Drug hypersensitivity reactions in skin: understanding mechanisms and the development of diagnostic and predictive tests

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Abstract

Cutaneous manifestations of drug hypersensitivity can be serious and potentially life threatening and may prevent effective drug therapy. T cells play an important role in the pathology of drug hypersensitivity reactions. Classical studies suggest that T-cell activation requires drug bioactivation, covalent binding to protein and antigen processing to stimulate an immune response. Recent studies have shown that drugs can also be presented to T cells in the absence of antigen processing and drug metabolism. In this article, sulfamethoxazole is used as a paradigm to describe the chemical mechanisms involved in the initiation and maintenance of an aberrant drug antigen specific T-cell response. Presentation of the same drug to different individuals can cause a variety of skin diseases. Such reactions have been classified according to the phenotype and functionality of the T-cell response. This review summarises the different forms of cutaneous hypersensitivity reactions and describes how T-cell clones generated from hypersensitive patients have been used to study the cellular mechanisms of anticonvulsant hypersensitivity. Potential uses of in vitro cell culture assays for patient diagnosis and drug evaluation are also discussed.

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1. Introduction

Drug hypersensitivity reactions, often referred to as type B or idiosyncratic drug reactions (Park et al., 1998; Uetrecht, 1999), cannot be predicted from the known pharmacology of the drug. Such reactions are not related to the dose administered; however, most drugs associated with a high incidence of hypersensitivity reactions are administered at high mass doses. Although rare, hypersensitivity reactions tend to be severe and because the chemical, cellular and molecular mechanisms of tissue pathology are not understood, drug hypersensitivity can be a major clinical problem.

The nature of the chemical (i.e., parent drug, stable metabolite, reactive metabolite or covalently modified autologous protein) responsible for stimulating hypersensitivity reactions and the subsequent effector mechanisms involved in the pathology of the reaction is not known or only beginning to be investigated; thus, hypersensitivity reactions are commonly defined in terms of clinical characteristics. Reactions can be classified as immediate or delayed. This manuscript will focus on delayed-type drug hypersensitivity reactions; these reactions commonly take 2–4 weeks to develop following initial exposure to the drug. Reactions can be severe and are responsible for many drug-induced
reactions are the most common and/or obvious signs of a drug hypersensitivity reaction. Other clinical features that may be present include fever, internal organ involvement, such as hepatitis, and eosinophilia. Toxic epidermal necrolysis and Stevens Johnson syndrome (bullous skin eruptions), which are thought to be variants of the same reaction, have the highest mortality rate. They are characterised by mucosal membrane involvement, widespread erythematous rash and epidermal detachment. Maculopapular rashes tend to be milder and have a lower mortality rate. Patients present with a red rash that has small, confluent bumps. Drugs also cause pustular skin reactions such as acute generalised exanthematous pustulosis. Pustular reactions are characterised by the appearance of sterile eruptions (pustules) and an accompanying red rash. In contrast to other skin reactions where eosinophilia is a common characteristic, pustular reactions are associated with the presence of elevated blood neutrophils (Sidotoff et al., 2001; Pichler et al., 2002). Many drugs cause hypersensitivity reactions that damage skin. With the exception of drugs used for the treatment of HIV and AIDS, antibiotics, non-steroidal anti-inflammatory drugs, sulfonamides and aromatic anti-convulsants are the most frequent causes of drug hypersensitivity. Interestingly, administration of certain classes of drug is associated with a full spectrum of cutaneous side effects in different individuals (e.g., sulfonamides); whereas, other classes of drug (e.g., anti-convulsants) cause a restricted clinical picture even though the drugs are not structurally related.

There is increasing evidence that T cells play an important role in the pathogenesis of drug hypersensitivity reactions. Mauri-Hellweg et al. (1995) demonstrated proliferation of lymphocytes from drug hypersensitive patients, following in vitro drug rechallenge, but not drug-exposed non-hypersensitive controls. More recently, in a prospective study, drug-specific lymphocyte proliferation was seen in 14/21 patients hypersensitive to several structurally unrelated drugs (Hari et al., 2001). In the acute phase of drug hypersensitivity T cells infiltrate skin and in recent studies T cells have been isolated and cloned from skin biopsies and blister fluid and have been shown to be drug specific (Yawalkar et al., 2000a; Nassif et al., 2002). The aim of this manuscript is to detail advances in our understanding of the mechanisms of drug recognition by T cells and how this leads to a cellular immune response and tissue damage in skin.

