In Vitro Methods for Diagnosing Nonimmediate Hypersensitivity Reactions to Drugs

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Abstract

Nonimmediate drug hypersensitivity reactions (DHRs) are difficult to manage in daily clinical practice, mainly owing to their heterogeneous clinical manifestations and the lack of selective biological markers. In vitro methods are necessary to establish a diagnosis, especially given the low sensitivity of skin tests and the inherent risks of drug provocation testing. In vitro evaluation of nonimmediate DHRs must include approaches that can be applied during the different phases of the reaction. During the acute phase, monitoring markers in both skin and peripheral blood helps to discriminate between immediate and nonimmediate DHRs with cutaneous responses and to distinguish between reactions that, although they present similar clinical symptoms, are produced by different immunological mechanisms and therefore have a different treatment and prognosis. During the resolution phase, in vitro testing is used to detect the response of T cells to drug stimulation; however, this approach has certain limitations, such as the lack of validated studies assessing sensitivity. Moreover, in vitro tests indicate an immune response that is not always related to a DHR.
In this review, members of the Immunology and Drug Allergy Committee of the Spanish Society of Allergy and Clinical Immunology (SEAIC) provide an overview of the most widely used in vitro tests for evaluating nonimmediate DHRs.

Key words: Nonimmediate. Drug hypersensitivity. Diagnosis. Lymphocyte. In vitro.

Resumen

En la práctica clínica diaria las reacciones de hipersensibilidad no inmediata a fármacos son difíciles de manejar, debido a la heterogeneidad de las manifestaciones clínicas y a la falta de marcadores biológicos selectivos. Los métodos *in vitro* son necesarios para establecer el diagnóstico especialmente si tenemos en cuenta la baja sensibilidad de las pruebas cutáneas y el riesgo para el paciente de las pruebas de administración controlada. La evaluación *in vitro* de las reacciones no inmediatas a fármacos incluye diferentes aproximaciones que se pueden realizar en diferentes fases de la reacción. Durante la reacción en fase aguda es posible monitorizar diferentes marcadores en piel y en sangre periférica que puede ayudar a discriminar entre reacciones de hipersensibilidad a fármacos inmediatas y no inmediatas asi como diferenciar entre reacciones cutáneas que teniendo síntomas clínicos similares se distinguen en el mecanismo inmunológico y por tanto tendrán un tratamiento y pronóstico diferente. Durante la fase de resolución, los tests *in vitro* detectan principalmente la respuesta de las células T tras la estimulación con el fármaco implicado y son de utilidad aunque con ciertas limitaciones, tales como la falta de estudios validados que evaluen la sensibilidad. Además, estos métodos *in vitro* pueden indicar una respuesta inmunológica no siempre relacionada con una reacción clínica.
En esta revisión miembros de los comités de Inmunología y Alergia a medicamentos de la Sociedad Española de Alergologia e Inmunología Clínica (SEAIC) proporcionan una visión general de los métodos *in vitro* más frecuentes utilizados en el diagnóstico de las reacciones de hipersensibilidad no inmediatas a medicamentos.

Palabras clave: No inmediata. Hipersensibilidad a fármacos. Diagnóstico. Linfocito. *In vitro*.

Introduction

Diagnosis of hypersensitivity reactions to drugs is complex, and in vitro methods are less frequent and less standardized than other approaches. In this review, members of the Immunology and Drug Allergy Committee of the Spanish Society of Allergy and Clinical Immunology (SEAIC) evaluate the most widely used in vitro methods for the diagnosis of nonimmediate hypersensitivity reactions to drugs.

1. Classification of Drug Hypersensitivity Reactions

Adverse drug reactions have typically been classified as type A, which are predictable and dose-dependent, and type B, which are unpredictable and not dose-dependent. The latter are less frequent and include drug hypersensitivity reactions (DHRs) [1]. DHRs have traditionally been thought to be mediated by immunoglobulin (Ig) E or T cells [2] and are included in the Gell and Coombs classification (Table 1) [3]. Recently, the European Academy of Allergy and Clinical Immunology and the World Allergy Organization proposed a revised nomenclature to distinguish between allergic (IgE- and T cell–mediated) and nonallergic DHRs [2, 4]. Allergic DHRs require prior exposure to the same or a cross-reactive compound, although in some cases this information is difficult to obtain from the patient history or clinical records [5].

