

Hypersensitivity Reactions to β -Lactams: Relevance of Hapten-Protein Conjugates

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■ Abstract

β -Lactams (BL) are the drugs most frequently involved in allergic reactions. They are classified according to their chemical structure as penicillins, cephalosporins, monobactams, carbapenems, and clavams. All BL antibiotics have a BL ring that is fused to a 5-member or 6-member ring (except in monobactams) and has 1, 2 or 3 side chains (except in clavams). Differences in chemical structure mean that a wide range of BLs are recognized by the immune system, and patients may experience clinical reactions to one BL while tolerating others. Diagnosis is based on skin and in vitro testing, although both display low sensitivity, possibly because they are based on drugs or drug conjugates that are not optimally recognized by the immune system. BLs are haptens that need to bind to proteins covalently to elicit an immune response. These drugs have a high capacity to form covalent adducts with proteins through nucleophilic attack of amino groups in proteins on the BL ring. Allergenic determinants have been described for all BLs, although benzylpenicillin is the most widely studied. Moreover, formation of BL-protein adducts is selective, as we recently demonstrated for amoxicillin, which mainly modifies albumin, transferrin, and immunoglobulin heavy and light chains in human serum. Given the complexity of BL allergy, understanding the immunological mechanisms involved and optimization of diagnostic methods require multidisciplinary approaches that take into account the chemical structures of the drugs and the carrier molecules, as well as the patient immune response.

Key words: Betalactams. Hapten. Carrier. Proteins. IgE.

■ Resumen

Las betalactamas (BL) son los fármacos implicados más frecuentemente en reacciones alérgicas. Se clasifican según su estructura química en penicilinas, cefalosporinas, monobactamas, carbapenems y clavamas. Poseen un anillo betalactámico que, excepto en las monobactamas, está fusionado a un anillo de cinco o seis miembros y, excluyendo las clavamas, tienen 1, 2 o 3 cadenas laterales. Las diferencias en las estructuras químicas resultan en un amplio rango de BLs, que puede ser discriminado por el sistema inmune, con inducción de reacciones clínicas a una BL y tolerancia a otras. El diagnóstico está basado en pruebas cutáneas e in vitro, aunque ambas presentan una baja sensibilidad. Esto podría deberse a que los fármacos o conjugados de fármacos empleados en estos tests que no se reconocen de manera óptima por el sistema inmune. Las BLs son haptenos que necesitan de su unión covalente a proteínas para inducir una respuesta inmunológica. Estos fármacos presentan una elevada capacidad para formar aductos covalentes con proteínas mediante el ataque nucleofílico de grupos aminos de proteínas al anillo BL. Aunque la bencilpenicilina ha sido la mejor estudiada, también se han descrito determinantes alérgicos del resto de BLs. Además, la formación de los aductos BLs-proteína muestra selectividad, así se ha demostrado recientemente para la amoxicilina, que principalmente modifica la albúmina en suero (HSA), la transferrina y las cadenas ligeras y pesadas en suero humano. Dada la complejidad de la alergia a BL, el conocimiento de los mecanismos inmunológicos implicados y la optimización de los métodos diagnósticos requieren de abordajes multidisciplinares teniendo en cuenta tanto la estructura química de los fármacos y de las moléculas portadoras, como las respuestas de los pacientes.

Palabras clave: Betalactamas. Hapteno. Portador. Proteínas. IgE.

Introduction

β -Lactam (BL) antibiotics remain the first choice for treating a large number of bacterial diseases. They have been classified according to their chemical structure as penicillins, cephalosporins, monobactams, carbapenems, and clavams (Table 1). The general chemical structure of BL antibiotics consists of a BL ring that, except in monobactams, is fused to a 5-member ring (thiazolidine, oxazolidine, and dihydropyrrole) or 6-member ring (dihydrothiazine). Both fused rings (bicyclic structure) constitute the core or nuclear region of the molecule. All the classes of antibiotics except the clavams have a side chain (R1) bound to the BL ring. Cephalosporins and carbapenems include other side chains (R2 and/or R3) bound to the non-BL ring (Table 1). Differences in the chemical structure of the nuclear region and the side chain have enabled the development of a wide range of BL antibiotics that can be recognized by the immune system [1]. This finding has important clinical implications, since some patients can develop reactions towards one BL while tolerating others.

The prevalence and incidence of allergic reactions to BLs in the general population are not known. Although penicillin allergy is self-reported by approximately 10% of the population [2], less than 24% of initial adult cases are finally confirmed [3], and this figure decreases to less than 10% in children [4]. Consequently, when only the clinical history is considered, the disease is overdiagnosed, with the subsequent unnecessary prescription of more expensive alternative antibiotics, more adverse effects, and development of bacterial resistance. Therefore, the public health implications of BL allergy are considerable, and major efforts must be made to ensure accurate diagnosis [2].

Although all commercialized BLs are capable of inducing allergic reactions, the prevalence of some reactions seems to be higher and correlates with consumption patterns. Differences have been detected between countries and in the same population over time. In general, benzylpenicillin (BP), initially the most frequent inducer, has been progressively replaced by amoxicillin (AX), cephalosporins (albeit to a lesser extent), and, more recently, clavulanic acid (CLV) [5-7].

Hypersensitivity reactions to BLs have been classified as immediate and nonimmediate based on the time to onset after intake [8]. Immediate reactions usually appear within 1 hour of intake and are mediated by specific IgE. The clinical symptoms are urticaria, which may be accompanied by angioedema, anaphylaxis, or anaphylactic shock. Nonimmediate reactions occur more than 1 hour after intake and can be T cell-mediated. The typical symptoms are maculopapular or urticarial exanthema, although more severe cutaneous and organ-specific reactions can occur. Reactions occurring in the overlap between immediate and nonimmediate reactions are known as accelerated reactions and were initially considered IgE-mediated reactions [9], although other studies indicate that they are T cell-mediated [10-11].

Diagnosis of BL allergy has been based mainly on skin testing with immediate and/or delayed readings. The classic BP reagents used for skin testing have been benzylpenicilloyl-poly-L-lysine (PPL) and minor determinant mixture (MDM), originally consisting of BP, benzylpenilloic acid, and benzylpenicilloic acid, although currently it only includes

benzylpenilloic acid [12]. When other BLs, such as AX, cephalosporins [5,7], or CLV are involved in the reactions, it is necessary to include them in the test [6]. The sensitivity of skin testing depends not only on the BL involved in the reactions and used for testing, but also on the type of reactions, and is less than 70% in the case of immediate reactions and even lower in nonimmediate reactions [7,13].