2. Drug stimulation of the immune system—chemical aspects

Our understanding of the mechanisms of how drugs activate the immune system is based on classical studies by Landsteiner and Jacobs (1935). In these studies, the authors use simple electrophilic contact allergens such as dinitrochlorobenzene. They were the first investigators to conclude that a covalent interaction between nucleophilic moieties on cutaneous protein and the chemical is a prerequisite for immune activation. The interaction between chemical (drug) and protein is referred to as haptenation, the electrophilic chemical is known as a hapten and protein conjugates that activate the immune system are known as antigens (Park et al., 2001; Naishitt et al., 2003a). The cellular processes involved in converting a chemical signal (i.e., the haptenated protein or antigen) to an immune response in contact sensitisation are well known and many of these processes relate directly to drug hypersensitivity. The haptenated protein is recognised and taken up by professional antigen presenting cells (e.g., Langerhans cells in skin), a major function of these cells is to monitor the cutaneous environment for the presence of antigenic stimuli (Gorbachev and Fairchild, 2001). Antigen-loaded antigen presenting cells migrate to local lymph nodes, where they process—a series of enzymatic reactions that breakdown the antigen to small peptide fragments (Kalish, 1995)—and present the antigen on major histocompatibility complex (MHC) molecules to naive T cells. Clonal T-cell expansion generates a population of antigen-specific T cells that migrate to skin following subsequent exposure to antigen. Secretion of cytokines and chemokines from inflamed skin and activated T cells control the nature of the cellular immune response and the extent of tissue damage (Sebastiani et al., 2002). Since the
chemical mechanisms of contact sensitisation are well known, reliable predictive tests such as the murine lymph node assay have been developed to assess the potential of a novel chemical entity to stimulate an immune response. The compound is normally administered topically and activation of local lymph node cells is measured by monitoring cell proliferation by monitoring the incorporation of $[^3]$H thymidine. The results obtained from treated mice are compared with mice administered vehicle alone (Kimber et al., 2002).

In an attempt to understand the nature of the cellular immune response to simple chemicals further, Kimber and colleagues measure cytokines secreted from lymph node cells of sensitised animals (Dearman et al., 2002). Sensitisation with respiratory allergens (immediate-IgE mediated hypersensitivity) such as trimellitic anhydride, stimulate lymph node cell production of high levels of type II cytokines such as IL-4, -5, -10 and -13 but only low levels of IFN-$\gamma$ (Dearman et al., 2000). In contrast, sensitisation with contact allergens (delayed hypersensitivity) such as dinitrochlorobenzene stimulates lymph node cells to produce the type I cytokines IFN-$\gamma$ and IL-12 (Dearman et al., 1996). The ability of simple chemicals to stimulate polarised cytokine secretion profiles is well defined and a recently published manuscript highlights:

1. potential experimental approaches available and
2. possible applications of cytokine profiling for hazard identification and characterisation of contact and respiratory allergens (Dearman et al., 2003).

The immune system regards exogenous and endogenous antigens differently. Although the differentiation is not absolute, most exogenous antigens are presented to CD4$^+$ T cells whereas endogenous antigens are presented to CD8$^+$ T cells (Kalish and Askenase, 1999). Thus, we have recently developed an in vitro assay to relate cytokine secretion profiles to the distribution of antigen formation. We utilised U937 cells, a monocyte-derived cell line, and the paradigm allergens trimellitic anhydride and dinitrochlorobenzene. We were able to demonstrate that trimellitic anhydride binds preferentially to serum proteins whereas dinitrochlorobenzene binds to cellular protein (Hopkins et al., 2003). IFN-$\gamma$ is produced by CD4$^+$ and CD8$^+$ T cells in dinitrochlorobenzene treated mice (Dearman et al., 1996). Thus, it is likely that dinitrochlorobenzene will bind to both membrane and cytosolic proteins, the membrane protein being "exogenous" and cytosolic protein "endogenous". It is also possible that the direct toxic effects of dinitrochlorobenzene may lead to cell membrane damage and leakage of haptenated intracellular proteins into the extracellular matrix—such proteins would be processed and presented as if "exogenous". Although these data are somewhat preliminary, there appears to be a relationship between the distribution of antigen formation and cytokine polarisation. Studies are underway to determine whether the distribution of antigen formation is an important factor in the development of type I and type II immune responses, which underlie chemical-induced hypersensitivity reactions in man.

In drug hypersensitivity reactions, the mechanism(s) of T-cell activation are less well understood. The reason for this is that most drugs are not protein reactive per se and metabolic activation (bioactivation) is required to generate an electrophilic intermediate with the potential to haptenate protein. The majority of drugs associated with a high incidence of hypersensitivity form chemically reactive metabolites and there seems to be some relationship between the extent of metabolic activation and the potential that administration of a drug will be associated with hypersensitivity (Williams et al., 2002; Table 1). There is, however, only circumstantial experimental evidence to relate the formation of a reactive drug metabolism
to immune activation. Halothane, a drug associated with immune-mediated hepatitis, is possibly the best example where there is direct experimental data to relate metabolic activation and the development of clinical symptoms of hypersensitivity. Approximately 20–50% of halothane is converted by CYP 2E1 to a reactive trifluoroacetyl chloride intermediate, which binds covalently to protein (Kenna et al., 1988). Structural modification of halothane, which reduces the extent of metabolic activation by over 95%, dramatically reduces the incidence of severe hepatotoxicity (Park and Kitteringham, 1994).