From a clinical point of view and based on the time between drug intake and development of symptoms, DHRs have traditionally been classified as immediate, accelerated, or delayed [6], and more recently as immediate and nonimmediate [7]. Immediate DHRs include mainly IgE-mediated reactions manifesting as urticaria, angioedema, bronchospasm, or anaphylaxis and appearing within 1 hour of administration. Nonimmediate DHRs, which are generally T cell–mediated, include urticarial, maculopapular, and more severe exanthema. Onset of clinical symptoms can be several hours after ingestion [8]. These types of reactions correspond to different immunological mechanisms (IgE or T cells), and different diagnostic approaches must therefore be applied [3,7,9,10]. As this classification has both mechanistic and clinical implications, some authors consider it problematic to separate DHRs based only on a cutoff of 1 hour [11]. Moreover, considering that the clinical history is often unreliable, it is difficult to establish exactly when the reaction started. In the case of nonsteroidal anti-inflammatory drugs (NSAIDs), a consensus has been reached on the classification of DHRs as acute (immediate to several hours) and delayed (>24 hours) [10], mainly because most reactions are not mediated by a

Table 1. Gell and Coombs Classification of Hypersensitivity Reactions to Drugs

Type	Denomination	Mechanisms	Manifestations
I	Immediate	IgE-mediated	Urticaria Angioedema Anaphylaxis Anaphylactic shock Bronchial asthma Rhinitis
II	Cytotoxic	Antibody-mediated	Immune hemolytic anemia Thrombocytopenia Blood diseases Organ-specific reaction
III	Immune complex	Immune complex-mediated	Serum sickness–like syndrome Vasculitis Organ-specific reaction
IV	Delayed	T cell–mediated	Maculopapular exanthema Delayed urticaria Stevens–Johnson syndrome Toxic epidermal necrolysis Organ-specific reactions Acute generalized exanthematic pustulosis Drug hypersensitivity syndrome with eosinophilia and systemic symptoms/drug-induced hypersensitivity syndrome Fixed drug eruption Contact eczema

Abbreviation: Ig, immunoglobulin.

specific immunological mechanism [12]. Difficulties in clinical evaluation arise when evaluating urticarial reactions, which can be immediate or delayed, thus indicating that the same clinical picture may be induced by different mechanisms (IgE, T cells, or even nonimmunological mechanisms, such as those involved in NSAID hypersensitivity) [3,10,13]. Therefore, in vivo and in vitro tests are necessary to demonstrate drug-specific IgE- or T-cell–mediated mechanisms [14].

In the present work, we define nonimmediate reactions [15] as those occurring more than 1 hour after the last dose of drug, involving 1 or more organs (with the skin being the organ most frequently affected), and inducing clinical manifestations such as exanthema, maculopapular exanthema (MPE), fixed drug eruption (FDE), or even severe cutaneous reactions [10]. Organ-specific reactions will also be addressed.

2. General Guidelines for Diagnosing Nonimmediate DHRs

Diagnosis of nonimmediate DHRs is complex and is usually made once the reaction has disappeared. The allergology workup (including clinical history, skin tests, and drug provocation tests) helps to identify the immunological mechanisms involved and the drug(s) responsible. In clinical practice, an accurate clinical history including the chronology of symptoms is necessary before selecting appropriate diagnostic tests [16]. However, the clinical history is particularly difficult in patients with nonimmediate DHR [17,18], and in many cases it is not easy to establish a temporal association between drug administration and onset of symptoms, especially in cases where a reaction appears more than 24 hours after intake. Moreover, the reaction can be confused with other skin disorders, such as autoimmune diseases [7], and may be similar to those induced by viruses, particularly in children; indeed, viruses can even act as cofactors by inducing symptoms and increasing both the duration and severity of the reaction.

Skin prick testing, intradermal tests with delayed reading, and patch testing have been widely used for diagnosis. Intradermal tests are usually recommended when drugs are available in injectable form; patch tests are recommended when drugs cannot be diluted. However, the sensivity of skin tests in nonimmediate reactions is somewhat low, especially in children [18-20]. Therefore, the drug provocation test is often the only test available, although it is risky, time-consuming, and contraindicated in severe reactions such as bullous eruptions or desquamative exanthema. In addition, the interval between drug administration and development of symptoms ranges from more than an 1 hour to several days, and the distribution and extension of skin manifestations can complicate evaluation of the results [21]. Accordingly, it is necessary to develop in vitro tests with sufficient sensitivity and specificity to confirm the diagnosis.

The difficulty in diagnosing nonimmediate DHRs once the reaction has resolved makes it necessary to evaluate the reaction while it is occurring. Thus, we can identify the suspected drugs, describe in detail the symptoms affecting the skin and other organs (including signs of poor prognosis such as bullous hemorrhagic lesions, mucosal involvement, or internal organ involvement), and, finally, analyze possible cofactors, such as viral disease. As the skin is the most commonly affected organ, it is easy to document the reactions by means of photographs and biopsy specimens, which could show the clinical picture and the mechanism involved [22]. Moreover, in cases of doubt between immediate and nonimmediate reactions, the tryptase level obtained during the acute phase should subsequently be compared with the baseline level.

3. The Skin as the Target Organ

In nonimmediate DHRs, the skin is the most commonly involved organ; other sites may or may not be involved [18]. Clinical manifestations vary depending on the immunological mechanism involved, although MPE and delayed urticaria are the most frequent reactions observed [23,24]. Other, more severe reactions include acute generalized exanthematous pustulosis (AGEP), drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome, bullous eruptions such as erythema multiforme, Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) [25,26], FDE, contact dermatitis [27], and serum sickness–like syndrome [28].

