDROMES

Drug hypersensitivity can be defined simply as a serious adverse drug reaction with an immunologic cause, to an otherwise safe and effective therapeutic agent.¹ Many delayed-type reactions manifest in the skin. Reactions vary in severity from self-limiting maculopapular eruptions to life-threatening Stevens-Johnson syndrome/toxic epidermal necrolysis. Pichler expanded the classic Gell and Coombs² classification through the categorization of delayed-type cutaneous reactions into 4 groups according to the phenotype and function of drug-responsive T cells (Table 2).³ The discovery of drug-responsive T cells from patients with delayed liver and kidney reactions to drugs including flucloxacillin,⁴ disulfiram,⁵ Augmentin (Katy Saide, 2014), and isoniazid (Toru Usui, 2014) indicates that noncutaneous reactions should also be categorized as a form of delayed-type drug hypersensitivity reaction. Thus, we have expanded Pichlers³ classification to include reactions targeting kidney and liver. However, this

Table 1 Pathways of drug-specific T-cell activation: main supporting experimental evidence and unmet needs		
Hypothesis	Main Experimental Observation	Unmet Needs
Hapten	Drug (metabolite)-protein adducts activate T cells	Chemical characterization of hapten-peptide conjugates naturally eluted off MHC and demonstration that they act as T-cell antigens Identification of drug metabolite-modified proteins that contain T-cell stimulatory peptide sequences
Pharmacologic interaction	Chemically inert drugs stimulate T cells in a readily reversible manner	Demonstration that activated T cells cause clinical reactions in patients and/or experimental models of drug-induced tissue injury
Altered self-peptide repertoire	Indirect T-cell activation by abacavir through MHC binding and alteration of the repertoire of self-peptides displayed on MHC molecules	Assessment of whether the altered self-peptide repertoire pathway can be used to describe the activation of T cells by other drugs

Abbreviation: MHC, major histocompatibility complex.

Table 2
Clinical and immunologic features of delayed-type drug hypersensitivity reactions

Clinical Reaction	Normal Time to Onset	Principal Drug-specific T-cell Response	Additional Features	Key References
Maculopapular exanthema	4–14 d	Th2-secreting CD4+ T cells	Account for many delayed-type drug hypersensitivity reactions, often associated with an eosinophilic inflammation. Self-limiting after drug withdrawal	77,78
Drug rash with eosinophilia and systemic symptoms	2–6 wk	CD4+ and CD8+ T cells secreting IFN- γ and IL-5	Multiple organs are damaged, viral reactivation often observed, and viral-specific T cells may be involved in the disease pathogenesis. Mortality approximately 10%	64,66,79
Pustular eruptions (eg, acute generalized exanthematous pustulosis)	<24 h–30 d	CD4 and CD8+ T cells secreting IL-8	Neutrophil infiltration is a common characteristic in pustules. Mortality approximately 5%	80,81
Bullous eruptions (eg, Stevens-Johnson syndrome)	1–3 wk	Cytotoxic CD8+ T cells, NK T cells	Blistering lesions leading to extensive skin detachment. Killing mediated by FasL, granzyme B, and granulysin. Fatality rate more than 30% for toxic epidermal necrolysis	46,82,83
Abacavir hypersensitivity	3–14 d	Cytotoxic CD8+ T cells	All patch-test–confirmed patients express HLA-B*57:01. Only reaction that does not involve CD4+ T-cell help. Genetic testing for B*57:01 prevents hypersensitivity	57–59,61
Liver reactions	7 d ++; frequently >80 d	Cytotoxic CD4+ and CD8+ T cells ^a	Broad spectrum of clinical syndromes. In flucloxacillin reactions, T cells express gut-homing chemokine receptors	4,84,85
Kidney reactions	1–3 wk	CD4+ T cells secreting heterogeneous cytokine pattern ^a	T cells do not secrete cytotoxic molecules, indicating they orchestrate a local inflammatory response in the target organ	5

Abbreviations: IFN, interferon; IL, interleukin; NK, natural killer.