Previous reviews have described possible pathways which explain how an orally administered drug might be metabolised to a reactive metabolite with the potential to activate the immune system and cause tissue damage in skin (Park et al., 2001; Naisbitt et al., 2001a). These pathways have been edited and are outlined briefly below:

1. Prior to systemic circulation, a drug may be metabolised by liver cells to a highly reactive intermediate that haptenates proteins at the site of metabolic activation. The haptenated protein would be taken up by dendritic cells in liver and presented to naïve T cells. For antigen-specific T cells to target skin they would have to express a highly conserved compilation of skin homing and/or chemokine receptors and at least partly by-pass the antigen presentation apparatus in liver and other organs. In addition to the antigenic signal, an individual would have to be exposed to a secondary signal in skin that up-regulates the expression of ligands that interact with skin homing/chemokine receptors expressed on antigen-specific T cells. CCL27 (CTACK), an example of a ligand released by epidermal keratinocytes, is involved in homing antigen-specific T cells to skin (Horney et al., 2002) and it’s expression has recently been shown to be up-regulated in patients with atopic dermatitis (Kakinuma et al., 2003). Whether drug(metabolite)s or some other form of stress up-regulate the expression of such ligands in skin at the time of a hypersensitivity reaction is not known.

2. An electrophilic drug metabolite formed in liver may be sufficiently stable to avoid binding to hepatic proteins. The free drug metabolite might circulate in the periphery and bind to cutaneous proteins. Similarly, it is possible that skin cells (e.g., keratinocytes), which represent approximately 10% of an individual’s body weight, might metabolise drugs locally at the site of tissue damage. Keratinocytes express high levels of certain P450’s (Janmohamed et al., 2001) and have been shown to metabolise sulfamethoxazole to the oxidative metabolite sulfamethoxazole hydroxylamine (Reilly et al., 2000). With both of the processes described above, cutaneous dendritic cells would process and present the drug(metabolite) modified cutaneous protein to naïve T cells (c.f. contact hypersensitivity). These processes would be dependent on relatively high concentrations of drug and/or drug metabolite accumulating in skin. In support of this hypothesis, we have shown that greater than 10% of the anti-convulsant lamotrigine is found in skin 4 h after a single i.v. injection (Maggs et al., 2000).

3. Hepatic metabolism may generate a pro-reactive metabolite, which under conditions of oxidative stress or in an environment containing low levels of thiols and/or anti-oxidants (e.g., skin) might undergo (auto)oxidation to an electrophilic species. Electrophilic drug metabolites stimulate T cells via two pathways (Fig. 1). Pathway 1 is a classical hapten mechanism. The covalently modified protein conjugate is taken up by antigen presenting cells, processed into peptide fragments, which translocate to the cell surface in the context of MHC for presentation to T cells (Naisbitt et al., 2000a). As yet, it is not clear whether T-cell receptor activation requires the presence of a haptenated drug bound to the peptide embedded in major histocompatibility complex. Drug metabolites also bind directly to MHC molecules expressed on the surface of antigen presenting cells. This pathway avoids the requirement of an antigen presenting cells processing machinery (Schnyder et al., 2000; Burkhardt et al., 2001). A recent study has shown that acid elution of pre-existing peptides on the MHC binding groove does not prevent drug presentation to T cells (Burkhardt et al., 2002); thus drugs might ignore the peptide embedded in MHC binding groove and bind directly to MHC itself.

Recently, Pichler has proposed an alternative pathway of T-cell receptor activation by drugs. Based on
findings from in vitro experiments with T-cell clones generated from the peripheral blood of hypersensitive patients, the authors proposed that drugs might bind directly in the absence of drug metabolism, covalent binding and antigen processing to MHC molecules. The resultant “pharmacological” bridging interaction between MHC and the T-cell receptor, although relatively weak in a chemical sense and readily reversible, is sufficiently stable to stimulate all the activation events of the T-cell receptor (proliferation, cytotoxicity and cytokine synthesis) (Schnyder et al., 1997, 1998; Zanni et al., 1998).

To study the chemical mechanisms of drug hypersensitivity reactions in skin we have used sulfamethoxazole as a paradigm. Sulfamethoxazole is a sulphonamide-containing compound that was first developed in the 1960s as an antibacterial agent and is still used today as a cost-effective alternative to the new generation expanded spectrum antibacterial agents to decrease or delay the development of resistance (Masters et al., 2003). Sulfamethoxazole is also administered in combination with trimethoprim, as co-trimoxazole, for prophylaxis and treatment of HIV-associated infections. Unfortunately, sulfamethoxazole administration is associated with the development of adverse drug reactions in between 1 and 5% of patients. The most common adverse event is the development of cutaneous symptoms. Such reactions are thought to be immune mediated. To generate a drug antigen, sulfamethoxazole is metabolised in liver, blood cells and keratinocytes to a hydroxylamine metabolite (Cribb et al., 1990, 1995; Reilly et al., 2000). These reactions are catalysed by CYP2C9 and/or myeloperoxidase. Sulfamethoxazole hydroxylamine is not chemically reactive, is sufficiently stable to circulate in the periphery, and is excreted unchanged in urine (Gill et al., 1997). Further (auto)oxidation of sulfamethoxazole hydroxylamine generates nitroso sulfamethoxazole, which is chemically reactive and has been shown to haptenate cellular protein (Naisbitt et al., 1999; Reilly et al., 2000; Summan and Cribb, 2002; Manchanda et al., 2002), including the surface of viable lymphocytes and keratinocytes. In further studies, we have used LC–MS and NMR technology to investigate the stability of nitroso sulfamethoxazole (Naisbitt et al., 2002). Here, we demonstrated that nitroso sulfamethoxazole is rapidly degraded in solution; degradation yielded products of oxidation (nitro)sulfamethoxazole, reduction (sulfamethazine, sulfamethoxazole hydroxylamine) and dimerisation (azo and azoxy adducts) (Naisbitt et al., 1996, 2002). Despite this, we have shown by flow cytometry, using a specific anti-sulfamethoxazole antibody, that nitroso sulfamethoxazole circulates in the periphery in
rats and binds covalently to epidermal keratinocytes (Naisbitt et al., 2001b). These data are somewhat paradoxical; however, it is our view that they provide experimental evidence to support the hypothesis that a futile redox cycle between hydroxylamine, nitroso and nitro metabolites of sulfamethoxazole is established in vivo in patients following the conversion of sulfamethoxazole to sulfamethoxazole hydroxylamine (Fig. 2).