In vitro testing can also be used to evaluate immediate allergic reactions to BL antibiotics (immunoassay and basophil activation test) [14-16] and nonimmediate allergic reactions (lymphocyte transformation test) [17-18]. Sensitivity differs between the tests, although it is usually lower than with skin testing. The reasons for the low sensitivity of skin testing and in vitro testing are not well understood, although it could be due to the use of a drug structure or conjugate that is not well recognized by the immune system.

Immunological Mechanisms

BLs are small molecules (<1000 Da) whose interaction with the immune system can be explained by 3 major working hypotheses (Figure 1): the hapten hypothesis, the danger hypothesis, and, more recently, the pharmacological interaction (PI) hypothesis.

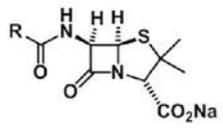
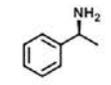
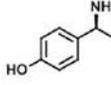
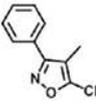
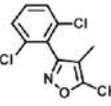
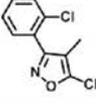
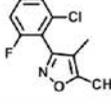
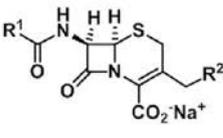
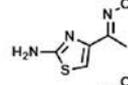
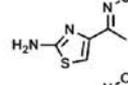
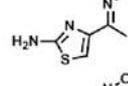
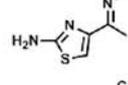
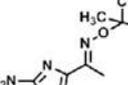
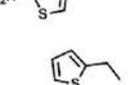
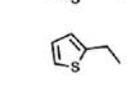
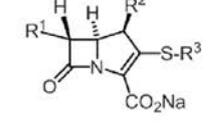
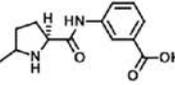
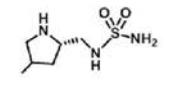
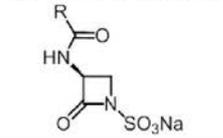
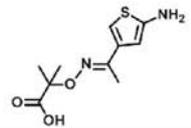
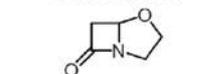
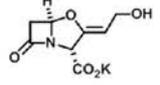
The hapten hypothesis is based on observations that small molecules did not induce an immune response unless they were covalently bound to a protein [19]. This concept has been widely established in allergic contact dermatitis caused by chemical agents [20-21] and in allergic reactions to BLs [22]. The other 2 hypotheses try to explain the interaction of the drug with the immune system without the need for covalent binding to proteins. The danger hypothesis is based on the fact that cell damage induces production of danger signals that interact with the immune system, thus activating antigen-presenting cells [23]. Drugs themselves or concomitant substances originating from viral infections can induce danger signals and thus initiate allergic reactions. The PI hypothesis, on the other hand, suggests that a reversible interaction between the drug and the T-cell receptor major histocompatibility complex (MHC) could take place. This would induce activation of T lymphocytes and thus initiate an immune response against the drug in an MHC-restricted and processing-independent pathway [24-26].

BL antibiotics have been used as models of the hapten hypothesis because of their high reactivity or capacity to bind to proteins through the nucleophilic attack on the BL ring by the amino groups in the protein [27].

However, for other non-chemically reactive drugs, it is difficult to identify the metabolites that act as haptens [28]. The danger hypothesis has also been used to explain nonimmediate reactions to BLs in the context of a viral infection [18,29], but the PI concept has never been confirmed in the case of BL antibiotics.

As stated above, allergic reactions to BLs can be mediated by different immunological mechanisms [30], which are normally IgE- or T cell-mediated. In the former, drug-specific IgE binds to high-affinity receptors (Fc ϵ RI) on the surface of tissue mast cells and circulating basophils. The binding of drug-carrier molecule adducts (multivalent antigen) to at least 2 adjacent IgE molecules induces degranulation of mast

Table 1. Chemical Structure of β -Lactam Antibiotics

	<i>R</i>	Name	<i>R</i>	Name	<i>R</i>	Name	
PENICILLINS 		Benzylpenicillin		Carbenicillin		Ampicillin	
		Penicillin V		Tircacillin		Amoxicillin	
		Meticillin		Oxacillin		Dicloxacillin	
		Ciclacillin		Cloxacillin		Flucloxacillin	
CEPHALOSPORINS 		-H	Cefalexin			Cefotaxime	
			Cefaloglycin			Ceftriaxone	
			Cefamandole			Cefepime	
			Cefonicid			Cefodizime	
		-H	Cefadroxil			Ceftazidime	
			Cefprozil			Cefalotine	
			Cefuroxime			Cefalonium	
CARBAPENEMS 		-H		Imipenem			Ertapenem
				Meropenem			Doripenem
MONOBACTAMS 			<i>R</i>	Name			
					Aztreonam		
CLAVAMS 					Potassium clavulanate		