The reason why a drug administered orally or parenterally mainly affects the skin is not known, although immunological mechanisms and/or the drug metabolism capacity of cutaneous cellular components may be involved [25,28]. Skin was traditionally thought to be no more than a physical and biochemical barrier protecting the organism from outside agents, but in recent years it has been shown to have a role in the immune response, since its cellular composition includes mast cells, macrophages, dermal dendritic cells, keratinocytes, and Langerhans cells, which act as static skin components to produce proinflammatory cytokines and induce recruitment of other cells that are part of the dynamic component [24,26]. These cells include antigen-presenting cells (APC), such as Langerhans cells, dendritic cells, monocytes, and macrophages, as well as T lymphocytes expressing skin-homing receptors, such as the cutaneous lymphocyte antigen (CLA) and various chemokine receptors (eg, CCR10, CCR4, CCR6), which represent the cellular basis of the immunological memory in the skin [23,25,27].

Cutaneous DHRs are often initiated as an immune response to a chemical interacting with the skin. For this interaction to occur, drugs must have a stable association with a protein so that hapten-protein conjugates can be produced. The classical pathway in skin sensitization could involve formation of these conjugates by keratinocytes, which are taken up by dendritic cells that process them for presentation to specific T lymphocytes [3,29,30]. However, not all the drugs involved in allergic reactions can form hapten–protein adducts in their native form, and reactive drug metabolites are sometimes necessary for conjugation with the protein. Metabolites are formed during the processes to eliminate the drug from the organism. Although these processes are normally associated with decreased toxicity (detoxification), the metabolites produced are sometimes more toxic and reactive than the parent drug (bioactivation), thus increasing the possibility of inducing DHR [31]. Cytochrome P450 enzymes [32] are one of the major families involved in metabolism and have the capacity to catalyze the oxidative biotransformation of xenobiotics, including drugs [33] (Figure 1). Although these

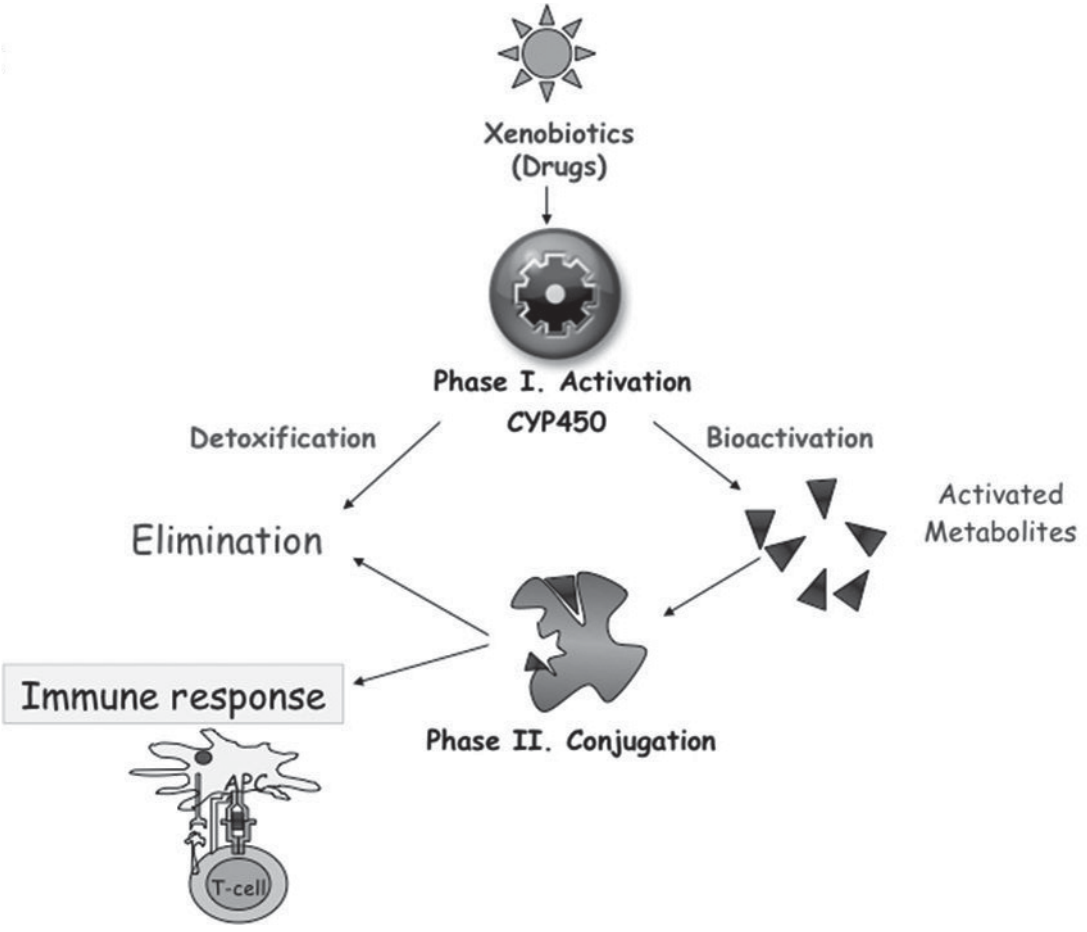

Figure 1. Drug biotransformation and adduct formation.

enzymes are mainly expressed in the liver [32], they have also been detected in the skin, where keratinocytes exhibit the highest drug biotransformation capacity, followed by Langerhans cells [34,35].