^a Data limited to 1 or 2 mechanistic studies focusing on a limited number of drugs.

classification is still outdated because it does not encompass new T-cell populations including Th9, Th17, Th22, and T_{reg}.

ACTIVATION OF DRUG-RESPONSIVE T CELLS

T cells are activated through the interaction of a major histocompatibility complex (MHC)–associated peptide with a specific T-cell receptor. In the appropriate cytokine microenvironment, the activated T cells divide and differentiate into multiple effector populations. In terms of drug hypersensitivity, the drug must in some way mimic the actions of the MHC-associated antigenic peptide.

There are currently 3 hypotheses that describe the way in which drugs act as antigens to activate T cells: the hapten hypothesis, the pharmacologic interaction (PI) concept, and the altered self-peptide repertoire hypothesis. Each hypothesis has been reviewed in detail elsewhere (Fig. 1)^{6–8}; this article briefly describes the

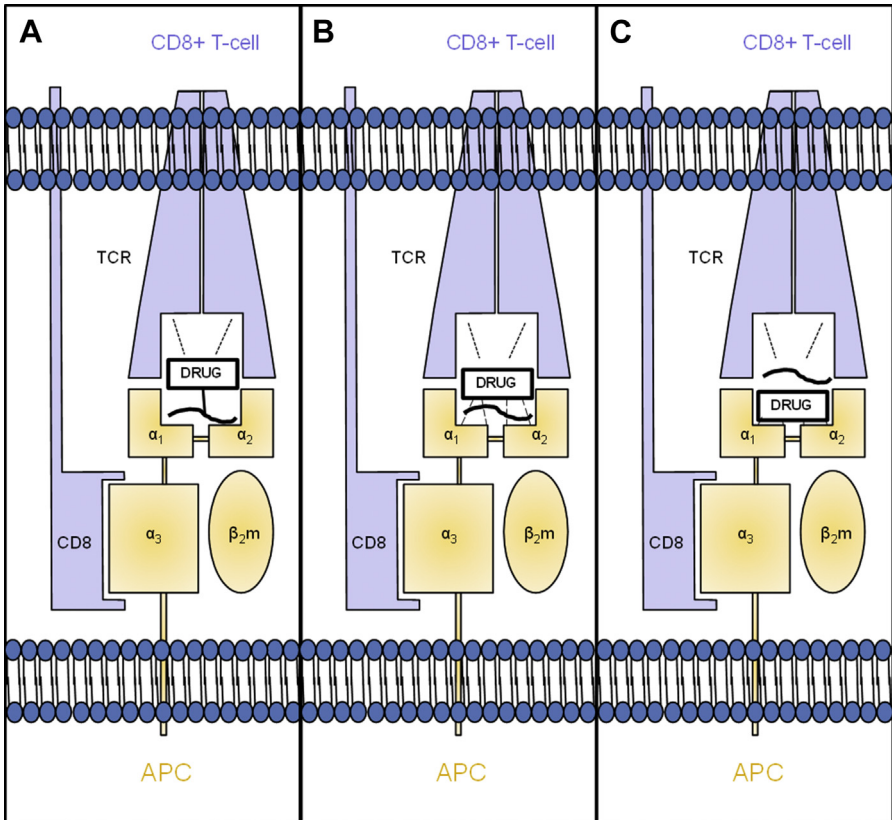


Fig. 1. CD8+ T-cell activation via hapten, PI, and altered self-peptide repertoire pathways. (A) Hapten hypothesis. The peptide embedded in the MHC-I molecule is covalently bound and altered by a protein-reactive drug (eg, flucloxacillin), creating an antigen. (B) PI concept. The drug (sulfamethoxazole, carbamazepine) is capable of binding directly to MHC and/or the T-cell receptor. (C) Altered peptide repertoire hypothesis. The drug is capable of binding directly to a particular MHC class molecule, allowing for presentation of altered self-peptides to CD8+ T cells (eg, only abacavir to date).

experimental evidence using T cells from drug hypersensitive patients and/or drug-naive human donors that supports each hypothesis. This information will aid the later discussion of the limitations of existing in vitro diagnostic assays.

