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REVIEW

In vitro analysis of metabolic predisposition to drug hypersensitivity reactions

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SUMMARY

Idiosyncratic hypersensitivity reactions may account for up to 25% of all adverse drug reactions, and pose a constant problem to physicians because of their unpredictable nature, potentially fatal outcome and resemblance to other disease processes. Current understanding of how drug allergy arises is based largely on the hapten hypothesis: since most drugs are not chemically reactive *per se*, they must be activated metabolically to reactive species which may become immunogenic through interactions with cellular macromolecules. The role of drug metabolism is thus pivotal to the hapten hypothesis both in activation of the parent compound and detoxification of the reactive species. Although conjugation reactions may occasionally produce potential immunogens (for example, the generation of acylglucuronides from non-steroidal anti-inflammatory drugs such as diclofenac), bioactivation is catalysed most frequently by cytochrome P450 (P450) enzymes. The multifactorial nature of hypersensitivity reactions, particularly the role of often unidentified, reactive drug metabolites in antigen generation, has hampered the routine diagnosis of these disorders by classical immunological methods designed to detect circulating antibodies or sensitized T cells. Similarly, species differences in drug metabolism and immune system regulation have largely precluded the establishment of appropriate animal models with which to examine the immunopathological mechanisms of these toxicities. However, the combined use of *in vitro* toxicity assays incorporating human tissues and *in vivo* phenotyping (or, ultimately, *in vitro* genotyping) methods for drug detoxification pathways may provide the metabolic basis for hypersensitivity reactions to several drugs. This brief review highlights recent efforts to unravel the bases for hypersensitivity reactions to these therapeutic agents (which include anticonvulsants and sulphonamides) using drug metabolism and immunochemical approaches. In particular, examples are provided which illustrate breakthroughs in the identification of the chemical nature of the reactive metabolites which become bound to cellular macromolecules, the enzyme systems responsible for their generation and (possibly) detoxification, and the target proteins implicated in the subsequent immune response.

Keywords hypersensitivity reactions metabolism P450 antigen

INTRODUCTION

The metabolic conversion of drugs to chemically reactive products (bioactivation) is now established as a prerequisite for many idiosyncratic drug reactions. Oxidative reactions are frequently involved, and several important groups of enzymes (cytochromes P450, flavin-containing monooxygenases, prostaglandin synthetase and various tissue peroxidases) have been implicated. If not adequately detoxified, the highly reactive products of xenobiotic bioactivation may interact (irreversibly) with cell macromolecules and mediate cellular necrosis, carcinogenicity or hypersensitivity reactions (Fig. 1). Idiosyncratic hypersensitivity reactions may account for 3–25% of all adverse drug reactions [1], and pose a constant problem to physicians because of their unpredictable nature (with respect to both chemical structure and apparent dose), potentially fatal outcome and resemblance to other disease processes [1,2].

The clinical manifestations of drug 'allergic reactions' are quite diverse, and include fever, urticaria, anaphylaxis, serum sickness and haematologic and tissue toxicities [2]. Our current understanding of how drug allergy arises is based largely on the hapten hypothesis: since most drugs are not chemically reactive *per se* (notable exceptions include penicillins, penicillamine and captopril), they must be activated metabolically to reactive species which may become immunogenic through interactions

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with cellular macromolecules. The drug–macromolecule complex may then act as an immunogen and elicit the production of specific antibodies (a humoral response) and/or the generation of specific T lymphocytes (a cellular response) towards the hapten and/or native macromolecule epitopes. The recognition of the native macromolecule would constitute a loss of self-tolerance. Although these reactions are, by definition, rare, they are of increasing concern due to the large number of individuals exposed to medication, and are not detected by routine animal toxicology screens or in early clinical trials (because of the limited number of human exposures). While the association between drugs and the symptoms of adverse reactions is enhanced by rechallenge with the suspected drug, such a procedure may be considered unethical given the life-threatening nature of some idiosyncratic reactions.

Clearly, the chemical nature of a drug, interindividual variation in drug metabolism (influenced by both genetic and environmental factors) and the properties of the target macromolecule all contribute to the manifestation of adverse drug reactions mediated by reactive drug metabolites. The role of drug metabolism is pivotal to the hapten hypothesis, both in activation of the parent compound and detoxification of the reactive species. For a given compound, toxicity may only occur once the precise balance between drug activation and detoxification is perturbed in favour of abnormally high (or prolonged) levels of the offending intermediate. This situation may arise as a consequence of increased levels of enzymes involved in bioactivation (inherited or produced by enzyme induction) and/or decreased (or compromised) detoxification mechanisms (Fig. 1).