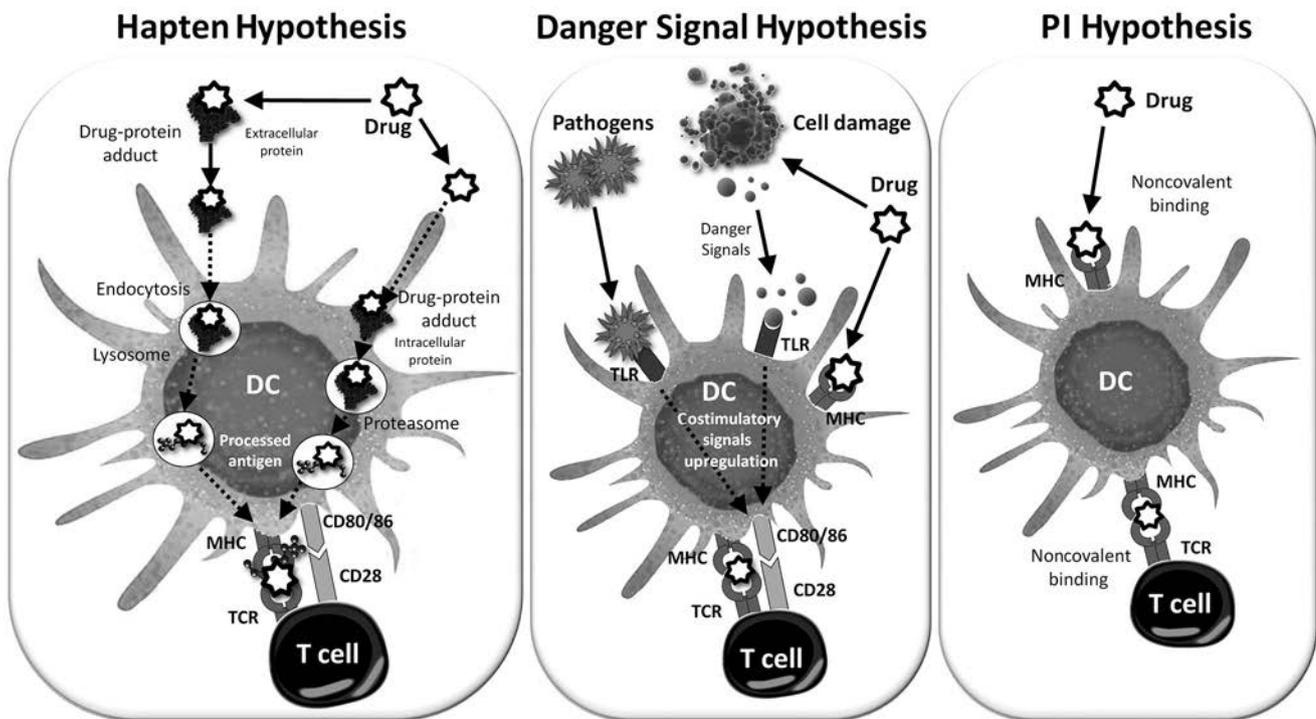


Figure 1. Representation of the 3 hypotheses that can explain drug immunological recognition: hapten hypothesis, danger hypothesis, and pharmacological interaction hypothesis. DC indicates dendritic cell; MHC, major histocompatibility complex; TCR, T-cell receptor; TLR, Toll-like receptor; PI, pharmacological interaction.

cells and basophils, leading to the release of inflammatory mediators, including histamine, leukotrienes, and cytokines. In T cell-mediated reactions, the drug-carrier molecule adducts interact with specific T cells via the T-cell receptor. These cells release proinflammatory mediators and cytokines, which attract monocytes, macrophages, and other T cells that are responsible for mediating the inflammatory response.

The antigenic determinants of BL have been investigated mainly in the context of IgE-mediated reactions. Studies performed with human IgE and murine monoclonal antibodies have shown that although small differences in the chemical structure are essential for specific recognition, the whole structure (including the BL and the protein carrier) is necessary for the formation of the complete antigenic determinant. The relevant parts of penicillins to be considered in recognition are the common BL ring and the side chain structure [31-35]. These findings are consistent with clinical evidence supporting the production of both selective responses and cross-reactive responses to different BLs [33].

Consequently, when studying the immunological mechanisms involved in BL allergy, it is necessary to take into account the chemical structure of the drug itself, the carrier molecule, and the patient's response.

β -Lactam Determinants

Identifying the drug metabolites involved in allergic reactions to BL is important, not only for understanding the underlying immunological mechanisms but also for improving

diagnostic tests. To this end, several studies have been performed with different BLs.

Penicillins

Benzylpenicillin

BP is the most widely studied drug and is considered the reference model for the study of allergy to BLs. The major determinant is benzylpenicilloyl (BPO), which results from the conjugation of BP with amino groups from carriers [27,33,36]. The reaction takes place quickly via opening of a BL ring. The effectiveness of this process lies in the high reactivity of the ring resulting from the high tension of the structure (4-member BL ring fused to the 5-member thiazolidine ring). This behavior is common to all penicillins. The stability of BPO has enabled its chemical characterization and its use as BPO acid or BPO amide in diagnostic studies. At least 3 epitopes have been recognized in the BPO molecule. These are the side chain, the whole molecule bound to a carrier by ring opening, and the bicyclic nuclear structure [34].

The BPO amide structure is also included in immunoassays, namely, the commercially available ImmunoCAP-FEIA [14] and in-house assays for the radioallergosorbent test (RAST) [37-38]. Comparison of these techniques revealed that the specificity of ImmunoCAP-FEIA ranged from 83.3% to 100% and the sensitivity from 12.5% to 25%; in the case of RAST, specificity ranged from 66.7% to 83.3% and sensitivity from 42.9% to 75% [37].

The generation of BP metabolites with different possibilities for conjugation led researchers to seek other determinants. The structures considered were benzylpenicilloic acid, benzylpenicillenic acid, benzylpenamaldate, benzylpenaldate, benzylpenicoyl, and benzylpenicanyl [39]. There has been speculation about the formation of some of these structures. For example, in the case of the penicanyl determinant proposed by Baldo [40], the BL ring is purported to remain intact, even in the presence of reactive amino groups. Moreover, advanced immunochemical analyses of IgE specificities are lacking [41].

Some of the determinants described above have been used for skin testing, with higher sensitivity than BP itself in some cases. Consequently, skin testing with the major and minor antigenic determinants of penicillin is recommended in diagnostic guidelines from Europe [42-43] and the United States [44]. The commercialized major determinant PPL is formed by the conjugation of BP to poly-L-lysine (PLL) [27] and the MDM consisting of BP, its hydrolysis product, penicilloate, and its corresponding decarboxylated product, penilloate (Allergopharma and Hollister-Stier) [45] (Figure 2).

The number of positive skin tests for PPL and MDM has decreased over time [7,46]. Initially, 78% of patients tested had positive skin test responses to PPL, MDM, or both; however, this percentage dropped to 42% (PPL) and 22% (MDM) after 10 years. Nevertheless, major and minor determinants of BP continue to play a key role in diagnosis, as they induce a positive response in 46% of patients with positive skin test results to penicillins, while only 14% of patients are positive to both these determinants [47]. Differences have been found between countries. Reports from the USA indicate that among patients with allergic reactions to penicillin confirmed by skin test, 75% were positive to PPL, 10% to MDM, and 14.8% to PPL and MDM [48].