HAPTEN HYPOTHESIS

The hapten hypothesis states that low-molecular-weight chemicals must form stable adducts with endogenous proteins to be able to induce an immune response. The hypothesis evolved from groundbreaking studies by Landsteiner and Jacobs,¹⁰ who were able to correlate direct reactivity of the dinitrohalobenzenes with sensitization potential.^{9,10} The hapten concept is still considered the primary pathway by which chemical sensitizers cause allergic contact dermatitis.¹¹ Protein-reactivity screens represent an important tool for the identification of skin sensitizers.^{12,13}

The hapten hypothesis is also used to describe the chemical basis of delayed-type β -lactam hypersensitivity reactions. The β -lactam nucleus binds irreversibly to amino acid residues within the active site of penicillin binding proteins (a group of bacteria cell wall proteins with high affinity for the β -lactam antibiotics), preventing cross-linking of the nascent peptidoglycan layer and disrupting bacterial cell wall synthesis. However, this intrinsic reactivity leads to covalent modification of other endogenous proteins, especially at lysine residues. Such drug-protein adducts are thought to be involved in a variety of adverse events including hypersensitivity.^{14–18} However, under cell culture conditions, adduct formation by the β -lactam antibiotics is extremely protein selective. All of the drugs analyzed to date (penicillin G, amoxicillin, flucloxacillin, piperacillin) modify extracellular protein, but essentially ignore cellular protein.^{4,19–21} This protein selectivity is restricted to the β -lactam antibiotics; other reactive chemicals and drug metabolites modify multiple cellular and extracellular proteins.^{22–28} Using mass spectrometric methods, we identified albumin as the major protein modified by β -lactam antibiotics and defined the profile of drug-protein conjugation at specific lysine residues with respect to dose and incubation time. Because human serum albumin accounts for most serum-bound penicilloyl groups in vivo, we also considered it a suitable model for investigating the chemistry underlying β -lactam adduct formation in patients. The β -lactam antibiotics piperacillin and flucloxacillin were bound in vivo to the same lysine residues that are modified in vitro.^{20,29} These data confirm that drug-protein adducts are formed in exposed patients and hence have the potential to be recognized by the hosts' immune system.

β -Lactam antibiotics stimulate CD4+ and CD8+ T cells from hypersensitive patients to proliferate and secrete a diverse cytokine secretion profile.^{30–35} Several studies have shown that β -lactam antibiotics conjugated directly to MHC molecules, and synthetic β -lactam–albumin constructs stimulate T cells from hypersensitive patients and drug-naive donors.^{19,20,36,37} Furthermore, penicillin peptide conjugates that have been designed to fit in specific MHC molecules have been shown to stimulate penicillin-responsive T cells³⁸; this proves that human T cells from hypersensitive patients are activated via a hapten mechanism.

Most drugs are not directly protein reactive (reactivity is designed out during drug development); however, protein adducts may form as a consequence of metabolic activation. Many drugs associated with a high incidence of drug hypersensitivity reaction form reactive intermediates, providing circumstantial evidence that they are involved in the disease pathogenesis. To study whether drug metabolites selectively activate T cells several groups have focused on patients with sulfamethoxazole hypersensitivity. Sulfamethoxazole is metabolized via a hydroxylamine intermediate to a nitroso metabolite, which binds readily to cysteine groups on protein.^{23,25,39} The

synthetic nitroso metabolite is a potent T-cell sensitizer in rodents^{25,40,41} and in vitro human T-cell priming assays.^{42,43} Furthermore, nitroso sulfamethoxazole-responsive T cells have been isolated from the blood and skin of 100% of hypersensitive patients presenting with reactions varying in severity from maculopapular eruptions to toxic epidermal necrolysis.^{44–47} A recent analysis found that more than 80% of drug-antigen-specific T cells from sulfamethoxazole-hypersensitive patients are activated with the nitroso metabolite.⁴⁴ Moreover, in sulfamethoxazole-hypersensitive patients with cystic fibrosis, the T-cell response is directed exclusively against the nitroso metabolite.^{48,49} Sulfamethoxazole hypersensitivity is one of only a few examples in which a synthetic metabolite is available that can be incorporated into in vitro studies. Detection of T-cell responses to the parent compound (discussed later) does not exclude the presence of drug metabolite-specific T cells.

