The Role Of Metabolism In Drug-Induced Hypersensitivity Reactions

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of Doctor in Philosophy

by

Joseph Peter Sanderson

October 2007

Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree

Joseph P. Sanderson B.Sc.(Hons)

For Charlie

Acknowledgements

Contents

Abbreviations

ABT 1-aminobenzotriazole

ADR adverse drug reaction

APC antigen-presenting cell

B-LCL B-lymphoblastic cell line

BSA bovine serum albumin

CBZ carbamazepine

cDNA complementary DNA

COX cyclooxygenase

cpm counts per minute

C_T cycles to threshold

CYP cytochrome P450

dH_2O distilled water

DMSO dimethylsulfoxide

DNA deoxyribonucleic acid

DNCB 2,4-dinitrochlorobenzene

DNFB 2,4-dinitrofluorobenzene

EBV Epstein-Barr virus

EDTA ethylenediamine tetraacetic acid

ELISA enzyme-linked immunosorbent assay

FBS fetal bovine serum

FITC fluorescein isothiocyanate

FMO flavin-containing monooxygenase

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GM-CSF granulocyte-monocyte colony stimulating factor

GSH reduced glutathione

GSSG oxidised glutathione

HBSS Hank's balanced salt solution

HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HIV human immunodeficiency virus

HOCl hypochlorous acid

HPLC high-performance liquid chromatography

H_2O_2 hydrogen peroxide

IL interleukin

LPS lipopolysaccharide

LTT lymphocyte transformation test

MHC major histocompatibility complex

MPO myeloperoxidase

MS mass spectrometry

NAC N-acetyl cysteine

NADP oxidised nicotinamide adenine dinucleotide phosphate

NADPH reduced nicotinamide adenine dinucleotide phosphate

NMR nuclear magnetic resonance

oxCBZ oxcarbazepine

PAMP pathogen-associated molecular pattern

PBMCs peripheral blood mononuclear cells

PCR polymerase chain reaction

PI propidium iodide

PMB polymyxin B

qRT-PCR quantitative real-time reverse transcriptase polymerase chain reaction

RNA ribonucleic acid

RPMI Roswell Park Memorial Institute

SI stimulation index

SMX sulfamethoxazole

SMX-OH hydroxyl-sulfamethoxazole

SMX-NHOH sulfamethoxazole hydroxylamine

SMX-NO nitroso-sulfamethoxazole

SMX-NO$_2$ nitro-sulfamethoxazole

TCR T-cell receptor

TEN toxic epidermal necrolysis

TLC thin-layer chromatography

TLR Toll-like receptor

TMP trimethoprim

Publications

● **Published experimental papers**

SANDERSON J. P., NAISBITT D. J., FARRELL J., ASHBY C. A., TUCKER M. J., RIEDER M. J., PIRMOHAMED M., CLARKE S. E., PARK B. K. (2007) Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J Immunol.* **178**, 5533-42.

WU Y., SANDERSON J. P., FARRELL J., DRUMMOND N. S., HANSON A., BOWKETT E., BERRY N., STACHULSKI A. V., CLARKE S. E., PICHLER W. J., PIRMOHAMED M., PARK B. K., NAISBITT D. J. (2006). Activation of T-cells by carbamazepine and carbamazepine metabolites. *J Allergy Clin Immunol.* **118**, 233-41.

● **Published review articles**

SANDERSON J. P., NAISBITT D. J., PARK B. K. (2006). The role of bioactivation in drug-induced hypersensitivity reactions. *AAPS J.* **8**, E55-64.

PARK B. K., SANDERSON J. P., NAISBITT D. J. (2007). Drugs as Haptens, Antigens, and Immunogens, chap PICHLER W. J. (ed): *Drug Hypersensitivity.* pp 55-65. Karger, Basel.

● **Published abstracts**

SANDERSON J. P., NAISBITT D. J., FARRELL J., DRUMMOND N. S., MATHEWS K. L., PIRMOHAMED M., CLARKE S. E., PARK B. K. (2006). Metabolism of sulphamethoxazole by dendritic cells: Implications for hypersensitivity. *Drug Metab Rev.* **38**, 182-3.

SANDERSON J. P., NAISBITT D. J., FARRELL J., DRUMMOND N., MATHEWS K. L., PIRMOHAMED M., CLARKE S. E., PARK B. K. (2006). Dendritic cell metabolism

of sulfamethoxazole leads to increased CD40 expression: a key determinant of immuno-genicity. *Br J Clin Pharmacol.* **61**, 645.

SANDERSON J. P., WU Y., FARRELL J., PICHLER W. J., PIRMOHAMED M., PARK B. K., NAISBITT D. J. (2004) Mechanisms of drug presentation to T-cells. *Immunology.* **113**, 104.

SANDERSON J. P., FARRELL J., COULTER E. M., CLARKE S. E., PARK B. K., NAISBITT, D. J. (2004) Investigation into the role of CD40 signalling in the induction of an immune response to a reactive metabolite of sulfamethoxazole. *Immunology.* **113**, 105.

● **Manuscripts in preparation**

SANDERSON J. P., HOLLIS F. J., MAGGS J., CLARKE S. E., NAISBITT D. J., PARK B. K. Non-enzymatic formation of a novel hydroxylated sulfamethoxa-zole compound in vitro: Implications for bioanalysis of sulfamethoxazole metabolism. *Manuscript in preparation.*

SANDERSON J. P., SYN W. K., FARRELL J., HANSON A., PIRMOHAMED M., PARK B. K., NAISBITT D.J. Characterisation of the T-cell response in a patient with trimethoprim-induced hypersensitivity with liver failure. *Manuscript in preparation.*

Abstract

Drug hypersensitivity reactions are believed to involve activation of the immune system, and in particular T-cells, although the mechanisms involved are not well understood. The studies reported in this thesis were intended to explore the possibility that drug metabolism and reactive metabolites are involved in the pathogenesis of these reactions.

There are two signals required for the activation of drug-specific T-cells, a primary event in the initiation of hypersensitivity, signal 1, or the antigenic signal, and signal 2, or danger signalling. Three paradigm drugs were used to explore the possibility that formation of reactive metabolites could supply one or both of these signals.

Firstly, the ability of sulfamethoxazole (SMX) or the protein-reactive metabolite nitroso-sulfamethoxazole (SMX-NO) to provide danger signals was explored. In this study, increased CD40 expression, but not CD80, CD83, or CD86, was demonstrated with dendritic cell surfaces exposed to SMX (250-500 μM) or SMX-NO (1-10 μM). Increased CD40 expression was not associated with apoptosis or necrosis, or glutathione depletion, but covalently modified intracellular proteins were detected when SMX was incubated with dendritic cells. Importantly, the enzyme inhibitor 1-aminobenzotriazole (ABT) prevented the CD40 upregulation with SMX, but not with SMX-NO or lipopolysaccharide (LPS). The enzymes CYP2C9, CYP2C8, and myeloperoxidase (MPO) were found to catalyse the conversion of SMX to sulfamethoxazole hydroxylamine, and MPO was found to be expressed in dendritic cells. SMX-NO immunogenicity was inhibited in mice with a blocking anti-CD40L antibody. In addition, when a primary SMX-NO-specific T cell response using drug-naïve human cells was generated, the magnitude of the response was enhanced when cultures were exposed to a stimulatory anti-CD40 antibody. These data indicate that local bioactivation by dendritic cells leads to dendritic cell maturation, an important event in SMX immunogenicity.

Secondly, T-cells isolated from individuals following carbamazepine (CBZ) hypersensitivity reactions were studied in order to explore their cross-reactivity with regards to several stable analogues of CBZ, and to determine the nature of the CBZ antigen to which they respond. PBMCs were found to proliferate in response to certain stable analogues, namely, CBZ-10,11-epoxide, 10-OH-CBZ, oxcarbazepine and N-acetyl-iminostilbene. Other derivatives, including CBZ-10,11-diol, iminostilbene, 2-OH-CBZ and 3-OH-CBZ were unable to induce a response. Several approaches were taken to determine the mechanism of CBZ presentation to CBZ-specific T-cell clones, including pulsing or fixing antigen-presenting cells, inhibiting oxidative metabolism by preincubation with ABT, determining the kinetics of presentation by TCR downregulation and combined pulse methods, and addition of glutathione to quench reactive metabolites. These all produced data suggestive of a metabolism-independent mechanism of presen-

tation, in that enzyme inhibition or co-incubation with glutathione did not alter the proliferative response, whereas pulsed antigen-presenting cells were incapable of presenting the drug to T-cells. In addition, the T-cells did not demonstrate a requirement for antigen processing, as fixation of antigen-presenting cells did not affect their ability to present the drug, and kinetic studies found that T-cells were activated with 2 h of drug addition, which is faster than antigen processing allows.

T-cell clones isolated from a patient following severe trimethoprim (TMP)-induced liver failure were similarly found to recognise the drug in the absence of metabolism and processing, as determined by a similar battery of tests.

In conclusion, it was found that SMX has the potential to provide the 'danger' signals required for the induction of hypersensitivity, as a result of localised bioactivation and covalent binding within the dendritic cell. Additionally, both CBZ and TMP were found to activate T-cells directly, with no prior requirement for covalent binding and hapten formation.

Chapter 1

General Introduction

Contents

1.1   Adverse Drug Reactions

The World Health Organisation definition of an adverse drug reaction (ADR) has existed for over 30 years, and is as follows,

> "A response to a drug that is noxious and unintended and occurs at doses
> normally used in man for the prophylaxis, diagnosis or therapy of disease,
> or for modification of physiological function" (WHO, 1972)

More recently, Edwards & Aronson (2000) suggested an alternative definition in order to remove ambiguities and to highlight the possibility of ADRs as a consequence of prescribing or administration errors,

> "An appreciably harmful or unpleasant reaction, resulting from an inter-
> vention related to the use of a medicinal product, which predicts hazard
> from future administration and warrants prevention or specific treatment,
> or alteration of the dosage regimen, or withdrawal of the product" (Edwards
> & Aronson, 2000)

However they are defined, ADRs are a common clinical problem that can often compromise good patient care. While most ADRs are relatively mild and self-limiting, and merely require discontinuation of the drug, some can be extremely severe and even life-threatening.

1.1.1 Classification of adverse drug reactions

The most commonly used system for the classification of ADRs is based on that devised by Rawlins & Thompson (1991) and later refined (Park *et al.*, 1992).

Type A, or 'Augmented' reactions, are those in which the adverse effect is simply an extension of the primary or secondary pharmacology of the drug, and so is typically predictable and dose-dependent. These reactions are generally reversible upon withdrawal of the offending drug. Examples of Type A reactions include postural hypotension induced by beta-blockers or NSAID-related damage to the gastric mucosa.

Type B reactions, known as either 'Bizarre' or 'Idiosyncratic' reactions, are those which cannot be predicted from the known pharmacology of the drug. These show no simple dose-response relationship, and typically only occur in a small proportion of those exposed to the drug. Host factors, particularly metabolic/immunological idiosyncrasies, are believed to play an important role in determining individual susceptibility to idiosyncratic reactions, although these factors have not been fully elucidated in most cases. Examples of these reactions are the agranulocytosis associated with clozapine administration or immune-mediated adverse effects such as hypersensitivity or anaphylaxis.