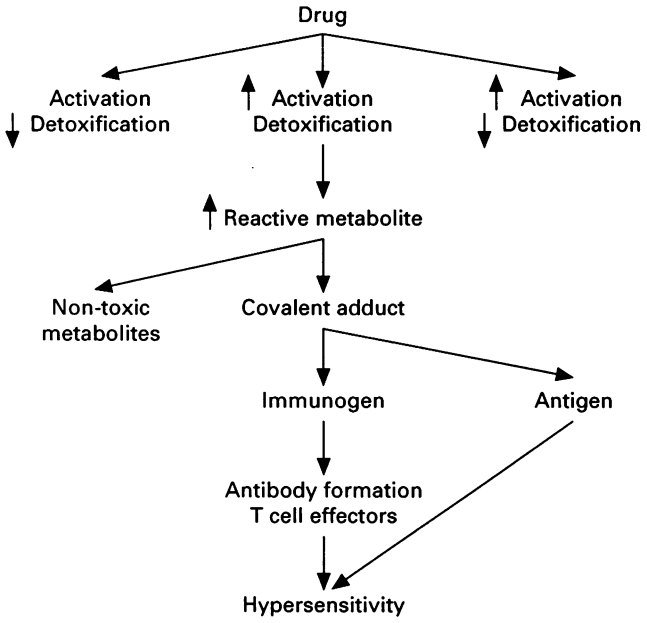

Fig. 1. Schematic representation of the classical 'hapten hypothesis' for the metabolic basis of drug hypersensitivity reactions.

CLASSICAL DIAGNOSIS OF DRUG HYPERSENSITIVITY REACTIONS

A limited number of assays have been described which confirm drug hypersensitivity reactions [1–3]. Specific humoral responses may be detected using the radioallergosorbent test (RAST) or an ELISA. The indirect Coombs' test has proven useful in the diagnosis of haemolytic anaemia, while complement fixation, platelet lysis or agglutination reactions have been employed to identify platelet antibodies. By contrast, except for the analysis of anaphylactic reactions to muscle relaxants and enzyme preparations, skin tests have proved of little value in the examination of drug allergy [3]. A cellular response involving drug-related T cell activity may be assessed using the lymphocyte transformation test or, more rarely, the macrophage migration inhibition test. However, the multifactorial nature of hypersensitivity reactions, particularly the role played by often unidentified, reactive drug metabolites in antigen generation, has hampered the routine diagnostic application of these techniques [1,3]. This brief review highlights recent efforts to unravel the bases for hypersensitivity reactions to several classes of therapeutic agents using a combination of drug metabolism and immunochemical approaches.

MORE RECENT APPROACHES

In the early 1980s, an *in vitro* cytotoxicity assay was developed, which uses human mononuclear leucocytes as surrogate, peripheral marker target cells, to identify individuals susceptible to drug toxicity produced by paracetamol, aromatic anticonvulsants and sulphonamides [4]. The co-incubation system employed relied on the metabolic activation of these commonly prescribed therapies by phenobarbitone-induced murine cytochrome P450 (P450) enzymes which provided '*in vitro* rechallenge' of patient cells to reactive drug metabolites. The use of inhibitors of lymphocyte detoxification enzymes (such as 1,1,1-trichloro-2-propane oxide (TCPO) for expoxide hydrolase) or the addition of exogenous cytoprotectants (such as reduced glutathione (GSH)), allowed limited mechanistic evaluation of the biochemical basis for these idiosyncratic reactions. For example, the observation that pretreatment of cells from control individuals with TCPO rendered them as susceptible to the toxic effects of aromatic anticonvulsant metabolites as cells from individuals who had experienced a toxic reaction to these compounds suggested that an inherited deficiency in epoxide hydrolase may predispose certain individuals to these adverse effects [4]. Although other laboratories have independently provided evidence that decreased epoxide hydrolase activity, either inherited [5] or produced through chemical inhibition by compounds such as valproic acid [6], can predispose certain individuals to toxicity produced by aromatic anticonvulsants [5] and increase alkylation of target proteins [6], the identity of the reactive metabolite(s) and the precise role of epoxide hydrolase in aromatic anticonvulsant hypersensitivity reactions have yet to be defined. Phenotyping sensitive individuals using carbamazepine as an *in vivo* probe for epoxide hydrolase activity [7] may prove useful, although this could theoretically constitute 'unethical' rechallenge.