There is an unmet need for identification of drug metabolite-modified proteins that contain T-cell-stimulatory peptide sequences.

THE PI HYPOTHESIS

The PI hypothesis states that drugs do not need to bind covalently to protein to become immunogenic and that some drugs can bind directly to MHC to activate T cells.^{8,50} This concept was devised through experimentation with human peripheral blood mononuclear cells and drug-specific T-cell clones from hypersensitive patients. The primary observation from these experiments was that T cells are reactive against a variety of drugs that do not bind covalently to protein.⁵¹ This concept is not compatible with a hapten mechanism. More detailed analysis revealed that the T cells were activated rapidly via an MHC-restricted, processing-independent pathway.^{52–56} Because reactive metabolites of most drugs associated with a high incidence of hypersensitivity are not widely available, most in vitro assays currently used to diagnose drug hypersensitivity rely on the direct activation of drugs via a PI mechanism.

ALTERED SELF-PEPTIDE REPERTOIRE HYPOTHESIS

Several drugs and chemicals have been shown to activate T cells via hapten and PI mechanisms. In contrast, only 1 drug (abacavir) has been shown to activate T cells indirectly through MHC binding and alteration of the repertoire of peptides that are ultimately displayed on the surface of antigen-presenting cells. Abacavir hypersensitivity is atypical in that skin test-confirmed reactions are only seen in individuals carrying the risk allele HLA-B*57:01⁵⁷; furthermore, the drug interacts exclusively with B*57:01 to activate CD8+ T cells.^{58–61} In all other forms of drug hypersensitivity reaction (including reactions strongly associated with HLA risk alleles), drug-specific CD4+ and CD8+ T cells are detectable and those that have been studied interact with multiple MHC molecules.

Elegant, integrated, structural, modeling, and biological studies describe the interaction of abacavir with endogenous HLA-B*57:01 and show that the binding interaction changes the confirmation of the peptide binding cleft, altering the repertoire of peptides displayed on the cell surface.^{58–60} These data imply that preexisting peptide-specific T cells are activated indirectly by abacavir through the display of cross-reacting altered self-peptides.

DIAGNOSIS OF DELAYED-TYPE DRUG HYPERSENSITIVITY REACTIONS

Diagnosis of delayed-type drug hypersensitivity is extremely difficult in the clinical setting. In vitro tests are not routinely available and hence assessment of causality

is usually based on a clinician's assessment of the relationship between drug intake and the time to onset of the adverse reaction. Because T cells are involved in the pathogenesis of all forms of delayed-type reaction, the ideal *in vitro* test would use a small volume of a patient's blood to detect a drug-antigen-specific T-cell response as a marker of sensitization.

The lymphocyte transformation test (LTT; also known as the lymphocyte proliferation assay) is the most established assay in this category (**Box 1**). The assay is based on incubation of freshly isolated peripheral blood mononuclear cells from hypersensitive patients (and drug-exposed tolerant controls) with titrated concentrations of the suspect drug. A stimulation index (counts per minute [cpm] in drug-treated wells/cpm in vehicle control wells) of 2 or 3 or more indicates a drug-specific T-cell response and can be used for causality assessment if other drugs render a negative result.