In patients with HIV infection, who are particularly susceptible to the development of sulfamethoxazole hypersensitivity reactions, a decrease in levels of both extracellular and cellular thiols has been demonstrated (Buhl et al., 1989; Eck et al., 1989; Walmsley et al., 1997). In Liverpool, we monitored reduced, oxidised, protein-bound, and total thiol levels in plasma from 33 HIV-positive patients and 33 control subjects using an HPLC method. Plasma thiols are an important non-enzymatic pathway in sulfamethoxazole hypersensitivity since sulfamethoxazole is largely excluded from cells (Naisbitt et al., 1999). These studies confirmed the previously observed finding that patients with HIV have low concentrations of plasma cysteine, when compared with non-HIV infected controls. Low plasma cysteine concentrations correlated directly with a decreased capacity to reduce nitroso sulfamethoxazole back to the hydroxylamine (Naisbitt et al., 2000b; Fig. 3).

To investigate the role of drug metabolism and covalent binding in the immunogenicity of sulfamethoxazole, we have developed an in vivo rat model. Here, male Wistar rats were administered the test compounds, sulfamethoxazole, sulfamethoxazole hydroxylamine and nitroso sulfamethoxazole four times weekly for 2 weeks. On completion of the sensitisation phase, splenocytes were isolated and drug(metabolite)-specific proliferation was measured by culturing the cells with either sulfamethoxazole or sulfamethoxazole metabolites. Splenocytes from rats administered the nitroso metabolite, but not the hydroxylamine or parent drug proliferated following in vitro stimulation with nitroso sulfamethoxazole (Fig. 4). No proliferation was seen following in vitro stimulation with sulfamethoxazole. Antigen-specific T cells were CD4+ and CD8+ and proliferated in the presence of nitroso sulfamethoxazole bound covalently to cellular, but not serum protein, in an MHC-restricted fashion (Naisbitt et al., 2001a). The antigentic threshold of nitroso sulfamethoxazole for T-cell activation was estimated to be between 0.5 and 1 μM, which is less than the concentration of sulfamethoxazole metabolites found in human plasma after administration of a therapeutic dose of sulfamethoxazole (Gill et al., 1997). Interestingly, we have recently observed similar results in mice and rabbits (Farrell et al., 2003) (Fig. 5). The essential aspect of these animal models is that there is a T-cell response to nitroso sulfamethoxazole but not the parent drug. Nitroso sulfamethoxazole was at least four orders of magnitude more immunogenic than the parent drug.

Covalent binding of nitroso sulfamethoxazole to rat splenocytes also led to a proportionate increase in cell death and indeed by flow cytometry we were able to demonstrate that the cells that were haptenated, above a threshold level, were the same as those that underwent necrotic cell death (Naisbitt et al., 2002). The threshold concentration of nitroso sulfamethoxazole for toxicity was one order of magnitude higher than that required to stimulate an immune response (i.e., between 5 and 10 μM). These data led us to hypothesise that the propensity of nitroso sulfamethoxazole to cause cell death in patients may determine individual susceptibility. These theories derive from animal models of contact sensitisation, where potent chemical antigens are not immunogenic in the absence of an irritant (toxic) signal. The irritant signal is dose-dependent and occurs at higher concentrations of the antigen than the antigenic signal and is the primary determinant of sensitisation (McFadden and Basketter, 2000; Zhang and Tinkle, 2000). Demonstration of increased microsome-derived, drug metabolite-specific killing of hypersensitive patient lymphocytes, when patient and control lymphocytes are compared (Friedmann et al., 1994; Neuman et al., 2000), is consistent with this proposal and provides a clinical parallel to our experimental observations and suggests that polymorphisms within drug metabolising enzymes may act as determinants of susceptibility. To this end, recent studies have shown that N-acetylttransferase-2 (NAT2) and glutathione S-transferase (GST) polymorphisms are not determinants of susceptibility (Pirmohamed et al., 2000; O’Neil et al., 2002). However, polymorphisms in other drug metabolising enzymes such as myeloperoxidase may be important but have not been investigated.
Fig. 2. Scheme depicting the chemical fate of nitroso sulfamethoxazole (from Farrell et al., 2003).
Traditionalists believe that the immune system has evolved to differentiate between self and non-self; the presence of “non-self” stimulates an immune response. Matzinger (1994) has recently proposed an alternative “danger” hypothesis. Matzinger’s theory states that the primary signal controlling whether the presence of an antigenic signal results in tolerance (ignorance) or immune activation is the presence of danger not non-self. Danger signals derive from damaged cells that release intracellular molecules such as heat shock proteins into extracellular matrix (Shi et al., 2000; Gallucci et al., 1999). Heat shock proteins interact with professional antigen presenting cells and up-regulate the expression of inflammatory cytokines.