By incorporating human liver microsomes into the assay, interindividual and interspecies variability in metabolic activation, interindividual variability in cellular detoxification mechanisms, and the chemistry and enzymology of these finely balanced processes were later examined [8,9]. Analysis of stable and chemically reactive metabolites generated in the toxicity assay aided mechanistic interpretation of the data

obtained for several drugs associated with hypersensitivity reactions. Hepatic enzyme induction by agents such as phenobarbitone was demonstrated to be a prerequisite for the bioactivation of phenytoin to reactive, cytotoxic intermediates by murine and, probably, human hepatic microsomes [8]. In addition, basic structural requirements for the bioactivation of aromatic anticonvulsants and the aldose reductase inhibitor sorbinil were proposed [10]. Human hepatic microsomal P450 enzymes selectively metabolized mianserin, a tetracyclic antidepressant, to a cytotoxic form [8,10] and were much more efficient at bioactivating dapsone than even induced murine microsomes [11], suggesting a predominant role of constitutive human P450 enzymes in the bioactivation of both these compounds. Indeed, Fleming and colleagues have since confirmed that a major human P450 isoform CYP3A4, catalyses the conversion of dapsone to its pro-reactive hydroxylamine metabolite [12]. Preliminary attempts to improve assay sensitivity using purified rat P450 enzymes as the source of reactive metabolites have been encouraging [13].

SULPHONAMIDE HYPERSENSITIVITY REACTIONS AND INFECTION

Using this *in vitro* assay, Carr *et al.* [14] proposed recently that the greater incidence of 'hypersensitivity reactions' to trimethoprim–sulphamethoxazole in HIV-infected patients may be related to the generation of the hydroxylamine metabolite of the sulphonamide, patient acetylator phenotype and perturbations in GSH metabolism. Several earlier investigations, including the use of cells from individuals deficient in GSH-synthesizing enzymes (expressed clinically as 5-oxoprolinuria), have also indicated a pivotal role of GSH and/or GSH-dependent enzymes in sulphonamide toxicity [4]. Sulphonamides (and dapsone) exert their toxic effects through hydroxylamine or nitroso metabolites, and their toxicity in this assay can be attenuated by the inclusion of exogenous GSH [4,11]. However, this tripeptide is unable to penetrate cells [15], and hence the cytoprotective effects observed in our studies [11] and that of Carr and co-workers [14] must be exerted extracellularly, either via conjunction of the reactive metabolites, or by preventing the oxidation of the hydroxylamines to more reactive species. Similarly, the role of trimethoprim (which may also be bioactivated to a hydroxylamine metabolite) in the patients identified by Carr and colleagues [14] was not addressed. Although limited in their repertoire of drug-metabolizing enzymes, human lymphocytes express the polymorphic glutathione-S-transferase μ enzyme (GSTMI) [16], and have proved to be a reliable surrogate for detecting hepatic GSTμ polymorphisms (expressed in some 50% of the population). However, we were able to demonstrate that an inherited deficiency in GSTμ, which catalyses efficiently the conjugation of several compounds containing hydroxylamine and nitr(os)o functions with GSH [17], is not a predisposing factor for sulphonamide hypersensitivity reactions [18]. Similarly, no significant correlation was observed between GSTμ activity and aromatic anticonvulsant toxicity, although this subfamily of GSTs also detoxifies epoxides (unpublished data). Subsequent experiments actually demonstrated that exogenous GSH acted by preventing further oxidation of sulphonamide hydroxylamine metabolites [19]. Is it possible that the 5-oxoprolinuria model simply mimics presentation of the target cells with exaggerated levels of reactive metabolites in a similar manner to the use of TCPO as a 'diagnostic' inhibitor for aromatic anticonvulsant toxicity? Alternatively, the supply of GSH for other transferase and/or peroxidase enzymes or its role in providing reducing equivalents for hydroxylamine reductases may be the limiting factor in these individuals.

THE ROLE OF N-ACETYLTRANSFERASES

An intriguing observation is the apparent preponderance of 'slow acetylators' in patients experiencing hypersensitivity reactions to sulphonamides [4], given that half the population express this phenotype. Indeed, the target cells employed in this *in vitro* assay express only the monomorphic *N*-acetyltransferase (NAT) enzyme, NAT1 [20]. Furthermore, the sulphonamide commonly employed as the substrate in this assay, sulphamethoxazole, is not polymorphically acetylated, and consequently NAT1 has been shown to exhibit a much higher affinity for this substrate than NAT2 [21]. Hence, the contribution of the NAT2 polymorphism to the increased sensitivity observed in these patients' cells is debatable, although it may be linked genetically to some other contributory process since slow acetylator phenotype has been shown to be a predisposing factor for several allergic diseases [22]. It is also conceivable that altered regulation of NAT1 activity (either related to detoxification or bioactivation given the dual role of these enzymes) may be involved.