Type C reactions, or 'Chemical' reactions can be explained by the chemical behaviour of the drug or drug metabolites. These include paracetamol hepatotoxicity due to generation of a reactive n-acetylbenzoquinoneimine species.

Type D reactions, or 'Delayed' reactions include such adverse effects as carcinogenicity or teratogenicity and often present some time after treatment is completed.

Type E reactions, or 'End of treatment' reactions, are reactions that occur following removal of a drug, especially following sudden withdrawal. These include the withdrawal symptoms seen after stopping many anxiolytics.

It is suggested that idiosyncratic reactions can be further divided into immune-mediated or non-immune-mediated, although it is not always clear which category

a particular reaction falls under. Subclassification of immune-mediated idiosyncratic reactions will be dealt with below (see 1.1.4.1)

1.1.2 Prevalence of adverse drug reactions

A large-scale meta-analysis by Lazarou *et al.* (1998) estimated that ADRs occur in as many as 15% of all hospital patients, although this estimate has been widely disputed (Kvasz *et al.*, 2000). In a more recent prospective study in the United Kingdom (Pirmohamed *et al.*, 2004), the proportion of hospital admissions directly caused by ADRs was 5.2%. Most of these reactions (76.2%-95%) were defined as Type A (dose-dependent) reactions, while a large proportion of the remainder were type B (idiosyncratic).

Estimates for the mortality rates of ADRs vary, although a figure of 0.13% incidence of fatal ADRs in patients admitted to hospital and 0.19% occurring in patients during their stay has been suggested (Lazarou *et al.*, 1998). Other studies have found similar figures (0.15% and 0.11% of admissions respectively; Pirmohamed *et al.*, 2004; van der Hooft *et al.*, 2006), and a large-scale prospective study to investigate ADRs in the UK is underway (Davies *et al.*, 2006). If these figures are accepted, then the overall mortality from ADRs is a major public health issue. It is estimated that the total mortality from ADRs in the US for the year 1994 was 106,000 (95% confidence interval: 76000-137000; Lazarou *et al.*, 1998), which would put ADRs as between the fourth and sixth greatest causes of death in the country. The largest study of its kind in the UK has estimated that total deaths from ADRs in new admissions to hospital was 5,700 annually (3800-7600; Pirmohamed *et al.*, 2004), although this is likely to be an underestimate of the total death rate from ADRs, as it does not include those occurring in hospitalised patients.

The cost, both financial and as bed occupancy, to the NHS has been evaluated in several of these studies. A systematic analysis of previous studies (Wiffen *et al.*, 2002) estimated the excess total bed-occupancy due to ADRs as admissions to be 2-6 400-bed hospital equivalents, and that due to ADRs in inpatients to be 14 400-bed hospital equivalents, although there is a wide range (3-27). Financially, this was estimated to cost the NHS around £380m/year. Pirmohamed *et al.* (2004) estimated

the burden on the NHS of ADRs in new admissions to be approximately 7 800-bed hospital equivalents, although they admitted that this extrapolation was uncertain. They derived a cost to the NHS from this of up to £466m/year, which is significantly higher than that estimated by Wiffen et al. (2002), as it does not include inpatient episodes. This suggests that the true picture is uncertain, and more studies may be required. In any case, it is clear from this that ADRs are a significant health problem, and anything that can be done to minimise their impact will be beneficial throughout the health service.

1.1.3 Impact of adverse drug reactions on drug development

In addition to the clinical consequences of ADRs, it is worth considering their impact on drug development. The attrition rates of novel chemical entities in preclinical testing, and that of lead compounds in clinical testing, remain exceedingly high, and have even increased over recent decades. To give some perspective on this, at present approximately 8% of lead compounds which enter clinical testing will eventually be successfully licensed, compared to the historical rate of 15% (FDA, 2004), despite the much greater intensity and concentration on preclinical screening and high-throughput analyses, which would be expected to reduce clinical attrition rates (Caldwell et al., 2001; Kassel, 2004). This is one of the major causes of the exceptionally high cost of development of each drug which reaches marketing, estimated recently (DiMasi et al., 2003) at $802m, an annual increase of 7.4% above inflation compared to a previous study (DiMasi et al., 1991).

The primary causes of attrition in drug discovery are either lack of efficacy (46% of compounds in clinical testing) or toxicological risks (27-40%; DiMasi, 1995; Kennedy, 1997). While many types of ADR can be predicted quite readily either from their primary pharmacology, or from relatively simple preclinical testing, idiosyncratic reactions pose two particular problems. Firstly, since idiosyncratic reactions are rare, they are unlikely to be identified until either phase III trials or post-marketing, which will obviously increase the overall cost. Secondly, our understanding of the mechanisms of idiosyncratic reactions is poor, which means that our ability to make predictions

based solely on mechanistic considerations is correspondingly limited. For this reason, preclinical screening has concentrated largely on eliminating candidate lead compounds which show potential for formation of protein-reactive metabolites (Caldwell & Yan, 2006; Williams & Park, 2003). However, this is a crude approach, and is probably responsible for unnecessary attrition of compounds which would not have produced problems, and so must be approached with an open mind (Evans *et al.*, 2004; Williams & Park, 2003). An improved understanding of the mechanisms of idiosyncratic reactions would aid immeasurably in the development of more robust and reliable screening tools.

1.1.4 Immune-mediated adverse drug reactions

The work in this thesis will be primarily concerned with the pathogenesis and mechanisms behind immune-mediated idiosyncratic reactions, in particular cutaneous delayed-type T-cell mediated reactions, often described as hypersensitivity reactions, although this does not accurately cover the heterogeneity of the reactions and their underlying pathologies.

1.1.4.1 Classification of immune-mediated ADRs

The clinical diagnosis of immune-mediated ADRs can be extremely problematic. In many cases, immune-mediated drug reactions may present in a similar fashion to many immunological pathologies, such as drug-induced lupus or rash, in which cases often the only indicator that the reactions are drug-induced is the temporal association between their incidence or termination and the period of drug exposure. Furthermore, even if the drug-associated nature of the reaction is unquestionable, it can be difficult to unequivocally identify the role of the immune system in the pathology. For instance, there is considerable evidence that the innate immune system is activated in paracetamol-induced liver injury (Liu & Kaplowitz, 2006), but whether this is primary or secondary, pathological or protective, or, indeed, merely epiphenomenological, is uncertain, and may vary from case to case.

These caveats notwithstanding, the most commonly used classification of immune-

Table 1.1: Relationship of clinical symptoms to type of immune response (adapted from Pichler, 2003)

Extended Gell and Coombs classification	Type of Immune response	Pathologic Characteristics	Clinical Symptoms
Type I	IgE	Mast-cell degranulation	Urticaria, anaphylaxia
Type II	IgG and FcR	FcR-dependent cell destruction	Blood cell dyscrasia
Type III	IgG and complement or FcR	Immunocomplex deposition	Vasculitis
Type IVa	Th1 (IFN-y)	Monocyte activation	Eczema
Type IVb	Th2 (IL-5 and IL-4)	Eosinophilic infiltration	Maculopapular exanthema, bullous exanthema
Type IVc	CTL (perforin and granzyme B)	CD4- or CD8-mediated killing of cells	Maculopapular exanthema, eczema, bullous exanthema, pustular exanthema
Type IVd	T-cells (IL-8)	Neutrophil recruitment and activation	Pustular exanthema

mediated ADRs is the Gell and Coombs scheme (see table 1.1), first published as Gell & Coombs (1963). This divides reactions on the basis of clinical presentation, and allocates them tentatively to a proposed pathophysiology. This scheme was further enlarged by the subdivision of type IV reactions (delayed-type reactions mediated by T-cells) into four subcategories based on variations in T-cell polarisation (Pichler, 2003).

While this scheme is useful for gaining an understanding of some of the underlying pathology, it doesn't necessarily accurately reflect the real-life situation. In particular, many delayed-type hypersensitivity reactions actually represent multiple overlapping pathologies. Furthermore, while the assignments of pathology are superficially plausible, there is insufficient evidence in many cases to unequivocally identify the fundamental pathophysiology.

1.1.4.2 Clinical features of type IV immune-mediated ADRs

Type IV immune-mediated ADRs most commonly affect the skin, and cover a wide range of symptoms, the majority involving mild, self-limiting urticaria or morbilliform

rashes. However, a small proportion of patients go on to develop a more severe reaction, known as drug hypersensitivity syndrome. This normally occurs 1-3 weeks after commencement of treatment, although it may occur some months later, and is characterised by the triad of rash, fever and internal involvement, often accompanied by lymphadenopathy (Knowles *et al.*, 1999). For this reason, it is also known as drug rash with eosinophilia and systemic symptoms, or DRESS. This is particularly common with aromatic anticonvulsants, but it has been seen with antimicrobials (particularly sulfonamides) and NSAIDs. Although this condition can be life-threatening, prompt withdrawal of the drug, accompanied by corticosteroid or intravenous immunoglobulin treatment (Mostella *et al.*, 2004; Scheuerman *et al.*, 2001), is normally sufficient for a complete recovery.

Erythema multiforme is a similar condition, associated with the administration of several drugs, but particularly anticonvulsants and antimicrobials. Erythema multiforme minor consists of target lesions with mild cutaneous involvement, although the patients remain healthy. More severe cases, however, form a spectrum from erythema multiforme major and Stevens-Johnson syndrome to toxic epidermal necrolysis. In Stevens-Johnson syndrome there is widespread skin toxicity, including sloughing of < 10% of total skin area, serious mucosal membrane involvement, and some degree of internal involvement, including lymphadenopathy. More severe cases, known as toxic epidermal necrolysis, involve massive skin sloughing (> 30% of body surface), with severe risk of secondary infection and sepsis. These conditions are often fatal, with mortality rates for toxic epidermal necrolysis in excess of 30% (Prendiville, 2002). High-dose corticosteroids are commonly used alongside supportive management, although there is some evidence that this can lead to increased mortality (Halebian *et al.*, 1986) through weakened immune responses to infection (Jones *et al.*, 1989).

1.2 Drug Metabolism

Most commonly used drugs are relatively lipophilic. This enhances their activity both by increased potency at their designated target, and by decreased excretion, hence allowing greater overall exposure to the drug. Therefore, in order to become readily

excretable, most drugs require structural alteration. This process, known as drug metabolism, is a major area of pharmacological investigation, as it plays an important role in determining both the effectiveness and toxicity of the drug *in vivo*.

The reactions which make up drug metabolism are typically divided into Phase I and Phase II reactions (Williams, 1959), although this does not necessarily imply they are temporally sequential. Phase I reactions are those which either introduce or unmask a reactive group upon the molecule, whereas Phase II reactions typically conjugate a polar endogenous compound onto the molecule in order to increase the rate of excretion of the compound. Phase I metabolism therefore can be said to prime the molecule for Phase II reactions, which actually have the desired effect of increasing excretion.