A PubMed (<http://www.ncbi.nlm.nih.gov>) database search (December 2013) using the terms drug hypersensitivity and lymphocyte transformation test generates 633 results. Many of the articles describe the successful application of the test for the diagnosis of drug hypersensitivity. In a detailed retrospective analysis of 923 probable/definite patients with drug hypersensitivity, the LTT had a sensitivity (ie, percentage of hypersensitive patients with a positive LTT result) and specificity (ie, percentage of drug-exposed tolerant patients with a negative LTT result) of 78% and 85%, respectively.⁶² More recently, Pichler and Tilch⁶³ cited a sensitivity of 60% to 70% for the diagnosis of β -lactam hypersensitivity. In our own retrospective analysis of β -lactam hypersensitivity in patients with cystic fibrosis, we found similar results, with the sensitivity of the assay being 76% when patients receiving oral steroids were excluded from the analysis.^{19,20} Specificity was close to 0%. In a separate cohort (patients with different forms of cutaneous hypersensitivity exposed to carbamazepine and lamotrigine) we again used the LTT for diagnosis and found a sensitivity of more than 80%.^{64–67} So the questions are: (1) why is the LTT not applied more widely in the clinical setting, and (2) what can be done to overcome the issues restricting its use. The main limitations of the LTT in its current form are discussed here.

- Technical limitations

1. The assay relies on the availability of freshly isolated peripheral blood mononuclear cells and/or a cryopreservation facility if assays are to be conducted at a later date.
2. A researcher must be trained in cell isolation, sterile technique, and cell culture methods.

Box 1**LTT**

The LTT measures the proliferation of memory T cells to a particular drug to which a patient has been exposed and provides an insight into the pathomechanism of the reactions. It has a high specificity with various drugs including the β -lactam antibiotics, sulfonamides, anticonvulsants and p-phenylenediamine. Sensitivity depends on the chemical properties of the drug.

The assay is performed using peripheral blood mononuclear cells isolated from sensitized patients over a density gradient. Cells (1.5×10^5 , 100 μ L) are cultured in triplicate in a 96-well U-bottomed microtiter plate with various concentrations of the culprit drug or drugs using tetanus toxoid and cell culture medium as positive and negative controls respectively at 37°C, 5% CO₂ for 6 days. [³H]-thymidine (0.5 μ Ci) is added during the final 16 hours of the incubation and T-cell proliferation evaluated using a scintillation counter. Lymphocyte proliferation is expressed as stimulation index, which is the ratio of the proliferative response with and without the drug.

3. Incorporation of the drug into the assay in an appropriate form is technically demanding. The sensitivity/specificity analysis discussed earlier focuses on a small number of compounds for which T-cell stimulatory concentrations have been defined over many years, largely through trial and error. For example:
 - a. Sulfonamides are solubilized in phosphate-buffered saline (PBS) at a high pH and diluted into medium before use. T cells are activated between 0.5 and 2 mM. Sulfonamide metabolites are dissolved in small quantities of dimethylsulfoxide (DMSO) before use. T cells are activated at low μM concentrations; 100 μM is toxic.
 - b. Anticonvulsants, such as carbamazepine, form a suspension in a PBS/DMSO (10%) mixture. Following dilution the drug is solubilized and concentrations of 50 to 400 μM activate T cells.
 - c. β -Lactam antibiotics dissolve directly in culture medium. T cells are activated at 0.5 to 4 mM.

The protocol used for new chemicals/drugs generates a stock concentration using small quantities of DMSO, which is then serially diluted (2-fold or 3-fold dilutions) starting with a concentration that causes approximately 25% peripheral blood cell death. When sufficient cells are available, 7 concentrations of the drug are assessed.

- Interpretation of the results

1. A stimulation index of 5 or more detected at 2 or more drug concentrations is considered in our laboratory as strongly positive. However, stimulation index results of 50 and more are seen regularly. In these patients, the positive LTT is reproducible and detectable over a period of several years. The outcome of the test is less clear when the LTT yields a stimulation index of 1.5 to 3. On repeated testing of the same individual, the result varies between these parameters. Thus, the relevance of the results is difficult to interpret without additional testing, namely T-cell cloning and analysis of drug-responses (Box 2).
2. The quantity of [^3H]thymidine incorporated in control cells is extremely variable. This variation is apparent when (1) samples from different donors and (2) repeated samples from same donor are analyzed. It is therefore difficult to conclude whether a donor with high counts in control wells (eg, 3000–5000 cpm) and a stimulation index of 3 to 4 is more strongly sensitized to the drug than a donor with low counts in control wells (eg, 200–500 cpm) and a stimulation index of 10.