METABOLIZING ENZYMES AS ANTIGENS

Our laboratories have also used this assay in tandem with immunoblotting to identify antimicrosomal antibodies in sera from patients experiencing hypersensitivity reactions to anticonvulsant therapy who gave a positive response in the *in vitro* cytotoxicity screen [23]. Subsequent analyses of these antisera have demonstrated that the circulating antibodies appear to recognize a human P450 enzyme [24], and the toxic reactions appear to have an immunological etiology similar to that described for tienilic acid [25], dihydralazine [26] and, possibly, halothane [27] (Table 1). Drug hypersensitivity reactions produced by these compounds are characterized by antibodies directed against metabolite-derived new antigenic determinants in the case of halothane and against P450 proteins (CYP2C8/9 for tienilic acid; CYP1A2 for dihydralazine) thought to be involved in the bioactivation of these compounds. Other laboratories have since proposed independently that human CYP2C and CYP3A enzymes catalyse the metabolism and, possibly, bioactivation of phenytoin [28] and carbamazepine [29,30]. Moreover, the major microsomal target for reactive phenytoin metabolites generated *in vitro* appears to be a 53-kD protein which may be a P450 protein [24]. These findings support our theory that these proteins may be involved in neoantigen generation encountered in hypersensitivity reactions to these compounds [23,24]. Autoantibodies against P450 proteins have also been identified in several non-drug related autoimmune diseases such as type II chronic autoimmune hepatitis (CYP2D6 [31] and, less frequently, CYP1A2 [32]), autoimmune polyendocrine syndrome type I (CYP11A1) [33] and Addison's disease (CYP17 and CYP21) [33,34]. The mechanism by which these autoantibodies to intracellular targets are generated remains unresolved, and although several

Table 1. Examples of enzymes implicated in the pathogenesis of drug hypersensitivity reactions and autoimmune disorders

Drug	Bioactivating enzyme	Postulated detoxification deficiency	Identified (neo)antigen (mol. wt)	Reference(s)
Drug hypersensitivity reactions				
Halothane	CYP2E1?	Antioxidant status, GSH metabolism?	GRP94/ERp99/endoplasmin (100 kD), ERp72 (80 kD), Calcireticulin (63 kD), microsomal carboxylesterase (59 kD), unknown (58 kD), protein disulphide isomerase (57 kD), unknown (54 kD)	[1,27,44]
Tienilic acid	CYP2C8/9	EH, GST?	CYP2C8/9 (50 kD)	[25]
Dihydralazine	CYP1A2	NAT?	CYP1A2 (50 kD)	[26]
Sulphonamides	CYP2C9/1A2?	GSH levels, GSH-dependent enzymes, hydroxylamine reductases? NAT?	?	[4,14,19]
Dapsone	CYP3A4	As for sulphonamides and G6PDH deficiency?	?	[11,12]
Aromatic Anticonvulsants	CYP2C/3A?	EH	CYP2C/3A? (53 kD)	[4,8,9,23,24]
Isoniazid	MAO-B?	?	MAO-B	[48]

Autoimmune disorder	Autoantigen(s)	Reference(s)
Type II chronic hepatitis	CYP2D6, CYP1A2	[31,39–41,32]
Autoimmune polyendocrine syndrome type I	CYP11A1	[34]
Addison's disease	CYP17, CYP21	[33,34]

GSH, (reduced) glutathione; EH, epoxide hydrolase; GST, glutathione-S-transferase; NAT, *N*-acetyltransferase; G6PDH, glucose-6-phosphate dehydrogenase; MAO, monoamine oxidase.

? Indicates that these details are at present unknown or have been extrapolated from the documented literature and have yet to be confirmed.

P450 proteins have been identified in hepatocyte plasma membranes [35], their presence on the outer cell surface remains debatable [36,37].

THE ROLE OF INFECTION AND 'MOLECULAR MIMICRY'

Recent data suggest that infection with an appropriate pathogen may stimulate T helper cells which could subsequently initiate a 'pseudo-graft-*versus*-host reaction' towards cells expressing an epitope which mimics a viral or bacterial protein [38]. Consistent with this hypothesis, the major epitope recognized by the CYP2D6 autoantibodies has been mapped independently by two groups [39,40] and confirmed by others [41]. The core sequence identified by these groups, (W)DPAQPPRD, bears striking homology to herpes simplex virus type 1 and hepatitis C virus (HCV) proteins, suggesting mimicry between viral antigens and CYP2D6 may occur. Although the relationship between HCV infection and type II autoimmune hepatitis remains to be clearly elucidated [42], the possibility of a viral etiology in the pathogenesis of this autoimmune disorder is intriguing.