1.2.1 Phase I metabolism

Phase I metabolism can be divided into three major classes of reaction, namely oxidation, reduction and hydrolysis reactions. Oxidative Phase I reactions are summarised in table 1.2.

1.2.1.1 Cytochrome P450s
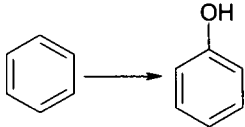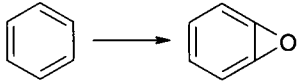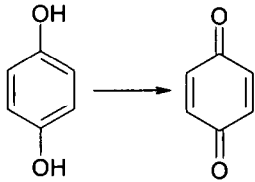
The quantitatively most important Phase I enzymes are the cytochrome P450 (CYP) enzymes. These are a family of structurally related haemoxygenases located in the endoplasmic reticulum. Although they are predominantly expressed in the liver, there is growing evidence that extrahepatic CYPs may be responsible for localised toxicity, which will be further discussed later (see 1.2.5).

CYP enzymes are classified according to amino acid sequence, with families being defined as CYPs which share greater than 40% sequence homology, and subfamilies sharing at least 59% similarity (Nebert *et al.*, 1987). Nearly all of the CYPs responsible for xenobiotic metabolism fall within families CYP1-4. Table 1.3 details the main isoforms responsible for drug metabolism.

CYPs enzymes are formed from a single polypeptide chain attached non-covalently via a conserved cysteine residue to the central iron atom of a haem group. Substrates bind to the iron centre, as Fe^{3+}, which initiates electron transport and oxygen binding.

9

Table 1.2: Phase I metabolism: Oxidative reactions

Type of Reaction	Mechanism
1. Aromatic Hydroxylation	benzene → phenol (OH)
2. Aliphatic Hydroxylation	$R-CH_3 \longrightarrow R-CH_2OH$
3. Epoxidation	benzene → arene oxide (O)
4. O,N,S-dealkylation	$R-O-CH_3 \rightarrow [R-O-\overset{H}{\underset{OH}{C}}-H] \longrightarrow ROH + HCHO$
5. Oxidative deamination	$R-\overset{CH_3}{\underset{H}{C}}-NH_2 \longrightarrow [R-\overset{CH_3}{\underset{OH}{C}}-NH_2] \longrightarrow R-\overset{CH_3}{\underset{O}{C}} + NH_3$
6. N-hydroxylation	$R-NH_2 \longrightarrow R-N\overset{OH}{\underset{H}{}}$
7. S-oxidation	$R-S-R' \longrightarrow R-\overset{O}{\underset{}{S}}-R'$
8. Dehalogenation	$R-\overset{H}{\underset{Cl}{C}}-Br \longrightarrow R-\overset{}{\underset{H_2}{C}}-OH$
9. Desulphuration	$R=S \longrightarrow R=O$
10. Alcohol oxidation	$H_3C-\overset{}{\underset{H_2}{C}}-OH \longrightarrow H_3C-\overset{OH}{\underset{H}{C}}-OH \longrightarrow H_3C-CHO$
11. Quinone formation	hydroquinone (OH, OH) → quinone (O, O)

10

Table 1.3: Characteristics of human cytochrome P450 metabolising enzymes

Isoform	Location	Substrate	Inhibitor	Inducer
CYP1A1	Primarily extra-hepatic	Benzo(a)pyrene	Napthoflavone	3-methylchol-anthrene
CYP1A2	Liver	Caffeine, Phenacetin	Furafylline	Benzo(a)pyrene
CYP1B1	Leukocytes	Benzo(a)pyrene, caffeine	Resveratrol	
CYP2A6	Liver	Coumarin	Tranylcypromine	Barbiturates
CYP2B6	Liver	Cyclophosphamide	Orphenadrine	Phenobarbitone
CYP2C8	Liver, intestine	Tolbutamide, diclofenac	Quercetin	
CYP2C9	Liver, intestine	Tolbutamide, warfarin	Sulfaphenazole	Rifampicin
CYP2C19	Liver	Omeprazole, proguanil	Tranylcypromine	Rifampicin
CYP2D6	Liver, intestine, kidney	Bufuralol, debrisoquine	Quinidine	
CYP2E1	Liver, intestine	Paracetamol, ethanol	Isoniazid	Ethanol
CYP3A4	Liver, intestine	Cyclosporin, carbamazepine	Ketoconazole, troleandomycin	Rifampicin, phenytoin
CYP3A5	Liver, placenta	Nifedipine	Troleandomycin	
CYP4A11	Lung	Testosterone		Clofibrate

11

Electrons are transferred to the iron from reduced nicotinamide adenine dinucleotide phosphate (NADPH) by cytochrome p450 reductase, the reduced iron (Fe^{2+}) binds and reduces molecular oxygen, in concert with a further electron from either cytochrome p450 reductase or cytochrome b_5. Loss of an atom of oxygen leaves the final oxidative complex, a high valent iron-oxo species. This chemically inserts oxygen into the substrate and decomposes to regenerate the resting form of the enzyme (Fe^{3+}). The overall reaction can be summarised as follows:

$$NADPH + O_2 + RH + H^+ \rightarrow NADP^+ + ROH + H_2O$$
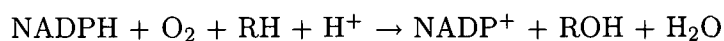
1.2.1.2 Other phase I enzymes

In addition to CYP enzymes, there are several other important enzymes which catalyse Phase I reactions. These can be roughly divided into oxidative, reductive and hydrolytic enzymes, of which only the oxidative enzymes are of importance for this discussion. We will briefly discuss two major classes of oxidative enzymes, the peroxidases and the flavin-containing monooxygenases (FMOs).

The general mechanism of action of peroxidases involves the reduction of a hydroperoxide (often, but not exclusively, hydrogen peroxide) to the corresponding alcohol, and oxidation of the enzyme to an intermediate known as Compound I. This is capable of oxidising xenobiotics directly, although in the presence of halide ions (Cl^- in the case of myeloperoxidase (MPO), Br^- for thyroid peroxidase), it produces significant quantities of hypochlorous acid (HOCl), a powerful oxidising agent. The native enzyme is regenerated via one or two further intermediates, Compounds II and III. Several peroxidase enzymes have been associated with xenobiotic metabolism, including MPO (Cribb et al., 1990; Liu & Uetrecht, 1995), cyclooxygenase (COX; Vogel, 2000), lactoperoxidase (Goodwin et al., 1996) and thyroid peroxidase (Doerge et al., 1997). Of these, MPO is the best studied, and will be discussed further here. MPO is largely stored in the lysosomes of neutrophils and leukocytes, and is released along with H_2O_2 as part of the respiratory burst. For this reason, reactive metabolites generated by myeloperoxidase are likely to be formed extracellularly, in contrast to those generated by the majority

of drug metabolising enzymes, with potential implications for the likely protein targets and the overall canon of modified proteins. While MPO-mediated oxidation is unlikely to make up a large part of the overall metabolism of a xenobiotic, it is thought to contribute to several ADRs. This will be discussed further later in the chapter (see 1.2.5.3).

FMOs are a family of microsomal oxidative metabolic enzymes, of which 5 are known to exist in humans, named FMO1-5. These enzymes are largely expressed in the liver, although there is also evidence for expression of most isoforms in kidney, intestine, lung (Cashman & Zhang, 2006) and skin (Janmohamed *et al.*, 2001). One of the key distinctions between reactions catalysed by these enzymes and reactions catalysed by CYPs is that FMO-catalysed reactions tend to be oxidations of nucleophilic centres, particularly N- and S-oxidations (Eswaramoorthy *et al.*, 2006), rather than oxidation of carbon atoms. While certain compounds are known to be metabolised by one or more of these enzymes (Cashman, 2000), little is known about any possible role they may play in the formation of reactive metabolites.

1.2.2 Phase II metabolism

Phase II metabolism involves the coupling of a large polar group to a reactive site on the target xenobiotic. With a few exceptions, this makes the molecule more hydrophilic, reduces its biological activity, and enhances its renal and biliary excretion.

The most important route of Phase II metabolism is glucuronidation, whereby a glucuronic acid moiety is transferred from uridine-5'-diphosphoglucuronic acid by uridine 5'-diphospho-glucuronosyltransferase to an exposed functional group on a xenobiotic, typically alcohol or amine groups, although conjugation to thiols and carboxylic acids has also been described. Unusually for a Phase II reaction, this has been associated with the formation of reactive metabolites, the acyl glucuronides. It has been suggested that this may be one of the mechanisms of the idiosyncratic hepatotoxicity seen with diclofenac administration (Kenny *et al.*, 2004). Other important pathways of Phase II metabolism are sulfation and acetylation. The latter is particularly important for aromatic amines, and is catalysed by the polymorphically expressed N-acetyl-transferase

family.

Glutathione conjugation differs from the above Phase II metabolic pathways in that its main role is to detoxify electrophilic reactive metabolites rather than simply enhance their clearance. This will be discussed in greater detail later in the chapter (see 1.2.4)

1.2.3 Reactive metabolites

Whilst the majority of metabolic reactions reduce the biological impact of xenobiotics, this is not universally the case. Both Phase I and Phase II metabolism can transform, or bioactivate, chemically inert compounds to compounds capable of directly reacting with cellular macromolecules. Examples of these reactions are listed in table 1.4.

Table 1.4: Reactive metabolites

Xenobiotic	Reaction	Reactive species	Toxicity
Benzo(a)pyrene	Epoxidation	Epoxide	Hepatocarcinogenicity
Paracetamol	Oxidation	p-Quinoneimine	Hepatotoxicity
Halothane	Dehalogenation	Trifluoroacetyl radical	Hepatotoxicity
Carbamazepine	Epoxidation, Oxidation	Quinoneimine, Arene oxide	Hypersensitivity
Clozapine	Oxidation	Nitrenium ion	Agranulocytosis
Amodiaquine	Oxidation	Quinoneimine	Agranulocytosis, hepatotoxicity
Sulfamethoxazole	N-hydroxylation	Arylnitroso	Hypersensitivity, thrombocytopenia
Diclofenac	Glucuronidation	Acyl glucuronide	Hepatotoxicity
Isoniazid	Acetylation, oxidation	Acetyl radical	Hepatotoxicity
Tamoxifen	Sulfation, oxidation	Carbocation	Hepatocarcinogenicity

The toxicity generated by reactive metabolites occurs by several different mechanisms, including direct toxicity and genotoxicity through protein and DNA adduction, and oxidative stress through either depletion of cellular antioxidant stores or direct production of reactive oxygen species.