Identification of reactive metabolites with the capacity to form adducts could help to prevent potential risks and improve drug safety. However, associations between metabolites and DHR have not been clearly defined [36], probably because of the poor accessibility of the target organ (mainly the liver) and the low concentration and half-life of the metabolites [37].

In Vitro Studies During the Acute Phase

Nonimmediate DHRs have very heterogeneous clinical symptoms, which reflect the different mechanisms involved, and although most reactions are T cell–mediated, many other cells could play a role in their development, for example, various cell subsets and inflammatory mediators. Reactions can be monitored in order to analyze the underlying immunopathological response by performing serial determinations in both peripheral blood and skin from the acute phase of the reaction through to the disappearance of clinical symptoms. Table 2 shows the main determinations performed during the acute phase of the reaction.

1. Evaluation of Peripheral Blood

The analysis of cell subpopulations and mediators in peripheral blood helps to characterize the underlying immunological mechanism. Comparisons between immediate and nonimmediate DHRs have revealed differences in effector cells, namel a type 1 helper T cell (T_H1) pattern with expression of interferon (IFN) γ, interleukin (IL) 12, and tumor necrosis factor (TNF) α, as well as downregulation of IL-4 in nonimmediate reactions and a T_H2 pattern with production of IL-4 and downregulation of IFN-γ in IgE-mediated reactions [38,39]. This cytokine production is the consequence of previous transcription factor expression, with T bet being characteristic of nonimmediate DHRs and c-maf and GATA3 being characteristic of immediate DHRs [38].

In nonimmediate reactions, the T-cell subset depends on the clinical manifestations. Studies have shown that CD4 T cells are involved in MPE, DRESS syndrome, and AGEP [3,40,41], whereas CD8 T cells are the main effector cells in FDE and

Table 2. In Vitro Tests for Evaluating Nonimmediate Drug Hypersensitivity Reactions During the Acute Phase and Resolution Phase

Reaction Phase	Test	Marker Determination	Mechanism
Acute	Flow Cytometry	Transcription factors	• $T_H1/T_H2/T_H17$/Treg
		Cell subpopulations	• Helper T lymphocytes • Cytotoxic T lymphocytes • Natural killer cells • B lymphocytes • Skin-homing T cells • Naïve or memory cells • Activation marker • Regulatory T cells • Immature dendritic cells • Mature dendritic cells
		Cytotoxic markers	• Cytotoxicity
		Cytokines	• $T_H1/T_H2/T_H17$/Treg
		Chemokines / receptors	• $T_H1/T_H2/T_H17$/Treg
	Real time RT-PCR	Transcription factors	• $T_H1/T_H2/T_H17$/Treg
		Cytotoxic markers	• Cytotoxicity
		Cytokines	• $T_H1/T_H2/T_H17$/Treg
		Chemokines/receptors	• $T_H1/T_H2/T_H17$/Treg
	ELISA	Cytokines Chemokines	• $T_H1/T_H2/T_H17$/Treg
Resolution	LTT (radioactivity)	H3-Thymidine incorporation	• Cell proliferation
	LTT (flow cytometry)	CFSE incorporation	• Cell proliferation
		Cell and cytokine marker expression	• Subpopulation phenotype
	ELISPOT	Secreting molecules	• Cell secreting mediators
	CD69 test	Activation marker expression	• Cell activation

Abbreviation: CFSE, carboxyfluorescein diacetate succinimidyl ester: ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot; LTT, lymphocyte transformation test; RT-PCR, reverse-transcriptase polymerase chain reaction.

TEN [42-44]. However, CD4 T cells may also be involved in TEN [45]. Moreover, a recent study revealed the participation of CD8 T cells in the epidermis of patients with MPE after patch testing [46], although further studies are needed to confirm whether the participation of this subpopulation is also observed during the acute phase of MPE.

Finally, determination of cytotoxic inflammatory markers, such as perforin, granzyme B, and, more recently, granulysin, which play an important role in MPE, DRESS syndrome, and SJS-TEN [42,47,48], can help to characterize the clinical manifestation. Levels of these markers increase in the acute phase and normalize once the process resolves [45,49]; the changes produced are associated with clinical severity [39]. Figure 2 shows the immunological mechanisms involved in the different clinical pictures of nonimmediate DHR.