Fig. 3. Patients with HIV infection have low levels of plasma cysteine and a decreased capacity to reduce nitroso sulfamethoxazole to sulfamethoxazole hydroxylamine, when compared with non-HIV infected controls. Cysteine levels were measured by HPLC following derivatisation with the fluorescent probe bromobimane. Sulfamethoxazole hydroxylamine (SMX-NHOH) was detected by LC–MS (from Naisbitt et al., 2000b).

Fig. 4. Nitroso sulfamethoxazole, but not sulfamethoxazole hyydroxylamine (SMX-NHOH) or sulfamethoxazole (SMX), specific prolif-eration of splenocytes in vitro from sensitised rats. Proliferation was measured by incorporation of [3H] thymidine for the final 16 h of a 3-day experiment (from Naisbitt et al., 2001a).
cytokines, chemokines and co-stimulatory molecules and thus prime the immune system to respond to an antigenic signal (Robert, 2003). Heat shock proteins also transport antigenic peptides to antigen presenting cells and assist MHC class I presentation to CD8+ T cells. In terms of drug hypersensitivity, we have recently shown that non-toxic and toxic concentrations of nitroso sulfamethoxazole up and down-regulate, respectively, the expression of CD40, a co-stimulatory receptor on antigen presenting cells (unpublished observation). In on-going experiments we are studying the complex relationship between covalent binding cell death and regulation of stimulatory receptors expressed on antigen presenting cells. Although certain aspects of Matzinger’s danger theory remain contentious (Vance, 2000), the hypothesis might explain why drug hypersensitivity reactions occur much more frequently with concomitant infection (e.g., HIV (Pirmohamed and Park, 2001) and human herpes virus 6 (Suzuki et al., 1998)).

To study the role of danger and co-stimulation in the induction of a drug or drug metabolite-specific immune response in vivo we administered sulfamethoxazole and nitroso sulfamethoxazole to rats in the presence of complete Freund’s adjuvant. Adjuvants up-regulate the expression of co-stimulatory molecules and cytokines and therefore prime the animal’s immune system to respond (Hadden, 1994). As expected, splenocytes from rats administered nitroso sulfamethoxazole proliferated following in vitro stimulation with nitroso sulfamethoxazole; however, and in contrast to naïve animals administered sulfamethoxazole alone, animals administered sulfamethoxazole with Freunds adjuvant proliferated on in vitro stimulation, but only with nitroso sulfamethoxazole (Naisbitt et al., 2001a). These data...
provide the first experimental evidence that nitroso sulfamethoxazole is formed in vivo following administration of the parent drug, in sufficient quantities to provide an antigenic signal to the immune system. The quantities formed per se seem to be insufficient to generate a toxic “danger” signal(s) that is required to activate the immune system. The presence of an adjuvant in our animal model, apparently overrides the requirement for high levels of nitroso sulfamethoxazole to provide secondary danger signals.

In recent studies, we have utilised the murine cytokine-fingerprinting assay (discussed in detail above) to test the potential of nitroso sulfamethoxazole to stimulate a polarised type I or type II cytokine secretion profile. Lymph node cells from mice sensitised with nitroso sulfamethoxazole secreted high levels of IL-5 and moderate/low levels of IFN-γ (Hopkins et al., 2002). These in vivo observations are consistent with certain characteristics of sulfamethoxazole hypersensitivity in patients. IFN-γ has been shown to up-regulate the expression of HLA on the surface of keratinocytes, which renders them more susceptible to perforin-mediated killing by sulfamethoxazole-specific T cells (Schnyder et al., 1998). The high levels of IL-5 secreted by nitroso sulfamethoxazole sensitised mice may relate to eosinophilia, which is a prominent feature of sulfamethoxazole hypersensitivity (Pichler et al., 2002; Yawalkar et al., 2000b). Nitroso benzene, a simple chemical hapten, formed by the reduction or oxidation of nitrobenzene and aniline, respectively (Ohkuma and Kawanishi, 1999; Wulferink et al., 2001), covalently binds to sulfhydryl containing proteins (Ellis et al., 1992). Exposure to nitroso benzene has been shown to stimulate the production of antigen-specific T cells in mice (Wulferink et al., 2001). In our laboratory, nitroso benzene was used to investigate whether the acidic sulfonamide containing side chain of sulfamethoxazole alters cytokine polarisation. Lymph node cells from nitroso benzene sensitised mice were found to secrete IFN-γ, but not IL-5 (unpublished observation). These data imply that the sulfonamide side chain of sulfamethoxazole, which is known to principally exclude passage of sulfamethoxazole across cell membranes (Naishitt et al., 1999; Manchanda et al., 2002), has important quantitative effects on the cytokine secretion profile of nitroso sulfamethoxazole.