It is not clear yet whether the toxicity that ensues from protein adduction is produced simply as a function of overwhelming nonspecific dysregulation of proteins, or requires damage to critical target proteins (Park et al., 2005). As certain nontoxic compounds are known to produce adducts at similar levels to structurally-related hep-

atotoxins (for instance, 3-hydroxyacetanilide vs. paracetamol; Roberts *et al.*, 1990), it would seem that simple quantity of adduct formation is unlikely to be the key determinant of toxicity. In addition, the reactive sulfamethoxazole (SMX) metabolite nitroso-sulfamethoxazole (SMX-NO) only induces cell death *in vitro* above a threshold of adduct formation; below that it is apparently well tolerated (Naisbitt *et al.*, 2002). It seems likely that certain sacrificial proteins, such as metallothionein (Liu *et al.*, 1999), are more readily adducted as part of the evolved cellular defense against chemical stress. A recent project to categorise and codify adducted proteins following reactive species treatment (Hanzlik *et al.*, 2007) will hopefully allow a better understanding of the relationship between patterns of adduction and toxicity.

1.2.4 Cellular defense

The body has evolved several approaches to minimising either the exposure to or the damage from reactive chemical species. Chemical species are detoxified either enzymatically or non-enzymatically, typically by reduction or conjugation reactions, and the damage induced by reactive species is carefully managed both by selective proteolysis and DNA repair, and ultimately programmed cell death or apoptosis. These processes are not just passive, and the cell actively responds to enhanced chemical stress (Goldring *et al.*, 2006).

Several low-molecular-weight compounds have an important role to play in reactive species detoxification. In particular, reduced glutathione (GSH), a tripeptide formed from cysteine, glycine and glutamate, acts as a powerful nucleophile to conjugate electrophilic species, either directly or when catalysed by a member of the glutathione-s-transferase family. This conjugate is either excreted following derivatisation of mercapturic acids, or they can react with unreacted GSH to produce the dithiol oxidised glutathione (GSSG), and release the reduced, and therefore less reactive, metabolite. GSSG is then reduced back to GSH by GSH reductase. Although this is initially protective, if the reduced metabolite is then oxidised, whether enzymatically or not, this can form a futile cycle, depleting intracellular stores of GSH and leading to cell damage. Other important small-molecules with protective roles in the cell are ascorbate

(vitamin C), tocopherol (vitamin E), and N-acetyl-cysteine.

1.2.5 Metabolism in drug hypersensitivity

The following section provides a brief overview of the importance of organ/tissue-specific metabolic drug activation in the development of drug hypersensitivity in some individuals but not others. We will focus specifically on metabolism in the liver, the skin, and the immune system. Although the liver is known to be the most important location for drug metabolism in the body, extrahepatic metabolism is also suspected to have an important role in the induction of hypersensitivity reactions.

1.2.5.1 Liver

Quantitatively, the liver is the most important organ for drug metabolism. Hepatic metabolism, primarily via CYPs, is the main route of bioactivation for drugs that have been linked to hypersensitivity, such as SMX (Cribb & Spielberg, 1992), carbamazepine (CBZ; Ju & Uetrecht, 1999; Pirmohamed *et al.*, 1992), phenytoin (Cuttle *et al.*, 2000), abacavir (Walsh *et al.*, 2002), and halothane (Njoku *et al.*, 1997). In most (although not all) cases, the reactive species formed are so reactive that they are unlikely to survive long in circulation, which implies that the liver will receive much greater exposure from reactive metabolites than other tissues. However, despite the increased exposure, the liver is rarely the main target for hypersensitivity reactions. There are 2 likely reasons for this discrepancy. First, the liver is very well protected from toxic insult (Park *et al.*, 2005) by cellular cytoprotective measures, such as high GSH and N-acetyl cysteine (NAC) levels, and readily activates further defenses via Nrf2 and NF-κB driven transcription. Second, the liver is an immunologically privileged organ (Ju & Pohl, 2005), and hepatic activation of T-cells by Kupffer cells, the resident hepatic antigen-presenting cells (APCs), is likely to lead to tolerance rather than a pathogenic immune response. This is believed to be at least partly due to increased expression of FasL in nonlymphoid hepatic tissue (French & Tschopp, 1996), which will drive T-cells to apoptosis rather than activation.

Halothane hepatitis is well studied as a model of drug-induced immune-mediated

hepatotoxicity. A transient increase in transaminases is seen in up to 20% of patients, whereas a severe reaction, characterised by massive cell necrosis, occurs in 1 patient per 35,000 on primary exposure, and 1 in 3700 on secondary exposure (Neuberger, 1990). Halothane is metabolised in the liver, predominantly by CYP2E1 (Eliasson et al., 1998), to trifluoroacetic acid, chloride, and bromide (Cohen et al., 1975). However, the reactive metabolite trifluoroacetyl chloride is also formed, which readily forms trifluoroacetyl adducts to free amino groups on hepatic proteins (Kenna et al., 1988a). The role of metabolism in the hepatitis associated with halothane administration is best illustrated by a global consideration of the relationship between the in vivo metabolism of general anesthetics and the observed incidence of ADRs in humans. Up to 50% of methoxyflurane and halothane is excreted as metabolites in human urine, and their administration is associated with severe toxicities. In contrast, less than 3% of enflurane and isoflurane is excreted as urinary metabolites, and human exposure is only rarely associated with hepatotoxicity (Park et al., 1998).

Antibodies to adducted neoantigens, particularly certain microsomal proteins (Gut et al., 1993), have been identified in the sera of halothane hepatitis patients (Kenna et al., 1988b), which has led some to conclude that these adducts are immunologically relevant. However, similar adducts are found in non-hypersensitive halothane-exposed patients, where they do not appear to be pathogenic (Gut et al., 1993), indicating that the major determinant of response may be idiosyncrasies in the immune system rather than generation of reactive metabolites and adducts. Additionally, although cellular reactivity to halothane has been identified both in humans (Mieli-Vergani et al., 1980) and in a guinea pig model (Furst & Gandolfi, 1997; Furst et al., 1997), most studies have concentrated on the role of autoantibodies and antibodies to neoantigens. It is therefore possible that an important pathogenic mechanism is being overlooked, and further work is required in order to address this.

1.2.5.2 Skin

Recently, there has been a lot of interest in the metabolic potential of the skin (Swanson, 2004), as skin is the most common site of hypersensitivity reactions (although it

must be noted that we do not know how many minor hypersensitivity reactions are associated with subclinical internal organ damage). Keratinocytes are metabolically active, expressing high levels of several CYP isoforms (Baron *et al.*, 2001; Yengi *et al.*, 2003). CYP mRNA has also been identified in other skin cell types, such as fibroblasts and melanocytes (Saeki *et al.*, 2002). When the activity of primary keratinocytes is compared with primary liver tissue (Swanson, 2004), conflicting data have been obtained, which is likely to be due to the wide inter- and intraindividual variation in both skin and hepatic CYP expression. Interestingly, although most important hepatic CYPs are expressed in the skin (including CYPs 1A1, 1B1, 2B6, and 3A4), there are several CYPs which are much more abundant in the skin, including several members of the CYP2 family that have never been identified in the liver (Du *et al.*, 2004). The relevance of this for hypersensitivity is not known, but it is possible that the relative proportion of metabolites produced in the skin may differ from those in the liver, with potential immunotoxicological implications.

One of the most important recent findings in this area was the demonstration that primary keratinocytes are capable of oxidative metabolism of both SMX and dapsone to their corresponding hydroxylamine metabolites (Reilly *et al.*, 2000). Intracellular SMX- and dapsone-protein adducts have also been identified in primary human keratinocytes when incubated with SMX, and these adducts co-localise on the cell surface with HLA-ABC (Roychowdhury *et al.*, 2005). Although the enzyme(s) responsible do not appear to be CYPs (Vyas *et al.*, 2006a), both peroxidases (but not MPO, lactoperoxidase or thyroid peroxidase) and FMOs, particularly FMO3, appear to play a role (Vyas *et al.*, 2006b), although uncertain inhibitor specificity make it hard to draw precise conclusions. It is not known as yet whether these adducts are actively presented in the context of HLA or are simply co-localised, for instance as part of lipid rafts. Furthermore, the ability of hapten-modified cutaneous protein to stimulate T-cells from hypersensitive patients has not been evaluated.

1.2.5.3 Immune System

There is less known about the metabolic activity of cells of the immune system than is known about the metabolic activity of cells of the liver or the skin. CYP expression in peripheral blood lymphocytes has been assessed (Dey et al., 2001; Krovat et al., 2000; McConnachie et al., 2003; Starkel et al., 1999), although a wide variability in findings (Finnstrom et al., 2002) makes interpretation difficult. Other immune cells are not as well studied, but there is some evidence that monocyte-derived dendritic cells (Sieben et al., 1999) and Langerhans cells (Saeki et al., 2002) are metabolically active. A common feature of CYP expression in the immune system is the high levels of expression of CYP1B1 (Baron et al., 1998), which is not expressed hepatically. Furthermore, studies have shown that this isoform has specificity for several xenobiotics, (Shimada et al., 1997) suggesting that there is the potential for specific immunological activation of drugs. Although CYP enzymes are the most widely studied in xenobiotic bioactivation, other enzyme systems, particularly peroxidases, are also capable of oxidative metabolism of small molecules. Two of these, MPO and cyclooxygenase (COX)-2, are highly expressed in cells of the immune system, particularly neutrophils and monocytes. Several drugs associated with a relatively high incidence of hypersensitivity reactions—including SMX (Cribb et al., 1990; Uetrecht et al., 1993), CBZ (Furst & Uetrecht, 1993), dapsone (Cribb et al., 1990), and trimethoprim (TMP) (Lai et al., 1999)—are metabolised to reactive intermediates by these systems. Neutrophils express both peroxidase systems (but low levels of CYPs) and can generate a powerful extracellular oxidising system when activated. For these reasons, and because of their sheer numbers in circulation, neutrophils have been described as "the greatest drug-metabolising engine outside of the liver" (Rubin & Kretz-Rommel, 2001). The role of neutrophils in drug-induced lupus has been widely discussed and has been linked to the ability of lupus-inducing drugs to be bioactivated by MPO (Jiang et al., 1994). Langerhans cells and dendritic cells express COX (Norgauer et al., 2003), although there is contradictory evidence regarding the expression of MPO (Pickl et al., 1996; Scholz et al., 2004). Although the specialised antigen-presenting nature and metabolic activity of Langerhans and dendritic cells would presumably enhance their potential for

initiating an immune response to haptenated proteins, this has not been unequivocally identified either *in vivo* or *in vitro*.

1.3 Immune Activation by Drugs

As discussed above, immune-mediated drug reactions are a major clinical problem. Despite extensive research, much is still unknown about the fundamental immunological mechanisms behind such reactions. From an immunotoxicological perspective it is essential that these fundamental mechanisms are elucidated in order to reduce this burden, in order to inform the physician with regard to the safer use of existing treatments and also to inform the medicinal chemist to facilitate the design of safer and better tolerated drugs.

Before considering the possibilities for a drug to activate the immune system, it is instructive to define some of the terms which will be used in this discussion, namely, hapten, antigen, and immunogen (Abbas & Lichtman, 2003; Park *et al.*, 1998).

Hapten: A small molecule which can only induce an immunological response when bound to a carrier protein, either exogenous or endogenous.

Antigen: Any substance which can be specifically bound by an antibody or T-cell receptor.