The MDM commercially available since 2003 (Diater SA) only contained BP and benzylpenicilloate, with several studies indicating that these components were equivalent to those from Allergopharma [49-51]. Recently, they have been substituted by purer and more stable products consisting of benzylpenicilloyl octa-L-lysine as the major determinant and benzylpenilloate (penilloate) as the minor determinant, with a sensitivity of 61.36% and specificity of 100%, as demonstrated in a multicenter clinical trial [12] (Figure 2).

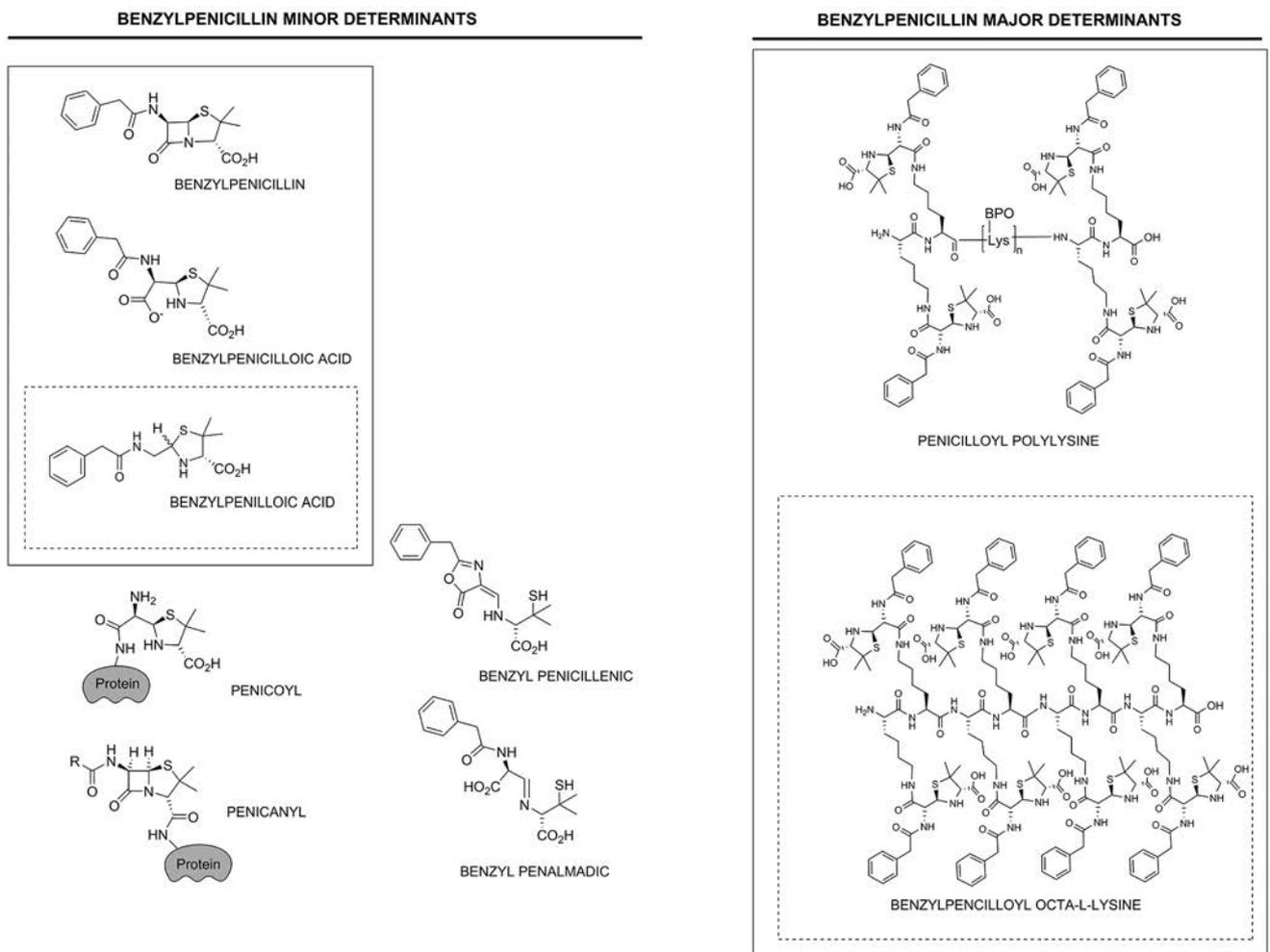


Figure 2. Minor and major determinants of benzylpenicillin. Previous commercially available structures are shown in the solid figure. Currently commercialized structures are shown in the dotted figure. BPO indicates benzylpenicilloyl.

Amoxicillin

The major antigenic determinant of AX is the amoxicilloyl (AXO) amide, which results from the opening of BL by amino groups, in much the same way as BP. The AXO structure is included in *in vitro* tests (ImmunoCAP [14] and in-house materials for diagnostic research studies [37,52,53]). However, in skin testing, the free BL, AX itself, is used with the assumption that it will be conjugated to a carrier protein after application on the skin. In fact, the addition of AX to the panel of haptens for skin testing in patients with suspected reaction to penicillins is currently recommended for routine skin testing by the European Network for Drug Allergy [8].

Complete solubility of the drug is required for correct performance of the skin test; however, the solubility of AX is open to debate [54], because there are 2 types of commercially available drug: the injectable type, a sodium salt, and the oral formulation, which is made in trihydrate form. Only the injectable sodium salt form can be easily dissolved in water at physiologic pH to reach the concentrations recommended for skin testing (20 mg/mL).

Until recently, the lack of commercialized AX diagnostic reagents meant that the injectable sodium salt was used in many countries [55], with several studies proving the validity of this approach for diagnosing immediate hypersensitivity to penicillins [7,13,46,55]. Since 2010, AX specifically designed for skin testing has been commercialized by Diater, with one study indicating that it is equivalent to the injectable form of AX for both skin testing and *in vitro* testing [54].