- Mechanistic limitations

1. Negative data in specific patient cohorts:
 - a. The pathogenesis of Stevens-Johnson syndrome/toxic epidermal necrolysis involves the activation of drug-specific T cells. However, the LTT is negative in many patients (even to drugs that yield positive results in patients with other forms of hypersensitivity),^{68,69} which may be because CD8+ T cells that do not proliferate readily are the primary effector cells in this patient group.⁷⁰ By changing the end point of the LTT to a functional marker of T-cell activation (discussed later) more sensitive assays are being developed for diagnosis.
 - b. Negative LTT data are obtained using cells from patients with flucloxacillin-induced liver injury.⁴ In these patients, sensitization to the drug was confirmed through analysis of flucloxacillin-specific peripheral blood mononuclear cell cytokine release and the generation of drug-responsive T-cell clones.

Box 2**Other assays that detect antigen-specific T cells**

- Flow cytometry

Flow cytometry is a technique used for the determination of multiple phenotypic parameters. Cell surface markers of T-cell activation (eg, CD69 CD25, CD40L, CD71, and HLA-DR [HLA D related]) can be used to measure drug-specific T-cell populations. Flow cytometry can also be used to evaluate T-cell proliferative responses. Cells are first stained with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE), which interacts with the amino group of intracellular protein. The intensity of CFSE is reduced by half following each cell division. Hence, the dividing population of antigen-specific T cells can be evaluated.

- Intracellular cytokine staining

Intracellular cytokine staining is a flow cytometry-based assay that evaluates the cytokine profile of activated cells. The method can detect cytokines secreted by different subsets of T cells following antigen stimulation. Drug-stimulated and control peripheral blood mononuclear cells are treated with either monensin or Brefeldin A to block Golgi-mediated transport. This process results in an accumulation of intracellular cytokines, thereby enhancing the cytokine signal obtained by flow cytometry. Cells are stained with anticytokine antibodies alongside antibodies against cell surface antigens in order to evaluate defined cell populations.

- Multiplex (Luminex) assays

Multiplex assays are expensive but are able to quantify soluble proteins secreted by a given cell population. Multiplex assays provide an accurate, quick, and efficient method to analyze multiple cytokines secreted from antigen-specific T cells. They are flow cytometry-based assays in which beads with known spectral characteristics are bound to particular capture antibodies and mixed with the cell suspension of interest. Using the spectral properties of fluorescent proteins conjugated to beads, the concentrations of specific proteins in each sample can be determined relative to a standard curve.

- T-cell cloning

T-cell cloning provides a valuable alternative to detect drug-specific T cells when other assays discussed earlier yield negative results. Cloning is performed by limiting dilution followed by repetitive mitogen stimulation. Several hundred single cell populations are generated and antigen-specificity assessed by culturing irradiated Epstein-Barr virus-transformed B cells with antigen and the clones (5×10^4 /well; 200 μ L) for 48 hours. Proliferation can be measured by the addition of [^{3} H]thymidine. Clones with a stimulation index of greater than 2 are expanded and then characterized in terms of cellular phenotype and function.

c. The LTT has limited value in the diagnosis of drug hypersensitivity in patients with acute reactions, possibly because the drug-responsive T cells are preactivated during the acute reaction or located primarily in the tissue. Polak and colleagues⁷¹ recently showed that the combined analysis of interferon gamma (IFN- γ) and interleukin (IL)-4 offers potential compared with the LTT for diagnosis in the acute setting.

2. Interpretation of a negative LTT result is extremely difficult because it is currently impossible to include all forms of drug antigen in the test. As mentioned earlier, the assay is almost always performed using the parent drug alone.