Immunogen: A molecule which can stimulate a specific cellular or humoral immune response.

It is important to note that individual compounds may have any of a number of combinations of these properties - while all immunogens are antigens, not all antigens are immunogens, for instance self-proteins are antigenic, but do not normally stimulate an immune response.

The following discussion will focus on the various mechanisms by which the immune system is activated, and how drugs can interact with these pathways to induce immune activation.

1.3.1 The induction and propagation of an immune response

In this section, the process of T-cell activation in both the induction and propagation of an immune response will be reviewed. In order not to preempt later discussion, several areas will be necessarily left brief, and the reader will be directed to relevant reading later in the chapter.

The immune system continually surveys the body, especially exposed parts such as the skin, lungs and digestive system, for the presence of pathogens. This process is performed by the innate immune system, and in particular immature dendritic cells, which, as Langerhan's cells, are continually present in skin. Detection of pathogens is performed either by detection of specific molecular patterns associated with pathogens (for instance, lipopolysaccharide; see 1.3.2.1) or by detection of the cellular damage which pathogens produce (see 1.3.2.2), and induces full maturation of the dendritic cells, including upregulation of several co-stimulatory markers, cytokine release, enhanced major histocompatibility complex (MHC) expression, and migration towards the lymph nodes. The overall effect of these maturational changes is to switch the dendritic cell's function from surveillance and antigen capture and processing, to antigen presentation. It is not clear what induces these maturational changes in drug hypersensitivity, or even if there is any commonality between different reactions. Convincing arguments can be made for the importance of the presence of bacterial components, concurrent viral infection, or cellular damage induced by the action of the drug or its metabolites (for a fuller treatment, see 1.3.4). However, these are all little more than speculation at present, and this remains one of the most important questions which must be answered in order to fully understand these reactions. In particular, it must be noted that, although the possibility that reactive drug metabolites can activate dendritic cells is *prima facie* plausible, there is no evidence as yet that it occurs.

Dendritic cell activation is an essential step, but it is incapable of inducing a response without the presence of a foreign antigen—that is to say, an antigen to which responding T-cells have not previously been selected out in the thymus. The antigen is recognised in the context of an MHC molecule by the highly variable T-cell receptor (TCR), which induces a series of biochemical events within the cell, leading to either tolerance (in

the absence of concurrent co-stimulation from the APC) or full activation of the cell (with co-stimulation). The requirements for T-cell activation by peptides, and the consequences of this are discussed later (see 1.3.5). How drugs can act as antigens is one of the key questions in the study of drug hypersensitivity reactions, and, despite considerable work, remains unclear. Two major hypotheses have been proposed (see 1.3.6 and 1.3.7 for a discussion of these), but conclusive evidence by which to choose one or the other, or to justify synthesising the two, is still elusive. A further complication is that the T-cell reactivity profile *in vivo* is known to develop over the course of a reaction. For instance, in several studies of the evolution of an immune response to peptide antigens it has been noted that over time the proportion of T-cells from high-affinity clones come to dominate over those from low-affinity clones (Amrani *et al.*, 2000). This process, known as avidity maturation, is due in part to competition and preferential expansion (Busch & Pamer, 1999), although other effects, such as early suppression of high-affinity responses by central and peripheral tolerogenic mechanisms (Han *et al.*, 2005) and secondary receptor revision (the modification of TCR-specificity by mature lymphocytes; McMahan & Fink, 2000; Serra *et al.*, 2002) appear to be important too. Therefore, it is perfectly plausible that the antigen to which the immune response is initiated is different from that to which T-cells taken later respond.

T-cell activation and the induction of an immune response to a drug lead to cellular damage through several known mechanisms. Migration of T-cells from the vasculature into tissue occurs by means of the expression of chemokines within the tissue, and presented on the endothelial surface, and chemokine receptors, which are up-regulated on activated T-cells. These induce transendothelial migration and movement along a chemotaxic gradient towards a site of inflammation. Several studies have looked for the chemokine receptors associated with specific tissue involvement. For instance, skin infiltration appears to be largely mediated by CCR4, CCR10 and CLA (Soler *et al.*, 2003), and indeed, both CCR10 and CCR4 have been found to be expressed on the T-cells involved in drug-induced skin reactions (Tapia *et al.*, 2004; Wu *et al.*, 2007). In contrast however, while the chemokine receptors involved in liver infiltration in response to viral infection have been investigated (for a good review, see Heydtmann *et al.*, 2001),

and certain chemokine receptors, in particular CXCR3 and CCR5 (Shields *et al.*, 1999), have been identified as important in this context, no studies have specifically looked for chemokine receptor involvement in liver-injury as part of a drug hypersensitivity reaction. A better understanding of why specific chemokine receptors are up-regulated on T-cells activated by drugs will hopefully allow greater insight into the causes of the specific pattern of organ involvement in drug hypersensitivity.

In addition to T-cell involvement, several other cell types are recruited, both as part of the innate and acquired immune systems. B-cell involvement leads to the production of drug-specific antibodies, although the pathogenic role of these is unsure. For instance, SMX antibodies have been detected both in SMX-hypersensitive and in SMX-tolerant individuals (Carr *et al.*, 1993a), and in many cases are not detectable at all (Cribb *et al.*, 1997; Gruchalla *et al.*, 1998). It is true that, in general, anti-SMX antibodies are more often detected in hypersensitive individuals, but it is hard to see how they can play a major role in the pathology when their presence is so variable. The main other immunological cell type which are recruited in drug hypersensitivity are eosinophils. These are recruited by the release of IL-5 and eotaxin (Yawalkar *et al.*, 2000), and play an important role in the development of cutaneous exanthems through the release of proinflammatory molecules such as histamine, prostaglandins and major basic protein.

Following migration to the extravascular site of activity, T-cells are believed to produce the majority of the observed pathological effects. There is some evidence that differences in the nature of the T-cell response are the key determinants of differences in the clinical picture (see the extended Gell & Coombs scheme; table 1.1). For instance, the cutaneous manifestations of bullous reactions, such as toxic epidermal necrolysis (TEN) or Stevens-Johnson syndrome, appear to be caused by CD8+ cytotoxic T-cells inducing widespread apoptosis of keratinocytes (Paul *et al.*, 1996) through both release of soluble Fas ligand (Abe *et al.*, 2003) and direct cell-mediated killing via the perforin/granzyme pathway (Nassif *et al.*, 2004). Other reactions, such as maculopapular eruptions, appear to be more dependent on CD4+ T-cells, although keratinocyte apoptosis is still a major feature. In addition to the expansion of a large

population of effector T-cells, a significant proportion of the activated T-cells become quiescent 'memory' T-cells, and it is these cells which react to a later rechallenge with the same, or structurally similar, drug.

In addition to these populations of effector T-cells, there is a further population of CD4+ T-cells which constitutively express CD25 (rather than solely when activated, as other T-cells do). These cells, known as regulatory T-cells or Tregs, play an important role in the control and limitation of immunological reactions, either through release of inhibitory cytokines, such as IL-10, or through cell-cell interactions such as CTLA-4 ligation of CD80/86 on effector T-cells. For a good review, see von Boehmer (2005). Regulatory T-cells have been found to play an important role in the suppression of T-cell responsiveness to typical allergens in atopy, and indeed, they appear to be a major determinant of allergy. An important study by Ling *et al.* (2004) found that PBMCs from atopic donors, but not non-atopic donors, responded vigorously to allergen (in this case, grass pollen) challenge. When CD4+CD25+ cells were depleted from the PBMC populations, however, cells from both atopic and non-atopic donors responded strongly. This appears to indicate that there is no lack of allergen-specific T-cells in non-atopic individuals, merely that there are kept under control by Tregs, and that it is actually differences in Tregs (or, possibly, differences in the response to Tregs) which determines whether or not allergy develops. A similar study (Cavani *et al.*, 2003) has found that nickel responsiveness is similarly repressed in normal individuals by the action of Tregs, and so it is tempting to suggest that this may also be a factor in the induction of immune responses in drug hypersensitivity. However, at present no study has found evidence to either confirm or disconfirm this proposition.

1.3.2 Danger signalling

The danger hypothesis, postulated by Matzinger (1994) as an extension of Janeway's work on the links between the innate and the adaptive immune systems (Janeway, 1992), holds that the nonself nature of a foreign antigen is not what induces an immune response; instead, it is 'danger signals,' such as cell damage or infection, that activate the immune system. Therefore, it can be said that effective activation of the adaptive

24

immune system requires 2 signals: signal 1, which is the TCR-mediated recognition of an MHC-restricted antigen; and signal 2, which represents the interactions between various co-stimulatory ligands and receptors between the T-cell and the APC, such as CD28:CD86 and CD40:CD154 (Naisbitt *et al.*, 2000a). In the absence of signal 2, signal 1 simply leads to tolerance, either by anergy or by apoptosis of responding T-cells (Appleman & Boussiotis, 2003). Danger signals act to enhance this signal 2 pathway, by upregulating co-stimulatory markers on professional APCs, such as dendritic cells.

1.3.2.1 Exogenous danger signals

Most studies of the activation of dendritic cells have concentrated on the effects of pathogen-associated molecular patterns (PAMPs). These typically produce dendritic cell maturation signals by interacting with Toll-like receptors (TLRs), a series of membrane-bound receptors. Thirteen individual mammalian TLRs have been identified to date, although only ten are known in man, numbered from TLR-1 to TLR-10 inclusive. TLRs have been identified in many other vertebrates, and highly homologous proteins are present both in bacteria and in plants, where they play a similar role in resistance to infection (Nurnberger *et al.*, 2004). TLRs therefore represent one of the most ancient components of the immune system.

The most commonly studied PAMP is the bacterial cell wall component lipopolysaccharide (LPS), which is known to interact with TLR-4 (Poltorak *et al.*, 1998), the first TLR to be identified (Medzhitov *et al.*, 1997), and induce dendritic cell maturation. Other well-known PAMP interactions are the interaction of unmethylated DNA CpG motifs with TLR-9 (Hemmi *et al.*, 2000) and the interaction of double-stranded viral RNA with TLR-3 (Alexopoulou *et al.*, 2001). See table 1.5 for an overview of known TLR functions.