It is important to note that results from the studies described thus far derive from animal models of drug immunogenicity and not hypersensitivity (i.e., there was no sign of tissue damage). Thus, we have recently changed the focus of our research to concentrate on the only currently available model of drug hypersensitivity lymphocytes isolated from the peripheral blood of hypersensitive patients. Lymphocytes from patients hypersensitive to sulfamethoxazole proliferate in the presence of nitroso sulfamethoxazole (Schnyder et al., 2000; Burkhardt et al., 2001; Farrell et al., 2003) (Fig. 5). These data show that patients are exposed to nitroso sulfamethoxazole at the time of the adverse drug reaction; however, and in contrast to our animal models of sulfamethoxazole immunogenicity, lymphocytes from hypersensitive patients also proliferated in the presence of sulfamethoxazole (Fig. 5). There are two possible explanations for this fundamental difference in animal and human cells. First, there may be ongoing metabolism in human cells from hypersensitive patients, which is below the limit of detection using available analytical techniques. Since the number of antigen molecules required to stimulate T cells is incredibly low—Irvine et al. (2002) estimated that T cells can be stimulated when as little as 10 antigenic ligands are present—it is not surprising that the immune system is more sensitive than analytical methodology. Secondly, it is possible that human T cells may be hyper-responsive in a chemical sense whereby non-covalently bound parent drug may bind in a “pharmacological-like” interaction with the MHC-restricted T-cell receptor. To study the importance of these different pathways of drug presentation to T cells we have cloned T cells from patients with sulfamethoxazole hypersensitivity. To avoid experimental bias, lymphocytes from hypersensitive patients were stimulated with either sulfamethoxazole or nitroso sulfamethoxazole prior to serial dilution and cloning. We were successful in generating over 200 T-cell clones, greater than 95% of which proliferated in the presence of the parent drug bound non-covalently to MHC (Schnyder et al., 2000). The few nitroso sulfamethoxazole-specific T-cell clones recognised nitroso sulfamethoxazole bound covalently to MHC in the absence of antigen processing. These data seem to suggest that sulfamethoxazole is the dominant antigenic stimulus in sulfamethoxazole hypersensitive patients; however,
Table 2
Identification of drug-specific T cells from hypersensitive patients

<table>
<thead>
<tr>
<th>Drug</th>
<th>Adverse event</th>
</tr>
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<tbody>
<tr>
<td>Abacavir</td>
<td>Hypersensitivity and pneumonitis</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Rash and nephritis</td>
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<tr>
<td>Carbamazepine</td>
<td>Hypersensitivity syndrome</td>
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<tr>
<td>Lamotrigine</td>
<td>Hypersensitivity syndrome</td>
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<tr>
<td>Nevirapine</td>
<td>Hypersensitivity syndrome</td>
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<tr>
<td>Phenobarbital</td>
<td>Hypersensitivity syndrome</td>
</tr>
<tr>
<td>Phenindione</td>
<td>Maculopapular eruption</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>Maculopapular eruption</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Hepatitis</td>
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</table>

In preliminary investigations using lymphocytes from sulfamethoxazole hypersensitive patients with HIV infection, we have generated eight T-cell clones. Six proliferated in the presence of nitroso sulfamethoxazole, while two proliferated with the parent drug. From these data it is apparent that our understanding of the nature of the drug antigen in sulfamethoxazole hypersensitive remains incomplete.

Using a lymphocyte proliferation assay, we have shown drug-specific proliferation of lymphocytes isolated from the blood of patients hypersensitive to a range of structurally unrelated drugs (Table 2; unpublished observations). Proliferation in all cases was dose-dependent and occurred at therapeutic drug concentrations in the apparent absence of drug metabolism.

3. Drug stimulation of the immune system—cellular aspects

In recent years, the role of T cells in the different forms of cutaneous drug hypersensitivity reactions has been evaluated using immunohistological data obtained from skin lesions and analysis of the phenotype and functionality of drug-specific T-cell clones from hypersensitive patients. These studies reveal that the different forms of cutaneous eruption can be characterised based on cellular and molecular pathophysiology (Pichler et al., 2002). The following section details the nature of T cells involved in maculopapular exanthema, bullous and pustular skin diseases and anti-convulsant hypersensitivity, a cutaneous adverse drug reaction with concomitant systemic symptoms.

3.1. Maculopapular reactions

Most drug-specific T cells isolated from patients with maculopapular skin eruptions are CD4+, drugs are presented on the MHC class II molecule HLA-DR (Schnyder et al., 1997). Following drug stimulation, T cells secrete a heterogeneous cytokine profile (Zanni et al., 1997; Brugnolo et al., 1999). Secretion of high levels of IL-5 and eotaxin are common characteristics of maculopapular reactions (Yawalkar et al., 2000b). IL-5 and eotaxin are both involved in the recruitment, growth and differentiation of eosinophils. During the acute phase of maculopapular reactions, levels of the skin homing receptor cutaneous lymphocyte antigen (CLA) are elevated (Blanca et al., 2000). Drug-specific T cells contain perforin and kill autologous keratinocytes pre-treated with IFN-γ, to up-regulate MHC class II expression (Schnyder et al., 1998).