Despite the inclusion of AX in hapten panels for skin testing, skin test sensitivity in patients with immediate allergic reactions to penicillins is not optimal, ranging from 50% to 70% [7,42,47,56]. This raises the question of whether the use of additional minor determinants of AX would improve sensitivity, as was initially reported with BP [57-58]. Two structures derived from AX were evaluated as minor determinants. These were amoxicilloic acid, which results from hydrolysis of BL and is equivalent to BPO acid, and

diketopiperazine, which results from intramolecular acylation by the amino group of the AX side chain. However, inclusion of these minor determinants did not improve the diagnostic capacity of either skin tests or *in vitro* tests [59].

It is not well understood why patients whose test results are positive to BP usually respond to several penicillin derivatives, including AX; however, most patients who are allergic to AX only respond to this compound. It would appear that both the hydroxyl and the amino groups in the side chain structure confer important recognition features on the AX molecule. Moreover, the specificity of IgE is highly related to the BL responsible for the first sensitization. Thus, IgE from patients originally sensitized to BP can recognize AX owing to the similarity of their whole structures [43], whereas IgE from patients first sensitized to AX mainly recognizes the AX side chain but does not cross-react with BP [60]. Therefore, patients first sensitized to AX can tolerate BP, whilst the administration of several doses of BP cannot induce the increase in specific IgE to AX [61].

Cephalosporins

Although the number of publications on allergic reactions to cephalosporins is relevant and growing, the antigenic determinants of cephalosporins are not yet completely elucidated. This is striking, given the fact that the determinants of penicillins are perfectly identified, and cephalosporins and penicillins share the reactive BL functional group.

The main difference between cephalosporins and penicillins is the ring to which the BL is fused, namely, a 5-member thiazolidine ring in penicillins and a 6-member dihydrothiazine ring in cephalosporins. These structural differences lead to differences in the electrophilic properties of the BL carbonyl and, therefore, in the potential to bind to proteins and form a determinant [31]. In cephalosporins, the lower reactivity of the BL ring slows carrier conjugation, and the R2 chemical structure modulates this reactivity depending on its ability to polarize electronic binding. The presence of a good leaving group at the 3' position increases the reactivity of the BL via elimination of R2 (Figure 3).

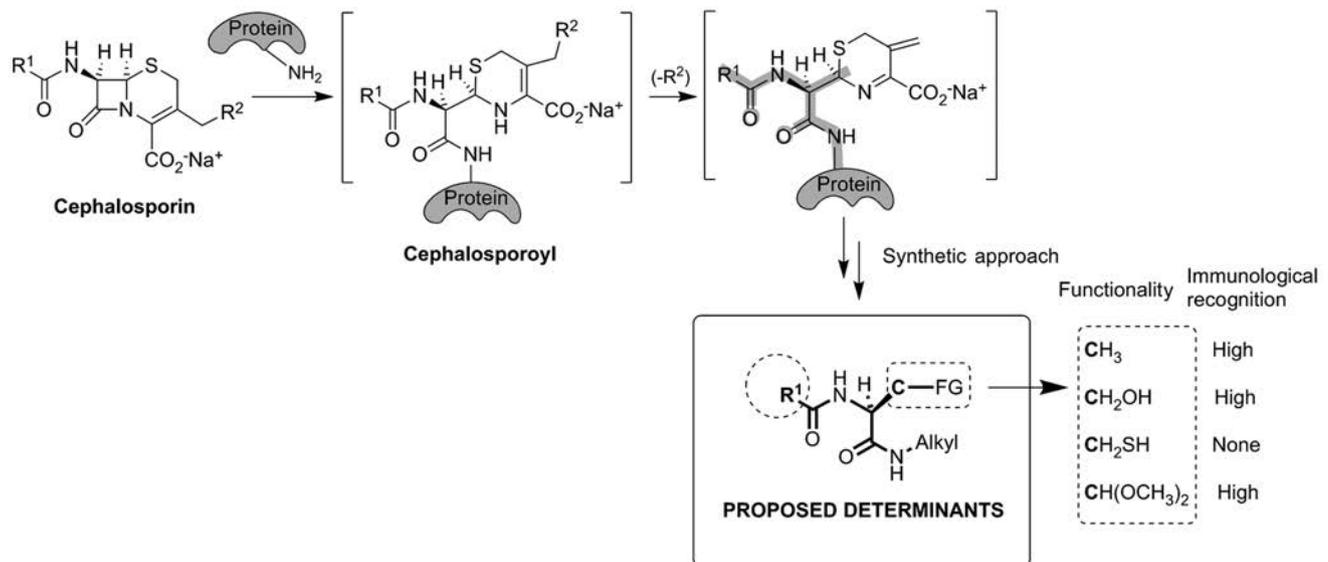


Figure 3. Proposed hypothesis of cephalosporin protein conjugation and description of the synthetic determinants of cephalosporins.

It is assumed that the formation of the determinants of cephalosporins requires nucleophilic attack at the BL carbonyl by the amino groups of the protein, thus leading to protein conjugation. The opening of the BL leads to a clear loss of the R2 substituent. The resulting conjugate is unstable and is degraded through dihydrothiazine ring rupture. The complex reactivity of cephalosporins and the high number of possible fragmentation structures is well described in the literature [31].

Lack of understanding of the chemical structure of cephalosporin determinants has hampered the development of commercial *in vitro* tests. In fact, only cefaclor is available for ImmunoCAP-FEIA, which is based on the reactivity of cefaclor with amines, although the resulting structure has not been identified. This strategy of reacting cephalosporins with amines is also used in *in-house* immunoassays for RAST [37,43], but the resulting determinant structures have not been described. A study of RAST inhibition with monomer structures consisting of this conjugation concludes that the R1 side chain, rather than the BL structure, seems to play a dominant role in determining the specificity of immunologic reactions to cephalosporins [43].

Taking into account both the evidence of the departure of the R2 group as a rule and the important role of the R1 structure in specific immune recognition, a series of determinants resulting from BL conjugation have been proposed. After the opening of the BL ring by proteins, the dihydrothiazine ring undergoes degradation and is lost. As a result, only R1 and part of the BL moiety of the cephalosporin remain linked to the protein. The evaluation of a series of proposed synthetic determinants including different R1 side chains and different functionalities in the remaining part of the BL (no derivatization [H], alcohol functionality [OH], thiol functionality [SH], and carbonyl functionality [C(OMe)₂]) have allowed us to gain insight into the chemical structure of the determinant. Specific IgE shows enhanced recognition of proposed epitopes with adequate functionality at C-3 and specificities mainly related to the R1 acyl side chain [32,62] (Figure 3).