1.3.2.2 Endogenous danger signals

In addition to these external sources of danger signals, several studies have focused on the ability of stressed, dead, or dying cells to provide maturation signals to dendritic cells. Initial studies revealed that cells killed necrotically, but not viable or apoptotic

Table 1.5: Overview of TLR specificity

Toll-like receptor	Known ligand
TLR-1	Triacyl lipopeptides (with TLR-2)
TLR-2	Lipoproteins; peptidoglycan; lipoteichoic acid
TLR-3	Double-stranded RNA
TLR-4	Lipopolysaccharide
TLR-5	Flagellin
TLR-6	Diacyl lipopeptides (with TLR-2)
TLR-7	Single-stranded RNA (in mice)
TLR-8	Single-stranded RNA
TLR-9	Unmethylated CpG DNA
TLR-10	Unknown

Refs: Alexopoulou *et al.* (2001); Diebold *et al.* (2004); Hayashi *et al.* (2001); Hasan *et al.* (2005); Heil *et al.* (2004); Hemmi *et al.* (2000); Poltorak *et al.* (1998); Schwandner *et al.* (1999); Takeda *et al.* (2002)

cells, activate dendritic cells (Gallucci *et al.*, 1999), although later studies have also found an effect from cells undergoing apoptosis (Janssen *et al.*, 2006). Heat shock proteins released from dying cells are obvious candidates as danger-signalling molecules, or 'endogenous adjuvants', and their ability to provide maturation signals to dendritic cells has been discussed in detail (Todryk *et al.*, 2000). More recently, ground-breaking studies by Shi *et al.* (2003, 2006) have used chemical analyses to define uric acid crystals as potent messengers that are released by injured and dying cells and that can stimulate dendritic cell maturation and enhance CD8+ T-cell responses *in vivo*, although the relevance of these findings in humans has been questioned (Gu *et al.*, 2004).

There is some evidence that signals from endogenous adjuvants are also transduced by TLR ligation, although this is less clear-cut than the evidence for PAMP-induced dendritic cell activation. It has been claimed that heat-shock proteins signal via TLR-4 (Asea *et al.*, 2000; Ohashi *et al.*, 2000), but other studies have suggested this may be due to LPS contamination (Bausinger *et al.*, 2002; Gao & Tsan, 2003). Certain other endogenous adjuvants have also been claimed to signal through TLRs, such as fibronectin via TLR-4 (Okamura *et al.*, 2001), hyaluronan via TLR-2 (Scheibner *et al.*, 2006) and TLR-4 (Termeer *et al.*, 2002), and fibrinogen via TLR-4 (Smiley *et al.*, 2001). However, as can be seen from the controversy regarding heat-shock protein signalling, there remains a risk that these signals are due to bacterial contamination.

1.3.3 Co-stimulatory signalling

Danger signals are primarily transmitted from the APC to the effector cell by means of co-stimulatory signalling. This refers to a range of ligand-receptor interactions between activated APCs and T-cells, including, but not limited to, CD40-CD154 and CD80/86-CD28 interactions. In addition to co-stimulation, there is increasing evidence of co-inhibition acting as a brake on the activation of the immune system. For a good review, see Subudhi *et al.* (2005). However, this discussion will concentrate largely on co-stimulation, and how it acts to provide modulation of immune-mediated drug reactions. In particular it will cover the two best-understood pathways, the CD28/CTLA-4:CD80/86 and the CD40:CD154 pathways.

1.3.3.1 CD28/CTLA-4:CD80/86 signalling

CD28 and CTLA-4 are structurally related T-cell surface receptors which interact with CD80 and CD86 on the surface of APCs. CD28, which is expressed on all T-cells, both active and naïve, transduces activational signals which lead, in concert with the ligation of the TCR by a specific antigen, to full activation. CD28 ligation is essential for cytokine release (Thompson *et al.*, 1989), enhances T-cell survival, prevents anergy (Harding *et al.*, 1992; Jenkins *et al.*, 1991), and stabilises expression of CD40L in order to provide signalling to dendritic cells and help to B-cells (Johnson-Lger *et al.*, 1998).

CTLA-4 ligation, however, leads to inhibition of T-cell activation. It is believed that this occurs both by actively inhibiting the biochemical steps required for T-cell activation, including blockade of cell cycle progression and inhibition of IL-2 production (Walunas *et al.*, 1996), and by competing for CD80/86 molecules (CTLA-4 binds to CD80/86 with much higher affinity than CD28) and hence inhibiting CD28 signalling (Saito & Yamasaki, 2003). CTLA-4 is not expressed on naïve T-cells, but is up-regulated on activated cells, and so acts as a 'brake' on excessive T-cell activation.

CD80/86 are both expressed on APCs, and although there is some degree of expression of CD86, but not CD80, on immature dendritic cells, significant upregulation of both occurs alongside maturation. They both transduce signals from APC to T-cell via CD28 and CTLA-4, but less is known about any signals which they receive from the T-cell. Recent studies have suggested that CTLA-4 ligation of CD80/86 leads to inhibitory signalling within the APC (Grohmann *et al.*, 2002; Munn *et al.*, 2004). However, the primary role of CD80/86 is still believed to be due to their effects on T-cells induced by ligation of CD28 and CTLA-4.

1.3.3.2 CD40:CD154 signalling

CD40 is a cell surface receptor of the TNF-α receptor family. It is expressed on APCs where it transduces activational signals from activated T-cells. Ligation of CD40 by its natural ligand CD40L (otherwise known as CD154) induces several changes within the APC through activation of Nf-κB (O'Sullivan & Thomas, 2002), such as release of inflammatory mediators, enhanced cell survival, up-regulation of MHC-II, CD80,

CD86, and release of pro-inflammatory cytokines (Caux *et al.*, 1994; Cella *et al.*, 1996).

CD40L is not expressed on the surface of resting T-cells, but becomes transiently expressed on the surface when T-cells become activated through the TCR. Both CD28 signalling (Johnson-Lger *et al.*, 1998) and cytokines, in particular IL-2 (Skov *et al.*, 2000), can stabilise this expression, and allow effective signalling to the APC.

The overall effect of CD40 signalling is to enhance the ability of APCs to present antigen to T-cells, and to drive an antigenic response from tolerance to immunity. As CD40 is itself up-regulated by APC activation (Verhasselt *et al.*, 1997), this produces a positive feedback loop which amplifies the original inflammatory signal. Indeed, CD40 interactions have been shown to be essential for persistence of immune responses *in vivo* (Miga *et al.*, 2001).

1.3.4 Dendritic cell activation by drugs

The source of co-stimulatory signalling for the induction of primary immune responses to drugs is still controversial. It is possible that these signals are supplied by concurrent infection or other source of endotoxin (Roth *et al.*, 2003), and indeed there is good evidence that viral infection is a major risk factor for hypersensitivity (Pirmohamed & Park, 2001). However, there is little evidence for a similar effect with bacterial infection, and there have been many documented cases of hypersensitivity in the absence of any overt infection. Therefore, it may be instructive to consider the possibility that drugs can directly induce innate immune activation.

It is possible for a drug to act pharmacologically to induce dendritic cell activation, as has been described for certain imidazoquinolene small anti-viral compounds such as imiquimod (Hemmi *et al.*, 2002), which are agonistic at the TLR-6/7 receptors, and lenalidomide, a thalidomide analog which supplies co-stimulation to T-cells via activation of the B7-CD28 pathway (LeBlanc *et al.*, 2004). Interestingly, both of these compounds have been associated with cutaneous reactions (Hadley *et al.*, 2006; Sharma *et al.*, 2006), although more work is required in order to determine whether these reactions are analogous to those seen in hypersensitivity. However, this seems to be an unlikely mechanism for most drugs associated with hypersensitivity, given the wide

range of chemical classes involved. A more plausible source of danger signals is the cell damage produced by reactive drug metabolites.

When considering the potential for drug metabolites to interact with dendritic cells and provide maturation signals, lessons can be learned from studies with contact sensitisers, which because of their intrinsic protein reactivity are often toxic to immune cells at relatively low concentrations. This toxicity can be the result of covalent binding, either to specific target proteins or non-specifically, potentially exposing hydrophobic residues and activating toll-like receptors (Seong & Matzinger, 2004). Alternatively, this toxicity could be due to the generation or impaired detoxification of reactive oxygen species, a major factor in activation of the pro-inflammatory NF-κB pathway (Kabe et al., 2005), which is essential for complete dendritic cell maturation (Ardeshna et al., 2000; Rescigno et al., 1998). It has been suggested that dendritic cell maturation in response to dinitrohalobenzenes may be due to inactivation of thioredoxin reductase and consequent impaired detoxification of reactive oxygen species (Nordberg & Arner, 2001). However, this direct activation is inhibited by thiol antioxidants (Bruchhausen et al., 2003) but not other antioxidants, suggesting that the effect of thiol antioxidants is not solely due to radical scavenging. This idea is supported by the finding that glutathione depletion enhances this activation (Mizuashi et al., 2005).

A wide variety of structurally unrelated contact sensitisers have been found to provoke signal 2 via direct activation of dendritic cells and monocytes, as determined by either up-regulation of cell surface markers, particularly CD80, CD86, CD40, HLA-DR, (Aiba et al., 1997; Coutant et al., 1999; Staquet et al., 2004; Tuschl et al., 2000) or chemokine receptors (Jugde et al., 2005); by activation of signal transduction pathways (Aiba et al., 2003; Arrighi et al., 2001; Becker et al., 2003; Bruchhausen et al., 2003; Iijima et al., 2003) or by functional effects, for instance, enhanced activation of allogeneic T-cells (Aiba et al., 2000; Coutant et al., 1999; Manome et al., 1999) or in vivo sensitisation (Aiba & Katz, 1990). Recently, Hulette et al. (2005) showed that contact sensitisers stimulate dendritic cell maturation only at concentrations associated with low levels of cell death, presumably via a classical 'danger' response (Matzinger, 1994) and the recognition of released endogenous signals (Gallucci et al., 1999; Shi

et al., 2003). However, a recent study using the hair dye allergen *p*-phenylenediamine as a paradigm (Coulter *et al.*, 2006), has shown that dendritic cell maturation can be induced at concentrations below those associated with cell death, as measured by increased expression of CD40 and stimulation of allogeneic lymphocyte proliferation.

Based on the observation that many protein-reactive drug metabolites bind covalently to thiol-rich protein, it is not unlikely that a similar effect may be driven by drug metabolites, although there is no published evidence at yet that this occurs.

1.3.5 T-cell activation by proteins and drugs

In addition to the signal 2 transduced by co-stimulation from activated dendritic cells, T-cells also require signal 1, antigen-specific ligation of the TCR, for activation. This typically involves recognition of a peptide antigen presented in the context of an MHC molecule, although there are some exceptions, such as superantigens.

The MHC molecules are divided into two main families, MHC-I (in humans, HLA-A,B,C) and MHC-II (HLA-DR,DP,DQ). The MHC region in the genome is exceptionally polymorphic, which is likely to be related to the evolutionary benefits of heterogeneity in pathogen immunity (Meyer & Thomson, 2001). MHC-I is expressed on all cell types, and presents antigens derived from intracellular proteins to CD8+ T-cells, along with some derived from extracellular proteins, through a process called crosspresentation. MHC-II, by contrast, is only expressed on professional APCs, and is up-regulated in response to APC maturation. This MHC presents antigens derived from extracellular proteins, taken up by the APC as part of its routine sampling of the surrounding milieu, to CD4+ T-cells.

Peptide antigens are specifically recognised by T cells via the TCRs interacting with the peptide bound non-covalently to the MHC molecule. Antigen-presenting cells process proteins into peptides, load them onto nascent MHC molecules and traffic them to the cell surface. The composite MHC-peptide surface then acts as a ligand for peptide-MHC-specific TCRs. Recent work has elucidated many of the structural requirements for the TCR-peptide-MHC interaction (for review, see van der Merwe & Davis, 2003).