Epitope mapping studies are presently underway to determine if such molecular mimicry is also involved in the generation of the antimicrosomal antibodies observed in drug hypersensitivity reactions. The classical hapten hypothesis may thus be modified, allowing incorporation of this concept as shown in Fig. 2. Recent work by Gut *et al.* [43] suggests that molecular mimicry may play a role in halothane hepatitis. Sera from halothane hepatitis patients contain antibodies which recognize various trifluoroacetyl (TFA)-protein adducts which may arise from the oxidative metabolism of halothane by CYP2E1 [44]. It has been proposed that mimicry may occur between lipoic acid, the prosthetic group of the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC), and TFA-protein adducts [43]. Lipoyl-containing E2 subunits of the PDC are major antigens recognized by autoantibodies in the sera of patients with primary biliary cirrhosis [45]. Burroughs and colleagues have proposed a novel hypothesis for the pathogenesis of this autoimmune disorder based on an association with recurrent urinary tract infections and homologies between HLA-DR-α and human and *Escherichia coli* PDC-E2 [46]. Whether this scenario is also applicable to halothane hepatitis is presently not known, but merits further investigation.

As with autoimmune diseases, there is probably an element of immune dysfunction involved in the pathogenesis of drug hypersensitivity reactions. The immunodeficiency associated with HIV infection is obvious; immunodeficiencies and immune dysregulatory effects associated with drugs such as the anticonvulsants phenytoin and carbamazepine [47] may be more subtle but still contribute to an altered response to drug-derived neoantigens or self-antigens.

If the pathogenesis of drug hypersensitivity reactions is so

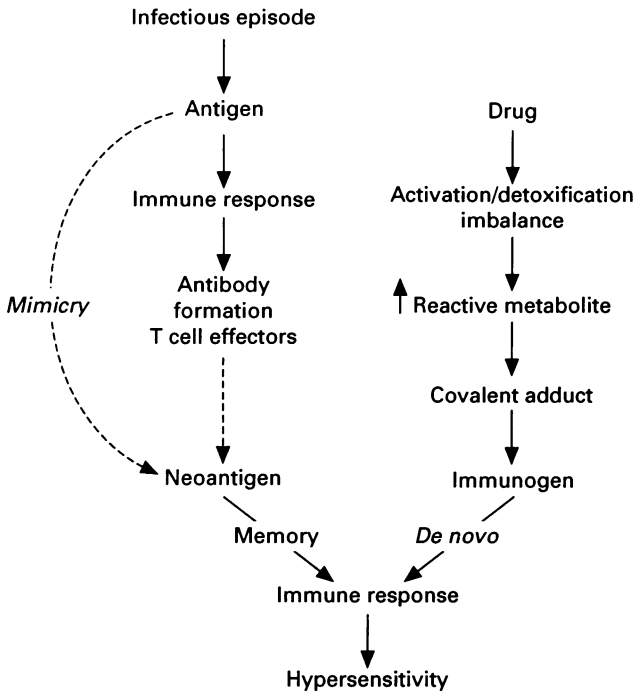

Fig. 2. Proposed revision of the hapten hypothesis encompassing the possible role of 'molecular mimicry' as a result of infection with an appropriate pathogen.

multifactorial, what is the significance of the *in vitro* challenge of peripheral blood mononuclear cells with cytotoxic drug metabolites? One possibility is that an imbalance in bioactivation and detoxification processes resulting in a net increase in reactive, cytotoxic intermediates is necessary, but not sufficient, for the development of drug hypersensitivity reactions. In most cases, this imbalance manifests as decreased detoxification capacity (as indicated by the lymphocyte assay data), although increased bioactivation in the presence of normal detoxification capacity is also possible. This could also explain why some patients with a high likelihood of a drug-induced toxicity from clinical observations test negative in the lymphocyte assay. The presence of other predisposing factors in susceptible individuals such as a previous infectious episode, MHC haplotype, immune dysregulation, cellular processing of the antigens or other unidentified factors may be necessary for ultimate expression of the adverse reaction.

CONCLUSIONS

In conclusion, despite several shortcomings and the limited drug-metabolizing capacity of the target cells, this *in vitro* assay continues to prove valuable in the identification of patients predisposed to hypersensitivity reactions to several classes of therapeutic agents. Recent advances in molecular biology should soon permit the inclusion of more appropriate activating systems (for example, purified human CYP3A4 for dapsone) into the assay, which may improve its sensitivity, and more detailed molecular analysis of candidate detoxifying enzymes or cellular defence mechanisms should ultimately identify the genetic basis for these adverse reactions.

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