3.2. Bullous reactions

Bullous and maculopapular skin reactions share several common characteristics. For example, a recent immunohistochemical study demonstrated massive cutaneous T-cell infiltration, up regulation of MHC molecules on keratinocytes and migrating T cells and IL-5 expression in skin (Yawalkar et al., 2000b). The major pathophysiological difference between maculopapular and bullous skin reactions is that drugs are normally presented on MHC class I molecules to cytotoxic CD8+ T cells (Hertl et al., 1995; Nassif et al., 2002). Drug-stimulated T cells kill autologous target cells via the perforin pathway (Nassif et al., 2002).

3.3. Pustular reactions

Pustular reactions are instigated by skin infiltrating MHC class II restricted CD4+ and CD8+ T cells that secrete high levels of IL-5 and the neutrophil attractant chemokine IL-8 (Britschgi et al., 2003). IL-8 secretion is not associated with other forms of cutaneous adverse drug reaction. Drug-specific T cells isolated from skin and blood of patients with pustular reactions are cytotoxic and kill target cells via both perforin and FAS-mediated pathways (Schmid et al., 2002).
3.4. Anticonvulsant hypersensitivity syndrome

Administration of the anti-epileptic drugs carbamazepine, phenytoin and phenobarbital, and more recently lamotrigine, is associated with serious and potentially life-threatening cutaneous adverse drug reactions. Maculopapular reactions are the most common form of cutaneous eruption, although approximately 30% of patients develop bullous reactions such as Stevens-Johnson syndrome (Sharma et al., 1998). Cutaneous eruptions often occur as part of a generalized syndrome, which is referred to as hypersensitivity (Leeder, 1998) or by the acronym DRESS (drug rash with eosinophilia and systemic symptoms; Bocquet et al., 1996); extra-cutaneous symptoms differentiate anticonvulsant hypersensitivity from other forms of cutaneous adverse drug reactions. Anticonvulsant hypersensitivity is relatively rare, with an incidence of between 1/1000 and 1/10,000 new exposures to a drug (Bessmertny and Pham, 2002). Nevertheless, severe reactions are a cause of patient morbidity and in rare cases mortality and can therefore prevent effective drug therapy, especially if a drug has to be withdrawn and there is no efficacious therapeutic alternative. Patients with a history of hypersensitivity to one anticonvulsant are more susceptible to the development of multiple drug hypersensitivities; the actual cross sensitivity rate for rashes involving carbamazepine and phenytoin is 40–58% (Hyson and Sadler, 1997). The mechanistic basis for the increased risk is not understood since anti-convulsants are not structurally related.

Lymphocytes from patients hypersensitive to carbamazepine, phenobarbital, phenytoin and lamotrigine have been shown to proliferate in the presence of the suspect drugs (Kahn et al., 1984; Zakrzewska and Ivanyi, 1988; Sachs et al., 1997; Troger et al., 2000), while immunohistochemical studies have shown the presence of activated CD4+ and CD8+ T cells (Friedmann et al., 1994; Mauri-Hellweg et al., 1995). We have recently used an in vitro lymphocyte proliferation assay to measure sensitisation to 18 anticonvulsant hypersensitive patients (13 carbamazepine, 3 lamotrigine, 2 phenytoin). Controls were patients exposed to the anti-convulsants long-term without the development of adverse effects. Fig. 6 summarises the proliferation data obtained from hypersensitive patients. In each experiment, proliferation was concentration-dependent; proliferation was seen at therapeutic drug concentrations, whereas super-therapeutic concentrations inhibited proliferation. Control patients did not proliferate in the presence of carbamazepine, lamotrigine or phenytoin. Based on the success of these preliminary studies, we now use a lymphocyte proliferation assay in Liverpool as part of a patient’s management programme. Fig. 7 shows data from one patient referred with carbamazepine hypersensitivity 21 days after the drug was first administered. Clinical features of the reaction included a widespread maculopapular rash, fever and raised liver enzymes. In the past 24 months, the patient has been administered various drugs to treat epilepsy; however, these drugs were either not effective or were associated with a recurrence of...
Fig. 7. Drug and drug metabolite-specific proliferation of lymphocytes from a carbamazepine hypersensitive patient.

symptoms of hypersensitivity. Thus, the aim of our investigations was two-fold: first, the identification of carbamazepine-specific T cells and therefore diagnosis of anticonvulsant hypersensitivity; and secondly, determination of whether the patients cells proliferated with the pro-drug oxcarbazepine (oxcarbazepine is rapidly converted to the active component 10-hydroxy carbamazepine in vivo (Eadie, 1991)), one of the few anti-epileptic drugs that the patient has never been exposed to. As expected lymphocytes proliferated in the presence of carbamazepine and carbamazepine-10,11-epoxide, a major stable circulating metabolite (Madden et al., 1996); however, despite the fact that the patient has never been exposed to oxcarbazepine, his lymphocytes proliferated in the presence of 10-hydroxy carbamazepine. The patient was not administered oxcarbamazepine; thus, preventing the development of a potentially serious adverse drug reaction.