2. Analysis of Skin Biopsies

The skin is the main target organ of DHRs, and its accessibility is of great importance for the evaluation of the underlying immunological process. The different clinical manifestations produced in nonimmediate DHRs result from a differential interplay between the immune system and skin tissue. Studies in MPE have demonstrated the presence of a mononuclear infiltrate in the perivascular dermis, with T lymphocytes, mainly CD4 T cells [45,50], neutrophils, and, occasionally, eosinophils [51]. Subcorneal pustules with dermal edema, spongiosis, leukocytoclastic vasculitis, and focal keratinocyte necrosis have been found in patients with AGEP [46], and the activated CD4 and CD8 T cells have an effector role, inducing keratinocyte death by the production of granulocyte macrophage colony-stimulating factor, IFN-γ, and TNF-α with the release of perforin/granzyme and Fas [52,53]. However, these T cells also produce IL-8 (CXCL8), which can activate neutrophils and chemoattract them to the skin, where they produce the characteristic clinical symptoms of AGEP [52,54-56]. In FDE, effector memory CD8 T cells are also responsible for necrosis of keratinocytes, most of which are intradermal [46,57,58]. TEN, one of the most severe DHRs, is characterized by massive necrosis of keratinocytes that is produced both by cytotoxic and apoptotic mechanisms induced by specific CD4 and CD8 T cells expressing the skin-homing receptors CLA and CCR10 and producing IFN-γ, TNF-α, perforin/granzymeB/granulysin, and Fas [50]. In DRESS

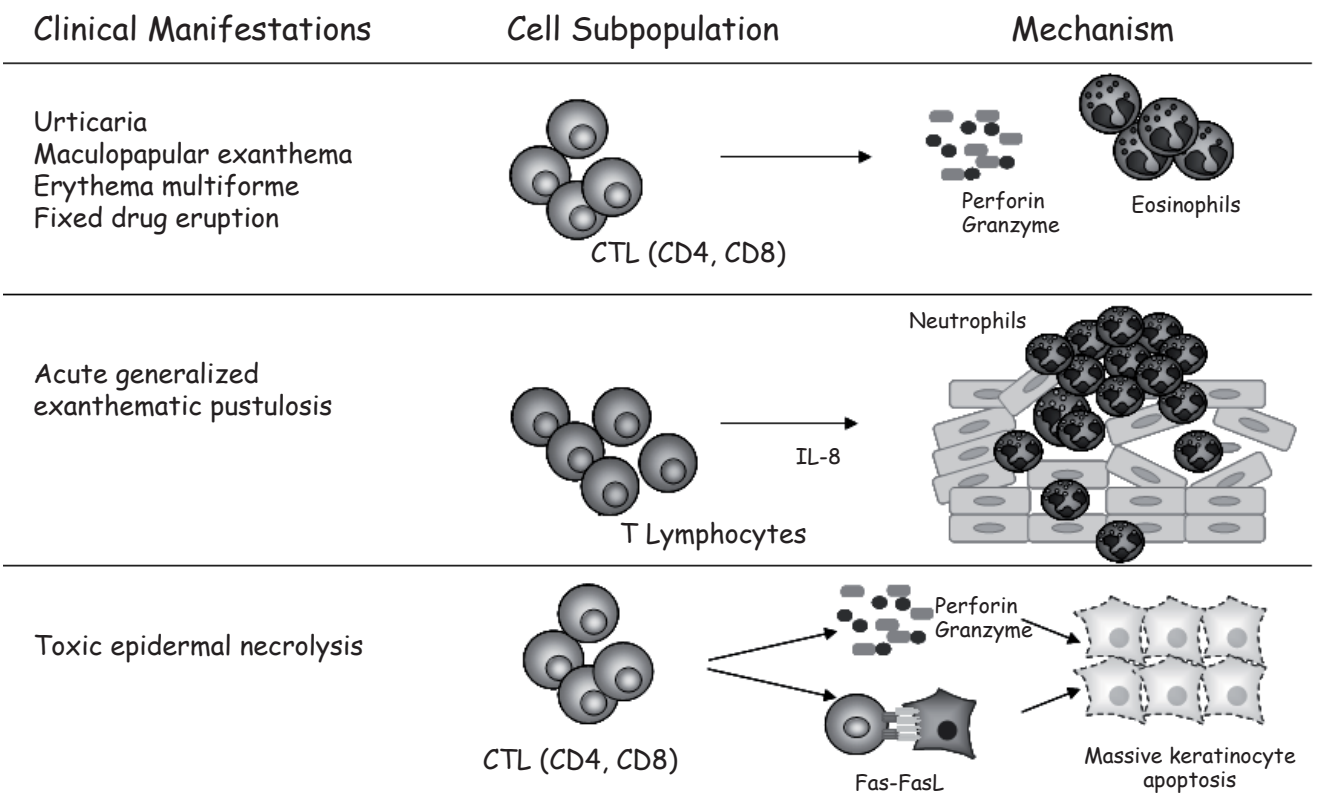

Figure 2. Clinical entities and mechanisms involved in nonimmediate drug hypersensitivity reactions. CTL indicates cytotoxic T lymphocytes.

syndrome, a severe drug-induced reaction, CD4 and CD8 T cells act as effector cells, and CD4 T cells with a T_H2 pattern produce IL-5, which is involved in eosinophil activation and recruitment to the skin [59].