In view of the high degree of recognition of IgE antibodies at the side chain of BL molecules, cross-reactivity between AX and cefadroxil, which contains an identical side chain, has been studied in patients who are selectively allergic to AX. The results indicate that the percentage of cross-reactivity between BLs with an identical side chain is high (38%) and that this critical part of the molecule seems to play a key role in these results [63].

Venemalm [64] studied amino cephalosporins and identified a pyrazinone allergenic degradation product of cefaclor and cephalexine. The formation of the structure involved intramolecular cyclization by the side chain amino group in intermediate products of aminolysis. In sera positive to cefaclor by ImmunoCAP, specific IgE to similar pyrazinone determinants was observed in 60% of cases

Carbapenems

Studies on the antigenic determinants of carbapenems are scarce. However, reactions between carbapenems and proteins have been described. These reactions result in a stable structure consisting of the open BL ring bound to the carrier proteins through an amide linkage. The specificity of the response to carbapenems is likely to be related to the relatively stable dihydropyrrrole ring [65]. The stability of this conjugate would lead to a high density of homogeneous epitopes.

A wide range of values for cross-reactivity between penicillins and carbapenems has been reported in various studies [66-67]; however, more recent prospective studies have shown the incidence of cross-reactivity between penicillin and carbapenem in skin tests to be around 1% [68-70].

Monobactams

Monobactams are BL compounds in which the BL ring is alone. The only commercially available monobactam is aztreonam, whose R substituent consists of the same side chain as ceftazidime. This molecule might be expected to form relatively stable homogeneous conjugates [65]. Three cell lines of hybridoma that produce antibodies recognizing aztreonam have been established [71]. Two of the monoclonal antibodies recognize aztreonam and ceftazidime, indicating that the same acyl side chain is a relevant part of the determinant. The monoclonal antibody from the third line recognized a new antigenic determinant and displays broad cross-reactivity with several BLs [68].

Clavams

CLV is the only BL included in this group and is prescribed in combination with AX. The complex chemistry of CLV has made it difficult to advance our knowledge of its antigenic determinants; diagnosis is incomplete when an AX-CLV combination is involved in the reaction [6]. A CLV skin test

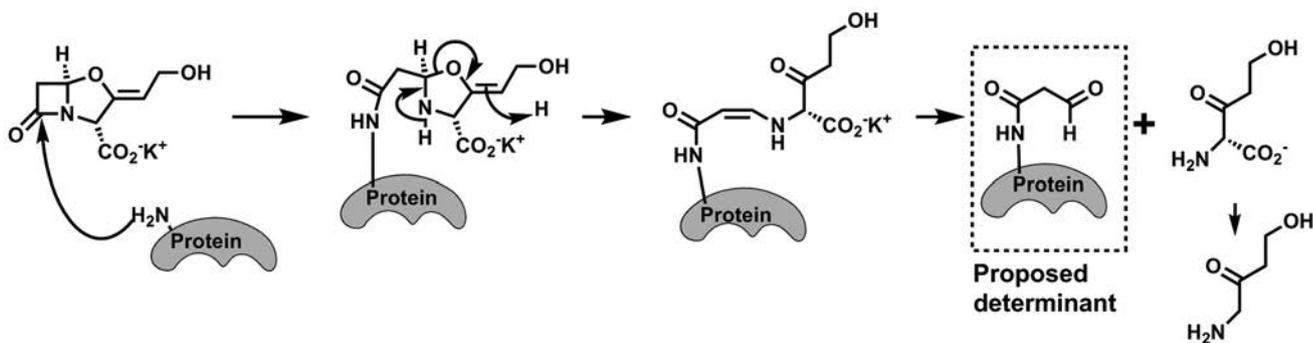


Figure 4. Proposed clavulanic acid pathway conjugation.

(DAP Clavulanic, Diater SA) was recently patented and commercialized for diagnostic purposes. Skin testing results indicate that selective immediate reactions to CLV do occur and account for around 30% of immediate allergic reactions to the combination of AX-CLV [6]. Of note, patients with an immediate positive response to CLV are younger than those who are positive to AX, probably because younger patients have had increased exposure to CLV; consequently, the number of patients allergic to CLV is expected to increase in the coming years [4].

The reactivity of CLV can be explained by its strained bicyclic structure (BL fused to an oxazolidine ring, which presumably reflects the substitution of an oxygen atom for sulfur), the lack of an acylamino substituent at C-6, and the presence of an *exo-p*-hydroxyethylidene function at C-2 [72]. These structural differences increase chemical reactivity to the CLV structure. It has been reported that CLV can generate stable products from an intermediate structure resulting from the opening of the 2 rings [73] through a mechanism similar to the formation of adducts with β -lactamases [74], as shown in Figure 4. These structures may be involved in IgE recognition [65]. However, the instability of the structure after protein conjugation may involve more complex degradation pathways, leading to multiple possible determinants. More in-depth studies using defined structures are still needed.

Candidate Carrier Proteins

The immune response to BLs is determined not only by the chemical structures of the metabolites, but also by the nature of the adduct itself and the characteristics of its uptake, processing, and presentation by antigen-presenting cells [75]. Therefore, studies have been performed to analyze binding of BLs to carrier molecules, as well as the ability of the adducts formed to activate the immune system and their immunopathogenic relevance. It is still not fully known how haptentation occurs *in vivo*, nor do we fully understand the amplification mechanisms that cause an allergic reaction quickly after intake and the severe clinical manifestations. One of the main limitations of *in vivo* studies is the difficulty involved in detecting the BL adducts produced after treatment. The first studies were performed to characterize the carrier proteins modified by BP *in vitro*, and the initial experiments were performed in alkaline pH (experimental conditions to increase protein modification) [76], because the formation of adducts is slower and detection is more difficult under physiological conditions. New studies have been performed under different experimental conditions to identify serum and cellular proteins that can act as carrier molecules (see below).

Human Serum Albumin as a Model

Human serum albumin (HSA) is the most abundant protein in plasma. It presents extraordinary ligand-binding capacity and has been shown to play a crucial role as a carrier for endogenous and exogenous compounds [77], including several drugs. Based on this evidence, HSA was traditionally

considered the main target protein in the haptentation process for penicillins, and most studies have focused on characterization of the penicilloyl-HSA adducts.