Peptides fit into a specific groove on the surface of MHC molecules where they are held in place by non-covalent interactions, particularly at certain conserved residues known as anchor residues. MHC-I molecules typically accept nonameric peptides, although some longer peptides have been seen. The N- and C-terminals are fixed in place, and longer peptides are accommodated by bulging of the peptide (Speir et al., 2001). In addition to the essential carboxy-terminal anchor position, MHC-I-bound peptides have up to three further anchor residues. MHC-II-bound peptides are generally held less tightly, and are less constrained in terms of peptide length (between 12 and 25 is common; Rammensee, 1995). The interactions between the peptide and MHC molecule are also less constrained, and involve more uniformly dispersed residues.

While anchor residues are particularly important for binding to MHC molecules, other residues make up a disproportionate amount of the binding to the TCR, especially for MHC-I-bound peptides. Indeed, in most studied MHC-I-bound 9-amino acid peptides, the residue at position 5 is responsible for over half of all TCR-peptide contacts (Rudolph & Wilson, 2002). As was the case for MHC interactions, peptides bound to MHC-II are less dependent on certain residues for TCR interactions, but they still only contact the TCR through a subset of available residues.

A striking finding of recent studies of TCR-MHC-bound structures is the heterogeneity of the TCR-peptide-MHC interaction, as there appear to be very few common features to all interfaces visualised so far (Rudolph & Wilson, 2002). However, it has been discovered that the overall portion of the total binding area represented by TCR-peptide interactions is significantly lower than that supplied by TCR-MHC interactions.

An important conclusion from these studies on TCR recognition of peptides is that although the peptides are relatively large, only a small portion of them is actually involved in binding to the TCR, and that the TCR-MHC interactions overshadow TCR-peptide interactions.

There is evidence that the T-cell is exquisitely sensitive to tiny quantities of antigen, with as little as a single peptide antigen being sufficient to activate its cytolytic activities (Sykulev et al., 1996), and a further study finding transient Ca^{2+} mobilisation following a single TCR ligation, and immunological synapse formation with as few as 10 (Irvine

et al., 2002). However, T-cell activation is believed to be triggered by the process of serial triggering (Valitutti *et al.*, 1995). Under this paradigm, simple ligation of TCRs is insufficient to activate the cell; what is required is multiple ligation of distinct TCRs. This involves accumulation of signalling molecules, most likely members of the ERK family (Borovsky *et al.*, 2002), until a threshold is reached, which allows full T-cell activation. This is due in part to the downregulation of TCRs following triggering, presumably a mechanism to prevent excessive activation. One of the consequences of this mechanism is that there is a relatively narrow window of affinity for TCR-antigen interactions, leading to a 'Goldilocks' effect: too low, and it cannot trigger the TCR; too high, and it cannot dissociate rapidly enough to allow serial triggering (Kalergis *et al.*, 2001).

1.3.6 Hapten hypothesis

In 1935, Landsteiner and Jacobs undertook a series of seminal studies on the sensitisation potential of low-molecular weight organic compounds (Landsteiner & Jacobs, 1935). They found a strong correlation between the sensitisation potential *in vivo* and the protein reactivity *in vitro*. Additionally, on the basis of studies with modified poly-l-lysine peptides (for instance, see Stupp *et al.*, 1971), it was not believed that small molecules (< 1000 Da) were able to act as an antigen. These findings have thereafter formed the basis of the hapten hypothesis, which posits that drugs–or more commonly, reactive metabolites formed by the normal processes of metabolising enzymes–are recognised by drug-specific T-cells only following haptenation to self-proteins (Park *et al.*, 2001). This leads to formation of a neoantigen that can be recognised by T-cells to override self-tolerance, and induction of a potentially pathogenic immune response. This mechanism has been well documented for contact sensitisers such as dinitrohalobenzenes (Cavani *et al.*, 1995; Park *et al.*, 1987; Weltzien *et al.*, 1996) and respiratory allergens such as trimellitic anhydride (Dearman *et al.*, 1991, 2000). More speculatively, it is thought to be a mechanism for the breaking of self-tolerance in autoimmunity (Doyle & Mamula, 2001; Kimber & Dearman, 2002; Palmer *et al.*, 2004).

There is little direct evidence for the importance of hapten formation in drug hyper-

sensitivity. That is not to say that there is no circumstantial evidence, which certainly appears to point to the importance of reactive metabolite generation and covalent binding in hypersensitivity reactions. In particular, there is convincing evidence that reactive metabolites are significantly more immunogenic *in vivo* than their parent compounds. This will be discussed as regards SMX hypersensitivity later (see 1.3.8.3; also 2.1). Furthermore, there is good evidence that human immunodeficiency virus (HIV) infection, which is associated with a greatly increased risk of hypersensitivity reactions (Lin *et al.*, 2006; Pirmohamed & Park, 2001), is also associated with thiol and ascorbate depletion (Naisbitt *et al.*, 2000b; Trepanier *et al.*, 2004; Walmsley *et al.*, 1997), which may thereby lead to a decreased ability to detoxify reactive metabolites (Naisbitt *et al.*, 2000b) and to increased metabolite-mediated cellular toxicity (Wijsman *et al.*, 2005). However, viral infection will also have significant immunological effects unrelated to metabolite detoxification, such as deranged regulatory mechanisms (Eggena *et al.*, 2005). Additionally, some studies have failed to find a link (Eliaszewicz *et al.*, 2002), so this remains controversial.

It is also worth pointing out that almost all drugs associated with a comparatively high incidence of hypersensitivity reactions are known to form reactive metabolites. It is not known whether this is simply observational bias, due to the increased research focus on these drugs, or is of genuine importance.

One of the difficulties with the hapten hypothesis is the generally poor association between exposure to reactive metabolite and incidence of reactions. Several studies have looked for associations of susceptibility to hypersensitivity with polymorphisms in drug metabolising enzymes, particularly CYPs (Pirmohamed *et al.*, 2000; Wolkenstein *et al.*, 2005), though also epoxide hydrolase (Gaedigk *et al.*, 1994; Green *et al.*, 1995) and N-acetyl-transferase (Alfirevic *et al.*, 2003; O'Neil *et al.*, 2002), but the results have been disappointing. A small association between SMX-hypersensitivity with N-acetyl-transferase polymorphisms has been claimed (O'Neil *et al.*, 2002), but attempts to replicate this have failed (Alfirevic *et al.*, 2003). Prospective studies have been performed in order to identify putative associations between exposure to reactive metabolites and hypersensitivity. In particular, a large-scale prospective clinical trial

of N-acetyl cysteine as prophylaxis against SMX hypersensitivity was undertaken, but was unsuccessful (Walmsley *et al.*, 1998).

1.3.7 p-i concept of T-cell activation by drugs

More recently, a hypothesis known as the p-i, or pharmacological-interaction, concept has been proposed. The p-i concept posits that drugs can activate T-cells directly in the absence of metabolism, covalent binding, and antigen processing, (Pichler, 2002) through a reversible interaction between the TCR, MHC, and the drug. Several independent lines of inquiry have led to this conclusion. Firstly, both peripheral blood mononuclear cells (PBMCs) from hypersensitive patients and T-cell clones cultured from the same patients respond to parent drug in the absence of appreciable metabolism (Naisbitt *et al.*, 2003a, 2005a; Schnyder *et al.*, 1997; Zanni *et al.*, 1997); second, washing the drug away from APCs prior to adding T-cells is sufficient to completely abrogate the T-cell response, irrespective of the length of time the APCs have been exposed to the drug (Depta *et al.*, 2004; Schnyder *et al.*, 1997; Naisbitt *et al.*, 2003b); third, glutaraldehyde-fixed APCs are still competent to present drug to T-cell clones (Naisbitt *et al.*, 2003b, 2005a; Schnyder *et al.*, 1997), although they are incapable of antigen processing (Schnyder *et al.*, 1997; Shimonkevitz *et al.*, 1983); and fourth, the time-course of activation of T-cell clones, as determined by several markers of T-cell activation, such as TCR downregulation (Zanni *et al.*, 1998), ERK phosphorylation (Depta *et al.*, 2004), and Ca^{2+} mobilisation (Zanni *et al.*, 1998), are too rapid to allow metabolism and antigen processing.

These findings have been demonstrated using *ex vivo* T-cell clones from patients hypersensitive to several drugs, including SMX (Schnyder *et al.*, 1997), lidocaine (Zanni *et al.*, 1997), lamotrigine (Naisbitt *et al.*, 2003b), and phenindione (Naisbitt *et al.*, 2005a). However, in all these studies, the T-cells have been derived from *in vitro* expansion of drug-specific T-cells prior to cloning, which may well affect the apparent make-up of drug-specific T-cells. Recently, this point was addressed by Nassif *et al.* (2004), who identified SMX-specific T-cells directly from blister fluid in TEN patients. However, their interpretations regarding the lack of a role for metabolism remain con-

troversial since blister fluid T-cells from all patients also responded to the reactive metabolite SMX-NO (Naisbitt *et al.*, 2005b).

One of the main concerns with the theory is that while relatively low-affinity antigens, as a non-covalently bound drug would presumably be, can activate memory T-cells, the initial T-cell priming event is more stringent with respect to TCR-antigen-MHC affinity. It has therefore been proposed that the drugs actually activate memory T-cells with unknown peptide-antigen specificity (Pichler, 2005). This may explain the propensity of certain compounds, such as radiocontrast media, to induce apparently immune-mediated hypersensitivity reactions within a few hours of the initial exposure to the compound (Christiansen *et al.*, 2000). An interesting corollary of this hypothesis is that it removes the need for co-stimulation as a requisite element of hypersensitivity reactions, since this is only required for induction of a primary response. However, although certain hypersensitivity reactions occur extremely quickly and without prior exposure, this is not the case for the majority of reactions, which require a relatively long exposure (2-3 weeks) to the drug. This would suggest that in most cases, there is sufficient time for the induction of a primary immune response. In addition, Engler *et al.* (2004) successfully induced a primary T-cell immune response *in vitro* against SMX in PBMCs from drug-naïve volunteers, but this required a significant period of incubation with the drug (3-4 weeks), which again does not appear to suggest a purely secondary response, and indeed suggests that non-covalently bound drug may indeed be able to act with sufficient affinity as an antigen to induce a primary response.

While the proposition that T-cells can recognise drugs as antigens in a non-covalent manner is still controversial, it is unquestionably recognised as the mechanism by which certain metal ions, such as nickel, are recognised. Nickel is one of the most commonly encountered contact sensitisers, and a common cause of allergy (Nielsen *et al.*, 2002). Ni^{2+} ions readily dissolve in sweat and are absorbed through the skin (Menne, 1996), where they bind to carrier proteins via four- and six-coordinate binding to electron donors such as oxygen, nitrogen and free thiols (Zhang & Wilcox, 2002).