To improve the sensitivity of the LTT for the diagnosis of delayed-type hypersensitivity reactions, the main strategy has been to alter the readout used to detect the

drug-specific activation of T cells. Flow cytometry-based methods have been developed to measure the number of dividing T cells (carboxyfluorescein diacetate succinimidyl ester staining³³), increased expression of surface markers of T-cell activation (eg, CD69,⁷² CD107a⁷³), and cytokine production⁶⁸ in drug-treated and vehicle-treated peripheral blood mononuclear cell cultures (see **Box 2**). When used in combination, these assays have an advantage compared with the LTT because the readouts provide information on the nature of the drug-specific T-cell response. However, because the frequency of drug-specific T cells in hypersensitive patients' blood can be as low as 1 in 5000,³³ flow cytometry-based methods lack sensitivity to accurately diagnose drug hypersensitivity reactions. Flow cytometry methods are more valuable for mechanistic studies in which it is easier to establish drug-specific T-cell lines and clones for assessment of phenotype and function.

The enzyme-linked immunosorbent spot (ELISpot) methodology has been developed to quantify the number of cytokine/cytolytic molecule-secreting cells in antigen-stimulated peripheral blood mononuclear cell cultures. The method is antibody based (**Box 3**) but has the advantage that low numbers of drug-specific T cells can be detected. IFN- γ ELISpot has been used to analyze the presence of drug-specific T cells in 23 amoxicillin-hypersensitive patients presenting with delayed-type maculopapular eruptions.³² The assay could detect drug-specific T-cell precursors as low as 1:30,000, allows the detection of cross-reacting drugs, and (3) is more sensitive than the LTT. ELISpot assays have recently been used to detect drug-specific T cells in the acute setting⁷¹ and in retrospective patients presenting with a range of conditions including Stevens-Johnson syndrome,^{68,74} drug-induced liver injury,⁴ and abacavir hypersensitivity. In each of these studies, the LTT had a low sensitivity because proliferating T cells were not readily detectable.

A cautionary note is that a single ELISpot readout cannot be used to detect all drug-specific T cells. This point is clearly shown (1) in our recent analysis of abacavir-specific T-cell clones that kill target cells through the release of either FasL or granzyme B,⁷⁵ and (2) during the application of a human T-cell priming assay to identify contact chemical allergens based on IFN- γ and tumor necrosis factor alpha (TNF- α) readouts.⁷⁶

Box 3 ELISpot assay

ELISpot assay is a quick, highly sensitive, and precise technique applied to the detection of low-frequency cytokine-secreting T cells following antigen stimulation. This type of assay provides an insight into the biological function of the T cells that mediate drug hypersensitivity reactions as well as quantification of specific cytokine-secreting T cells. Activated T cells can secrete an array of cytokines as well as other effector molecules like granzyme, perforin, and Fas ligand. ELISpot kits are available to detect a wide range of proteins; a panel of assays can be conducted simultaneously to characterize the nature of the drug-specific response.

The assay is performed by coating nitrocellulose plates with a capture antibody of choice and incubating them overnight at 4°C. The following day, wells are washed and blocked before use. Peripheral blood mononuclear cells (5×10^5 to 1×10^6) are then cultured with various concentrations of the culprit drug or drugs using Phytohaemagglutinin (PHA) and culture medium as positive and negative controls respectively at 37°C in 5% CO₂. Plates are developed after 48 hours according to the manufacturer's instructions. Spots (representing cytokine-secreting cells) are visualized by the addition of nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate (BCIP/NBT) substrate. When thoroughly dried, the plate is counted on an ELISpot reader. Results are expressed as either spot-forming units per million cells or as a stimulation index relative to control.

SUMMARY

For the effective diagnosis of delayed-type drug hypersensitivity, our laboratory currently uses the LTT and a panel of ELIspot assays selected from IFN- γ , IL-4, IL-5, IL-13, IL-17, IL-22, FasL, TNF- α , granzyme B, and perforin (individual panels depend on the volume of blood available and the nature of the clinical reaction). The availability of multiple readouts has improved our ability to diagnose reactions. However, most studies published to date, originate from well-defined test and control patient groups. We are currently collecting samples longitudinally from patients with cystic fibrosis exposed to β -lactam to characterize markers of the effector and regulatory immune response in hypersensitive and tolerant patients. A component of this analysis is a detailed assessment of the sensitivity and specificity of the LTT for the diagnosis of β -lactam hypersensitivity.

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