In an early study by Lafaye et al [78], the number of penicillin molecules, which are covalently bound to HSA, was directly proportional to the drug concentration. In fact, the detection of penicilloyl groups decreased exponentially over time after interruption of drug treatment, and the half-life of penicilloylated HSA proved to be shorter than or equal to that of nonmodified HSA [78]. Some studies have explored the detection and identification of HSA residues modified by BL antibiotics. Early studies by Yvon et al [79,80] revealed BPO-HSA adducts in samples of serum from patients treated with BP or samples generated *in vitro* and in which binding of BPO was observed in 6 of the 59 lysines of HSA (Lys 190, 195, 199, 432, 541, and 545) using separation of trypsinized peptides based on high-performance liquid chromatography and peptide sequencing by Edman degradation.

More recently, the modification of HSA by flucloxacillin [81], piperacillin [82-83], BP [84-85], and AX [21,86] has been characterized using tandem mass spectrometry coupled to a liquid chromatography system performed with serum from patients treated with drugs or samples modified *in vitro*. In addition, the grade of HSA modification is dependent on the drug concentration and the incubation time used for the *in vitro* modification [82-85], with BLs showing a preference for some HSA-specific residues (Table 2 and Figure 5). Interestingly, modified residues appear to vary widely between patients in *in vivo* assays, although in the case of flucloxacillin, 3 residues always appear along with other residues that vary from one individual to another [81] (Table 2). Although the factors that determine which amino acids are modified by BLs are not known, binding to lysine residues may be favored by the presence of a serine close to the polypeptide chain or the tertiary configuration of the protein [79-80].

Other Serum Proteins

Serum proteins other than HSA could be involved in the haptentation process and in the induction of an immune response; however, very little is known about their nature or their role in the development of a hypersensitivity reaction. In a study by Lafaye and Lapresle [78] based on blood samples from patients treated with BP, BPO groups were detected in a fraction of serum proteins where HSA had been removed, but the modified proteins detected were not identified.

In a later study, HSA and transferrin were identified as target proteins for ampicillin using 2-dimensional electrophoresis and immunological detection with plasma from patients treated with this drug [87].

In a study by our group based on immunological and proteomics methods, we identified serum proteins modified *in vitro* by AX. We observed that serum proteins other than HSA, such as transferrin and immunoglobulin (light and heavy chains), were also modified by AX [22,86]. The fact that other relatively abundant serum proteins did not form detectable adducts under the experimental conditions used suggests that factors other than plasma protein concentration could determine which serum proteins were targets for BL antibiotics.

Table 2. HSA Residues Modified by Amoxicillin, Benzylpenicillin, Flucloxacillin and Piperacillin^a

Reference	Amoxicillin		Benzylpenicillin			Flucloxacillin	Piperacillin	
	[21]	[86]	[79-80]	[84]	[85]	[81]	[82]	[83]
Lys 4					+ ^b		+ ^c	
Lys 12					+ ^b		+ ^c	
Lys 20				+ ^{b,c}				
Lys 73					+ ^b			
Lys 137				+ ^{b,c}	+ ^b	+ ^b	+ ^c	
Lys 159				+ ^{b,c}	+ ^b			
Lys 162						+ ^{b,c}	+ ^c	
Lys 190	++ ^b	++ ^c	+ ^b	+ ^{b,c}	+ ^b	++ ^{b,c}	+ ^{b,c}	+ ^b
Lys 195			+ ^b	+ ^{b,c}	+ ^b	+ ^{b,c}	+ ^{b,c}	+ ^b
Lys 199	++ ^b		+ ^b	++ ^{b,c}	+ ^b	+ ^{b,c}	+ ^c	+ ^b
Lys 212				+ ^{b,c}	+ ^b	++ ^{b,c}	+ ^c	
Lys 351	+ ^b			+ ^{b,c}	+ ^b	+ ^{b,c}	+ ^c	
Lys 359					+ ^b			
Lys 372				+ ^b				
Lys 414					+ ^b			
Lys 432	+ ^b		+ ^b	+ ^{b,c}	+ ^b	+ ^{b,c}	+ ^{b,c}	+ ^b
Lys 436				+ ^b	+ ^b			
Lys 444					+ ^b			
Lys 525				+ ^{b,c}	+ ^b	+ ^{b,c}	+ ^c	
Lys 541	++ ^b		+ ^b	+ ^{b,c}		+ ^{b,c}	+ ^{b,c}	+ ^b
Lys 545	+ ^b		+ ^b	+ ^{b,c}		+ ^c	+ ^c	
Lys 560					+ ^b			

^aThe most reactive residues, which were identified in the shortest times of incubation or with the lowest concentrations of drugs, are shown as ++.

^bResidues identified in HSA-drug adducts generated in vitro.

^cResidues identified in HSA purified from the serum of patients who have received drug treatment (see the corresponding articles for the route of administration).

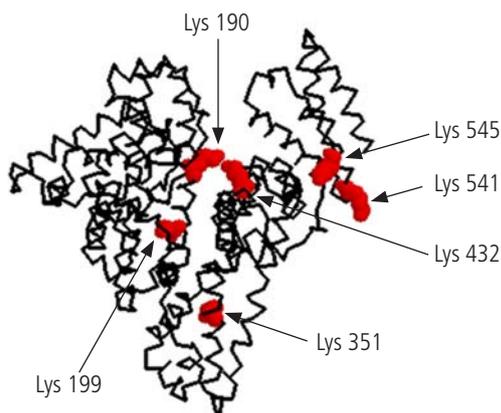


Figure 5. HSA model with the lysine residues modified by amoxicillin, benzylpenicillin, flucloxacillin, and piperacillin identified by mass spectrometry in several of the studies referenced in the text. Atomic coordinates are taken from code 1A06 [105] of the Protein Data Bank.