3. Cell Trafficking Between Compartments

Cell trafficking between compartments is not completely understood. An antigenic stimulus originating in the skin is believed to trigger a specific immunological process with the arrival of lymphocytes via peripheral circulation and the interplay of different ligands and receptors, including adhesion molecules and chemokine receptors. Depending on the clinical symptoms, DHRs can be analyzed in peripheral blood and skin simultaneously by studying the participation of different T-cell subsets and inflammatory mediators [60].

In MPE, which is the most frequent reaction and therefore the best studied, T cells express the skin-homing and chemokine receptors CLA, CCR6, and CCR10 [61] in both peripheral blood and skin, whereas their corresponding chemokines, CCL20 and CCL27, are only found in the skin, where they are produced. These chemokines are responsible for the recruitment of the T cells expressing their specific ligands. Moreover, T cells expressing the T_H1 chemokine receptor CXCR3 have been found in both compartments, whereas their corresponding ligands CXCL9 and CXCR10 have only been found in the skin of patients with drug-induced MPE [61]. Chemokine production in the skin correlates with high cutaneous expression of TNF-α and IFN-γ produced by keratinocytes that upregulate CCL27 [62] and CXCL9 and CXCL10, respectively [63].

In both DRESS syndrome and MPE, an increase in the levels of T_H2 cytokines (eg, IL-5), several chemokines (eg, RANTES), and eotaxins has been observed, thus explaining the involvement of eosinophils expressing CCR3 [64]. In AGEP, IL-8 produced by activated T cells is involved in neutrophil activation and migration [54,56]. In TEN, levels of T cells expressing CCR10 and its corresponding chemokine CCL27 are increased in the skin during the acute phase [65]. In FDE, intraepidermal CD8 T cells have been shown to be cytotoxic for keratinocytes [58]; these cells can be activated by mast cells in the epidermis, leading to a wheal and flare–like reaction [58].

Monitoring the acute response of DHR can help discriminate between immediate and nonimmediate reactions—particularly important in urticaria—and help to characterize the cutaneous response and distinguish between reactions that, whilst having similar clinical symptoms, have different immunological mechanisms and therefore vary in their treatment and prognosis. Although these studies cannot identify the drug involved in the reaction, they still have an important role, as some drugs induce specific types of DHR more often (eg, DRESS syndrome induced by anticonvulsants).

In Vitro Studies Once the Reaction Has Disappeared

Once the reaction subsides, the in vitro studies aimed at assessing the nonimmediate DHR try to enhance a T-cell immune response after exposure to the culprit drug(s). Table 2 shows the most important techniques that can be applied once the reaction has resolved.

1. Lymphocyte Transformation Test

The lymphocyte transformation test (LTT) has been used for the past 20 years for the in vitro diagnosis of nonimmediate DHRs. This test has several advantages over in vivo tests, including a better safety profile and the option to evaluate different drugs at the same time. A World Allergy Organization international survey revealed that the LTT was more widely used in Europe for evaluating DHRs than elsewhere, especially for DHRs induced by ß-lactams (77.8%), non–ß-lactam antibiotics (58.3%), and NSAIDs (36.1%). The reactions most commonly evaluated using the LTT were drug-induced hypersensitivity syndrome in 65.6%, SJS in 65.5%, MPE in 46.9%, and TEN in 46.9% [66].

With this technique, mononuclear cells isolated from the patient's peripheral blood are incubated with increasing concentrations of the suspect drug under appropriate culture conditions where T cells undergo blastogenesis and generate cytokines such as IL-2, which then proliferate. The process of incubation lasts 6 days, after which time the proliferative response is evaluated by incorporation of 3H-thymidine, as a sign of drug-specific T-cell recognition. In recent years, flow cytometry with carboxyfluorescein diacetate succinimidyl ester (CFSE) staining has been applied to LTT. The nonradioactive approach, the CFSE proliferation assay, enables proliferating cells to be characterized in terms of their subpopulation and cytokine production [50]. This method has revealed heterogeneous subpopulations in the drug-specific proliferative response, which may involve both effector and regulatory T cells [67]. Therefore, evaluation of a DHR enables more accurate characterization that can in turn help to establish a diagnosis.

Several studies have shown that the sensitivity and specificity of the LTT depend on the drugs and clinical entities involved in the DHR [68-72]. The most frequent drugs studied are ß-lactam antibiotics, followed by antiepileptics, particularly carbamazepine [40,68,71,73]. In the case of immediate and nonimmediate reactions induced by ß-lactams, sensitivity was 64.5% and 57.9%, respectively, and specificity 92.8% [68,74]. In the case of hypersensitivity to anticonvulsants, the LTT showed a sensitivity of around 70%, with high positive and negative predictive values in highly imputable cases [75]. However, since nonimmediate DHRs are often severe reactions, final confirmation of the culprit drug using the gold standard drug provocation test cannot be performed, thus preventing us from obtaining accurate figures for sensitivity and specificity. It is noteworthy that the LTT enables identification of the drug involved and cross-reactivity, but it is also important to bear in mind that a negative LTT result does not always indicate the absence of a nonimmediate DHR and a positive result does not necessarily reflect an effector response. As stated above, both effector and regulatory T cells can proliferate in the LTT, and this may be the reason why patients with good tolerance to a drug can have a positive LTT result.