The aim of our recent studies has been to elucidate the cellular nature of the T-cell response in anticonvulsant hypersensitivity and therefore begin to understand the mechanism by which T cells cause serious tissue injury in man. To address these issues, T cells from carbamazepine and lamotrigine hypersensitive patients who developed maculopapular exanthema as part of a generalised hypersensitivity reaction were cloned and characterized in terms of their phenotype, functionality and mechanisms of antigen presentation and cytotoxicity (Naisbitt et al., 2003b, 2003c). Over 80 drug-specific T-cell clones were generated; most of which were CD4+ with occasional CD8+ cells. All clones expressed (a) the αβ T-cell receptor with use of a single Vβ chain. Usage of Vβ 5.1 seemed to be of particular importance in anticonvulsant hypersensitivity reactions since a number of T-cell clones from carbamazepine, lamotrigine and phenobarbital (Hashizume et al., 2002) hypersensitive patients were T-cell receptor Vβ 5.1 positive. Lamotrigine- and carbamazepine-stimulated T cells killed autologous target cells and secreted perforin, IFN-γ, IL-5 and MIP-1α, MIP-1β and I-309. RANTES was expressed constitutively. Secretion of IFN-γ, MIP-1α, MIP-1β and RANTES alongside ATAC has recently been shown to constitute a group of “type I cytokines” that act together as a functional unit by cells of the innate and adaptive immune system to drive antigen-specific responses in vivo (Dorner et al., 2002). I-309 is a regulatory chemokine that controls the duration and strength of the inflammatory response (Sebastiani et al., 2001). T-cell recognition of carbamazepine and lamotrigine was dependent on the presence of HLA class II (DR/DQ) matched antigen presenting cells, but seemed to be independent of drug metabolism and antigen processing. The T-cell receptor of certain clones could accommodate some stable drug metabolites, but no cross reactivity was seen with other anti-convulsants or structural analogues.

These data characterise drug-specific T cells in anticonvulsant hypersensitive patients that are phenotypically different to T cells involved in other serious cutaneous adverse drug reactions and show that Th1 secreting T cells mediate anticonvulsant hypersensitivity. Demonstration of IL-5 secretion and perforin-mediated killing are consistent with the clinical features including eosinophilia and the observed tissue damage in skin (Fig. 8). However, the reason(s) why skin is so frequently affected in anticonvulsant hypersensitivity is not fully understood. It is likely that trafficking of antigen-specific T cells, which is
regulated by differential cell surface expression of chemokine and tissue homing receptors, plays a key role in tissue-specific disease including drug hypersensitivity. Using lymphocytes isolated from drug hypersensitive patients, increased levels of the skin homing molecule, cutaneous lymphocyte antigen has been detected on activated T cells, which parallels the severity of the disease (Leyva et al., 2000). Using flow cytometry and an anti-CLA antibody we have reported that drug-specific T-cell clones from patients with anticonvulsant hypersensitivity also express high levels of CLA, when compared with tetanus toxoid-specific T-cell clones from the same patient (Naisbitt et al., 2003c). Homey et al. (2002) and Soler et al. (2003) in independent reports have shown that most CLA positive T cells from patients with allergic dermatitis express skin-homing chemokine receptors, such as CCR10 and CCR4. In preliminary studies, on a limited cohort of lamotrigine-specific T-cell clones, we have shown relatively high expression of CCR10, but not CCR4 (unpublished observations). Further studies of skin homing receptors in anticonvulsant hypersensitivity might allow us to identify T-cell receptor gene polymorphisms that act as determinants of susceptibility, which will aid the development of therapeutic tools for the treatment of T-cell mediated diseases involving the skin.

4. Conclusions and future perspectives

Drug hypersensitivity reactions are T-cell mediated, they can be severe and cause patient deaths. Characterisation of drug-specific T cells from peripheral blood of hypersensitive patients, reveals the presence of T cells that recognise drugs in the presence and absence of drug metabolism. By characterising the cellular basis of drug hypersensitivity reactions in skin, we have classified the different forms of cutaneous adverse drug reaction based on cellular and molecular pathophysiology. These recent advances in our understanding of drug hypersensitivity provide a framework
to develop in vitro cell culture assays that might be useful for patient diagnosis and drug evaluation. In this respect, we are validating an in vitro induction protocol using lymphocytes from drug naive volun-
teers to study the ability of the drug sulfamethoxazole and the drug metabolite nitroso sulfamethoxazole to induce a primary T-cell response. Lymphocytes were stimulated weekly for 5 weeks with autologous irradiated blood mononuclear cells and IL-2 in the presence of either sulfamethoxazole or nitroso sul-
framethoxazole. Lymphocytes from 10/10 individuals reacted in response to the reactive metabolite nitroso sulfamethoxazole, but only 3/10 reacted in the pres-
ence of sulfamethoxazole (Engler et al., submitted for publication). Following future development, we hope that this in vitro induction protocol might be used to predict the potential of a new chemical entity to cause T-cell mediated reactions in man.

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