Cellular Proteins

The formation of antigenic determinants with cellular proteins is well documented. BP derivatives have the ability to bind to the cellular membranes of macrophages [88-89] and monocytes [90-91]. Formation of these antigenic determinants is slower with cellular proteins than with serum proteins [92]. A recent study carried out using piperacillin and T-cell culture revealed no cellular adducts [83]. Covalent binding of AX to HLA class I molecules on the surface of B cells has been reported to cause overexpression of these molecules [93]. Moreover, we recently showed that confocal fluorescence microscopy with a biotinylated AX analog (AX-B) revealed the presence of intracellular protein adducts and modified proteins in extracts from AX-B-treated cell lines (monocytes, B-lymphoma cells, and macrophages) with different patterns, showing that the haptentation process may be cell type-dependent [94].

Relevance of Hapten-Protein Conjugates in In Vitro Diagnostic Tests

In order to improve the sensitivity of diagnostic tests, it is necessary to take into account the identity of the BL determinants, the carrier proteins, and the characteristics of the haptenation process.

Quantitation of Specific IgE

Immunoassay is the most widely used method for the quantitation of specific IgE to BLs. In the routinely used commercial fluoroimmunoassay platform ImmunoCAP-FEIA (Phadia), haptens are bound to spacers on a solid phase with high surface capacity. Specificity is 83.3%-100% and sensitivity 12.5%-45%, depending on the clinical manifestations and skin test results [14]. In a recent study, a general decrease in the sensitivity of this technique was observed over time [95].

Noncommercial radioimmunoassay is also used for the determination of specific IgE antibodies. This approach consists of a solid phase (sepharose, cellulose discs) on which the BL structure is conjugated to a carrier molecule (such as PLL, HSA, or aminospacers).

The best-known approach for the study of BL allergy is RAST, in which a hapten-carrier conjugate is anchored to a functionalized cellulose solid phase. The sensitivity of RAST with BL-PLL is around 50%, with a specificity of around 80-95% [7,8]. The use of different carrier molecules has been investigated in 2 comparative studies [96,97]. Blanca et al [96] analyzed the influence of 2 carrier molecules (PLL and HSA) in the detection of specific IgE to BP and found that the nature of the carrier influenced the capacity for binding IgE antibodies in RAST and that BPO-PLL was the best-recognized structure [96]. García et al [97] subsequently confirmed these results by testing the detection of BP-specific IgE bound to 3 different carrier molecules: PLL, HSA, and aminospacers. The test revealed better diagnostic sensitivity and specificity for BPO-PLL and BPO-aminospacer than for BPO-HSA. These results may be explained by the importance of hapten density in relation to assay sensitivity [98-99], because HSA has a much lower capacity for binding to penicillin molecules than PLL. Furthermore, not all the BPO determinants bound to the HSA are accessible for antibodies [100].

These results highlight the importance of the molecules used as carriers for BLs in the detection of specific antibodies and therefore in the sensitivity and specificity of immunoassays.

In order to improve the sensitivity of these tests, recent studies have focused on the use of dendrimer structures as carrier molecules for BLs [101]. Montañez et al [102] proposed nanoconjugates consisting of polyamidoamine dendrimers bound to BP as an effective tool for the study of adverse immune responses to drugs in humans. The same authors also proposed new strategies for the functionalization of solid phases, which would enable a higher density of antigen determinants on the same solid phase and thus improve the sensitivity of the test [38,103].

Other studies have focused on the search for new solid phases that would enable increased surface density of the immobilized nanoconjugates. Zeolite crystals [103] and silica nanoparticles [53] have been tested as solid phases to which

dendrimeric structures conjugated to BL structures are bound. These approaches enhanced the sensitivity of the test without appreciably decreasing specificity, thus indicating that these materials are promising candidates for improving in vitro clinical diagnostic practice.

Cellular Tests

Basophil Activation Test by Flow Cytometry

The basophil activation test has been used as a diagnostic test for IgE-mediated reactions to drugs and is based on the in vitro stimulation of basophils by the culprit drug in peripheral blood. Several studies have analyzed the value of the test in the evaluation of hypersensitivity reactions to BL and revealed a sensitivity of 49% and specificity of 93% [15-16]. It has been observed that the sensitivity of this assay is higher when haptens (drugs) not previously conjugated to a carrier molecule are used [16]. Although the mechanism by which these haptens interact with specific IgE bound to the surface of basophils eliciting their activation remains unknown, the kinetics of conjugation of BL with serological proteins suggest that a yet unknown BL-carrier adduct produced during the test procedure interacts with the specific IgE.

Lymphocyte Transformation Test by Flow Cytometry

The lymphocyte transformation test is an in vitro test based on the measurement of T-cell proliferation in cell culture in the presence of the drug of interest. In vitro proliferation suggests a previous in vivo reaction due to sensitization. This test can be used for the in vitro diagnosis of T cell-mediated reactions to BLs, although it is not routinely recommended and is still considered a research tool.

In a study performed by Whitaker et al [82], HSA modified by piperacillin generated during a lymphocyte transformation test was detected in the supernatant. In order to confirm the antigenicity of piperacillin-HSA adducts, the authors generated them in vitro under physiological conditions and observed a positive proliferation in peripheral blood mononuclear cells and T-cell clones from piperacillin-allergic patients.

Similarly, Meng et al [84] observed lymphocyte proliferation using BP-HSA conjugates generated in vitro, although stimulation was also detected when unconjugated BP was used [84]. These results are consistent with those of other studies [85,104], indicating that HSA conjugated to different BL structures, as well as to the free drug, can stimulate the proliferative response of lymphocytes from patients with hypersensitivity to BLs. More studies are needed in order to determine whether the inclusion of well-characterized drug-carrier adducts can improve the sensitivity of this test.

Concluding Remarks

BLs are small molecules that act as haptens and must bind to a protein carrier to form BL-protein adducts with the ability to induce an immune response that can vary: some patients react to a specific BL and show tolerance to others, whereas other

patients react to all BLs. This pattern is continuously changing as BLs with different chemical structures are commercialized. In order to characterize the different phenotypes of BL-allergic patients, it is crucial to identify the BL (or its metabolites) recognized by the immune system and the protein carrier.

Nanotechnology is a promising approach that will enable better and more reliable IgE detection. The identification of BL derivatives, suitable dendrimer structures, and well-recognized proteins as more efficient carrier candidates will increase our knowledge of IgE recognition and B-cell response and thus enable personalized diagnosis and more appropriate alternative treatment with BL antibiotics. The benefits of nanotechnology in this setting should be addressed using a multidisciplinary approach involving chemists, immunologists, and allergists.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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