Sensitivity can be markedly improved when LTT is performed at the optimal time point [76]. This timing varies depending on the clinical manifestations: LTT should be performed within 1 week for patients with MPE and SJS/TEN, whereas 5-8 weeks is optimal for patients with DRESS syndrome [76]. As the immune system is still strongly activated during the acute stage, there may be high background proliferation [77]. In contrast to in vitro testing for immediate reactions, whose sensitivity is highly influenced by the disappearance of specific IgE antibodies over time, LTT can measure memory T cells, thus maintaining the possibility of having positive responses for many years. However, although positive responses have been found 12-20 years after the DHR [68,72,78], in other cases reactivity has disappeared after 3-4 years. Accordingly, and because it is not possible to predict this disappearance, LTT is recommended 2-3 years after the reaction [74].

Since the LTT is a cellular test, it is more complex than serum tests, and many factors affect its reproducibility and sensitivity. Good performance requires antigen presentation to T cells, which is usually achieved in the conventional LTT (based on peripheral blood mononuclear cells) by the presence of monocytes and B cells. In recent years, however, the LTT has undergone modification, with the inclusion of monocyte-derived dendritic cells as APCs. This variant offers higher sensitivity and specificity, as demonstrated in DHRs induced by amoxicillin, heparins, corticosteroids, and contrast media, and provides the possibility of detecting a response over a longer period of time, thus preserving sensitivity to the culprit drug [71,78-80].

Another important issue for the evaluation of DHRs using LTT is the influence of drug metabolism. Although this process is normally associated with detoxification for better secretion, during processing the drug metabolites become more reactive and gain the ability to bind to macromolecules and therefore to cause a DHR [31]. It has been demonstrated that nitroso sulfonamide metabolites are strongly recognized by T cells from patients experiencing DHR to sulfonamide [81]. The importance of including drug metabolites to increase the T-cell proliferative response has also been shown in nonimmediate DHRs to anticonvulsants using an in vitro cell system including drug-metabolizing cells (CYP-transfected-HaCaT) and dendritic cells [82]. Therefore, knowledge of the drug metabolites that interact with the immune system and induce DHR can help to increase the sensitivity of in vitro tests.

2. Enzyme-Linked Immunosorbent Spot

Determination of cytokines is a promising in vitro readout system in the diagnosis of DHR [83]. Production after T-cell activation, which occurs at 48-72 hours, could also make the incubation time shorter than that required for cell proliferation in the LTT while conserving its advantages. The enzyme-linked immunosorbent spot (ELISPOT) assay determines the increase in the number of cells producing a specific cytokine after their activation. This technique, which is similar to a conventional

enzyme-linked immunosorbent assay, is based on detection of the cytokine by a plate-immobilized specific antibody and identification by an enzymatically labeled secondary specific cytokine-antibody [84]. The resulting plate contains a number of spots, each corresponding to a different single cell-secreting cytokine or cytotoxic marker [46,48,77,83] and, in theory, any other secreted molecule. ELISPOT is higly sensitive and can detect fewer than 25 secreting cells per million peripheral blood mononuclear cells [85].

ELISPOT has been used to detect lymphocytes secreting cytokines such as IFN-γ, IL-5, or IL-13 from allergic patients in the presence of the culprit drug. Recently, this test was also used to evaluate the cytotoxic response in DHR by determining the release of granule content (granzymes and perforin) and cytokines (IFN-γ) by cytotoxic cells after activation with the culprit drug, showing differences between allergic patients and tolerant individuals [46,48]. The test showed high sensitivity and specificity, although in some cases results did not correlate with the LTT, probably because cytotoxicity-based tests measure effector cell function, which is different to the proliferative response, where the cell subpopulation may be heterogeneous.

A recent study comparing ELISPOT with skin testing in the diagnosis of cephalosporin-induced MPE showed that determining both IFN-γ and IL-5 is more sensitive than skin testing for the diagnosis of cephalosporin allergy [86]. Therefore, quantification of cytokines such as IL-2, IL-5, IL-13, and IFN-γ is a promising diagnostic tool in most DHRs [83], although further studies are needed with larger series of allergic patients and controls to evaluate the sensitivity and specificity of the technique, together with the cutoffs to be used.

3. Detection of CD69

After activation, lymphocytes express several molecules on their surface. One of these markers is CD69, which is expressed early after cell triggering. Some studies have shown that expression of CD69 after in vitro stimulation correlates with cell proliferation; therefore, detection of CD69 could be useful as an in vitro marker of DHR [87], although no firm consensus exists on this matter [88]. In a study published in 2008, Beeler et al [89] evaluated the diagnostic utility of CD69 in 15 patients with nonimmediate DHR, finding that all those cases with a positive LTT result also had increased expression of CD69 on T cells after 48 hours of stimulation exclusively with the drugs implicated in the reaction. These results suggest that CD69 is a promising marker for the detection of drug-reactive T cells in the peripheral blood of patients with nonimmediate DHR. The same study also demonstrated that the frequency of CD69 T cells after stimulation with the drug was much higher than the frequency of proliferating drug-specific T cells evaluated by CSFE staining and the number of cytokine-secreting cells assessed using ELISPOT. Importantly, further analysis revealed that drugs are able to stimulate a few truly drug-specific T cells, which are responsible for the activation of other non–drug-specific bystander T cells (via IL-2 secretion), which reacted with upregulation of CD69 [83]. As this response only occurs if drug-specific T cells are present, the specificity of the test would not be affected, although sensitivity would increase. The main limitation of this approach is that some drugs can induce upregulation of CD69 even without specific recognition. Consequently, the drugs used in either assay should be evaluated carefully in nonallergic individuals [83].

Expression of CD69 is a measure of exposure and immune recognition, with the advantage over the LTT that it is faster. However, systematic research analyzing its diagnostic capacity compared with tolerant individuals is necessary.

Of note, all the tests that evaluate DHR during the resolution phase have limitations that need to be taken into account when interpreting the results. Many of the studies using these methodologies are based on small case series and even case reports; moreover, larger series usually report heterogeneity in drugs and clinical symptoms. In addition, it is important to remember that positive in vitro test results indicate an immune response that is not always related to a DHR. Further studies are needed for clinical validation.

Organ-Specific Reactions: A Special Issue

Most organ-specific reactions, excluding those appearing as part of generalized reactions, can be considered T-cell–mediated reactions [90]. The organs most frequently involved are the liver, lung, kidney, and blood. Unlike the skin, it is difficult to obtain biopsy specimens. In addition, skin testing is not useful, and drug provocation testing is contraindicated because of the associated risk. Therefore, in vitro testing can aid in the diagnosis of these reactions, as it is risk-free and can be used to evaluate different drugs simultaneously and determine the T-cell mechanism involved [74].

1. Liver DHR

Although allergic hepatitis is less frequent than toxic hepatitis, it has a higher clinical impact because it can occasionally be fatal. Clinically, it is characterized by the presence of malaise and increased liver enzyme values. Liver DHR is usually T-cell–mediated with implication of CD4, CD8, and NK cells, accompanied in some cases by antibodies to cellular components in autoimmune hepatitis [73,91-94].

Diagnosis is complex and is based mainly on the clinical history and liver biopsy findings. As for specific immunological tests, the LTT is the most commonly used diagnostic method [95-97]. Results are variable because, as in cutaneous reactions, the lymphocyte response depends on the type of drug, drug presentation, and the interaction between T cells and dendritic cells. However, this test is now the diagnostic method used in drug-induced allergic hepatitis [74,83,98] and in drug-induced allergic pancreatitis [99].

2. Lung DHR

Different drugs can induce lung infiltrates with eosinophilia, especially antibiotics and NSAIDs, and some induce progression to fibrosis. LTT [100], identification of the chemokines and cytokines produced by sensitized T cells after specific drug stimulation [100,101], and flow cytometry [102] have all been used for diagnosis.

LTT is the most widely used in vitro method for identifying drug-induced sensitization, although it cannot differentiate

sensitization from clinical symptoms [74,103,104]. While its sensitivity and specificity are not known, positive LTT results have been reported in drug-induced eosinophilic interstitial lung diseases [105]. However, in desquamative interstitial pneumonia [106,107] and minocycline-induced pneumonia [108], the LTT was not useful for diagnosis, probably because, in the latter disease, minocycline induced a decrease in T-cell proliferation [109,110]. In the case of methotrexate [111], the LTT was unable to confirm the diagnosis [112].

3. Kidney DHR

The most frequent reactions include acute interstitial nephritis, glomerulonephritis, and nephrotic syndrome. These reactions are probably mediated by drug-specific T-cell infiltration of kidney cells, indicating that the drug can induce an immune response in the kidney or reactivate T cells that migrate to this organ. This reaction can produce eosinophilia in urine instead of in blood [113]. The mechanisms involved are similar to those involved in DHR affecting the skin. It is not known whether the drug inducing a reaction affecting only the kidney needs to be metabolized in this organ in order to induce the specific metabolite finally recognized by the immune system. If so, this may explain why it is so difficult to obtain a positive LTT result in these reactions and why the reaction only affects the kidney [113].

4. Blood DHR

This group of DHRs mainly includes eosinophilia, thrombocytopenia, neutropenia, and hemolytic anemia, which may or may not appear together with other manifestations of DHR. LTT is rarely positive (<10%) in these reactions [8].

To summarize, in nonimmediate DHR, in vitro methods are necessary to establish a diagnosis, particularly considering the low sensitivity of skin tests and the risks associated with drug provocation testing. These reactions can be evaluated both in the acute phase and once the reaction has resolveds these approaches that are complementary. Currently, no single method is able to diagnose nonimmediate DHR with sufficient accuracy.

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Conflicts of Interest

The authors declare that they have no conflicts of interest. Research is part of their daily activity. All authors had full access to all the data (including statistical reports) and can take responsibility for the integrity of the data and the accuracy of the data analysis.

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