Nickel-specific T-cells have been cloned on a number of occasions (Moulon *et al.*, 1995; Pistoor *et al.*, 1995; Sinigaglia *et al.*, 1985), and these have allowed detailed

investigations into the nature of the nickel antigen. It appears that for certain clones, nickel acts in a similar fashion to a classical hapten, in that it is not possible to wash the bound nickel away from antigen-presenting cells and eliminate T-cell reactivity (Moulon *et al.*, 1995). In certain clones that react to nickel in this fashion, it appears that Ni^{2+} binds directly to the surface of a peptide-MHC complex (Lu *et al.*, 2003), whereas a functional antigen-processing pathway is required for other clones, suggesting that Ni^{2+} remains bound to the peptide as it is processed (Moulon *et al.*, 1995). In both of these cases, however, it is clear that the bonds Ni^{2+} forms to the MHC peptide are of sufficient strength to allow the ion to act as a hapten. Alternatively, certain clones appear to depend on the continual presence of free Ni^{2+} ions for activation, and in these clones it appears to act in a peptide-independent superantigen-like fashion (Gamerdinger *et al.*, 2003). In these cases, it seems likely that Ni^{2+} does not bind tightly to the MHC or peptide, but forms a relatively weak interaction that can be removed by washing.

An important implication of these findings is that it is not so much covalent vs. non-covalent which is the issue of importance, but more the summation of the interactions in question—certain non-covalent interactions, such as the co-ordinate linkages involved in nickel binding, can act either as haptens or as free compounds, depending on the T-cell clone which responds.

1.3.8 Examples of xenobiotic immunogenicity

In order to further elucidate some of the previously discussed themes, we will briefly discuss four important paradigm compounds, namely, nitrohalobenzenes, penicillins, SMX and CBZ. This will illustrate the different possible ways by which the immune system can recognised drugs, and it also offers insights into the common features which they all share.

1.3.8.1 Dinitrohalobenzenes

The most widely studied classes of model chemical immunogens are the nitrohaloben-zenes, particularly 2,4-dinitrochlorobenzene (DNCB), 2,4-dinitrofluorobenzene (DNFB), and 2,4,6-trinitrochlorobenzene. These are highly potent topical sensitisers, although

they are rarely encountered amongst the population at large. However, their relatively simple chemistry and effectiveness for *in vivo* sensitisation has made them essential tools for the investigation of fundamental immunochemistry.

Nitrohalobenzenes are powerful electrophiles which readily react with nucleophilic residues on proteins, particularly cysteine and lysine groups, through an S_NAr nucleophilic substitution mechanism (see figure 1.1A). This involves nucleophilic attack at the halogen-bonded carbon, which is stabilised by resonance structures involving the electron-withdrawing nitro groups, followed by preferential ejection of the halide ion as a good leaving group. This has been seen to occur with cysteine and lysine, both *in vitro* (Habig *et al.*, 1974; Tingle *et al.*, 1990) and *in vivo* (Kitteringham *et al.*, 1985; Maggs *et al.*, 1986). The biological relevance of chemical reactivity with other nucleophilic amino acid residues, such as tyrosine and histidine, is less certain. Some studies have shown that DNFB reacts with these residues (Jackson & Young, 1986; Liu *et al.*, 1993), whereas a recent analysis found extremely limited reactivity for DNCB to histidine (Gerberick *et al.*, 2004). It is not known whether this reflects genuine differences between the two compounds or simply differences in experimental method. It may also reflect the variable reactivity of amino acid side chains in different proteins. Chemical reactivity of individual amino acid residues is a function of their microenvironment within the protein, which, in addition to steric effects, can have a profound influence on pKa (Bello *et al.*, 1979). Importantly, all dinitrohalobenzenes generate the same dinitrophenyl adduct on proteins, although the residues to which they attach may differ, as can be seen by the controversy regarding their reactivity to histidine residues. Trinitrohalobenzenes generate trinitrophenylated derivatives of proteins, again typically on lysines and cysteines.

Early studies focused on the ability of nitrohalobenzenes to induce humoral immune responses to haptenated proteins. In a series of elegant studies, Landsteiner & Jacobs (1935, 1936) found that the sensitising potential of a series of nitrohalobenzenes in guinea pigs correlated with their chemical reactivity *in vitro*. Further work confirmed that in addition to the effects of the parent compounds, nitrophenylated cells and proteins were also potent immunogens (Soeberg *et al.*, 1978a,b), although this was cell

38

type and protein dependent.

T-cell responses to both nitrohalobenzenes and nitrophenyl-modified proteins and peptides have also been well characterised. T-cell clones have been generated from hypersensitive individuals which respond to DNCB in a processing and covalent binding-dependent manner (Pickard *et al.*, 2007), suggesting that DNCB must be bound solely to the peptide. This is in keeping with several other studies which have found that trinitrophenyl-modified peptides are sufficient to activate *in vivo* primed CD4+ and CD8+ T-cells (Cavani *et al.*, 1995; Martin *et al.*, 1993). Additionally, trinitrophenyl-modified peptides have been shown to be capable of priming trinitrophenyl-specific T-cell responses *in vivo* (Kohler *et al.*, 1995). These studies have found a precise requirement for trinitrophenyl-modification of a specific residue, albeit on a given peptide. Whether this is a general feature of trinitrophenyl reactivity is not known at present.

An interesting development in our understanding of how contact hypersensitivity to sensitisers such as dinitrohalobenzenes occurs is the finding that they are capable of directly activating dendritic cells and supplying requisite co-stimulatory signals for primary T-cell activation (see earlier discussion (1.3.4); Aiba *et al.*, 1997; Coutant *et al.*, 1999; Jugde *et al.*, 2005; Manome *et al.*, 1999).

Therefore, it is clear that dinitrohalobenzenes may activate the immune system both by acting as an antigen, in a haptenic fashion, and by direct activation of the innate immune system to drive active T-cell priming. Indeed, this newly discovered function may be the mechanism behind the therapeutic immunomodulatory effects of DNCB as used topically in dermatology (Holzer *et al.*, 2006). A full consideration of the interactions of this class of compounds will allow an understanding of how it can act as a complete immunogen.

1.3.8.2 Penicillin

Penicillin is a directly reactive drug associated with a high incidence of allergic reactions. It has a reactive structure, with the capacity to modify amine, hydroxyl, mercapto and histidine groups on protein. The site and extent of protein modification is influenced by concentration (of penicillin and protein), pH and the presence of certain metal ions
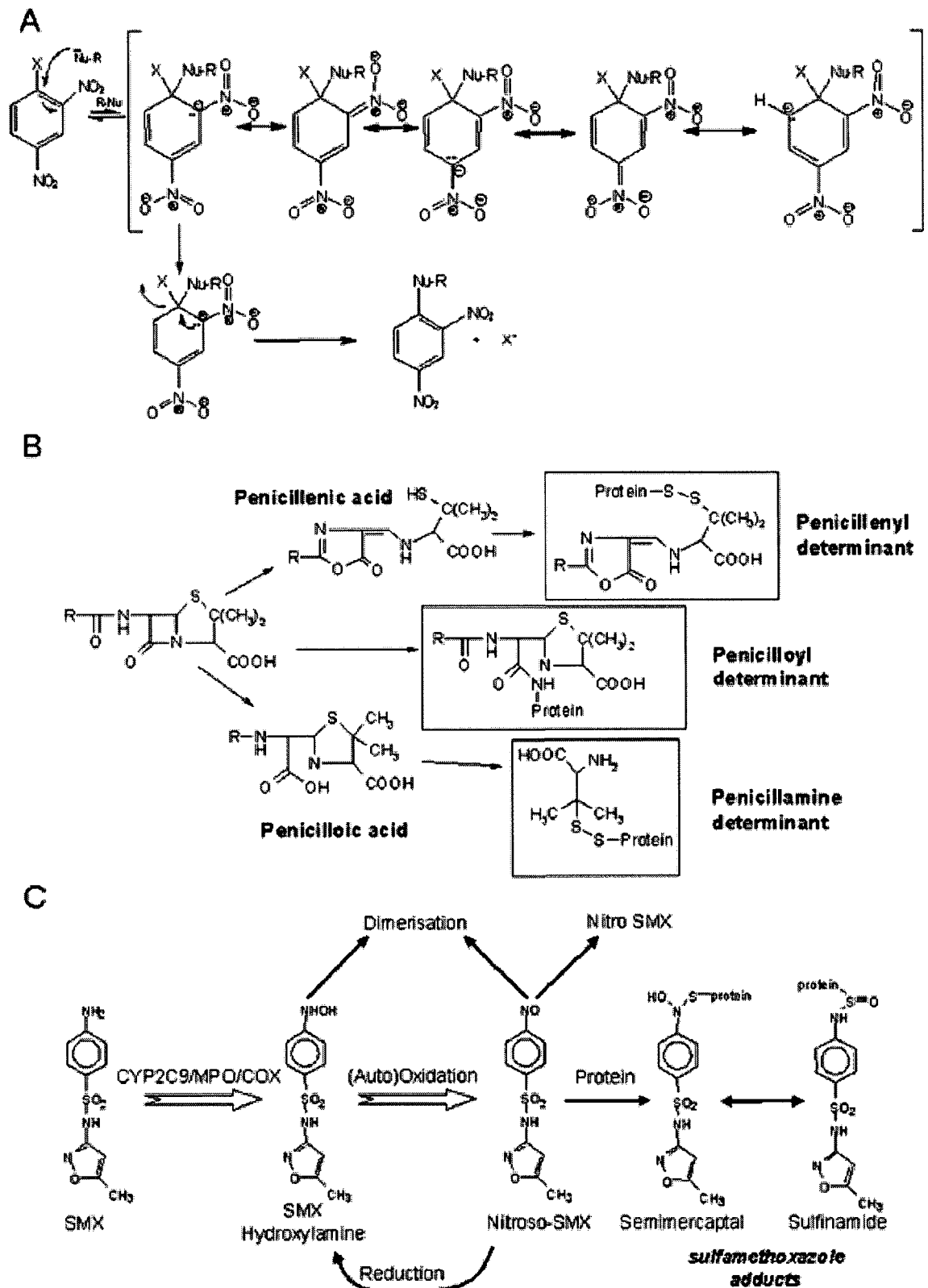
Figure 1.1: Mechanisms of protein-adduct formation by (A) dinitrohalobenzenes, (B) penicillin, and (C) SMX

(Ahlstedt & Kristofferson, 1982).

Nucleophilic attack of the β-lactam ring by free amino groups of protein leads to a covalently linked penicilloyl determinant, often referred to as a major antigenic determinant for antibodies and T-cells. This derivative represents greater than 90% of penicillin-modified protein. Other penicillin-related adducts are formed through the conversion of penicillin in solution to penicillenic or penicilloic acid, both of which are thought to modify cysteine residues in protein. Resultant penicillenyl and penicillamine conjugates, called minor antigenic determinants, are formed in low levels, but may contribute towards the induction of allergic reactions (see figure 1.1B).

T-cells from allergic patients are mainly CD4+ and stimulated with penicillin presented in the context of MHC class II (Padovan et al., 1996). T-cells are penicillin- specific (i.e. they are not stimulated with structurally unrelated drugs); however, certain clones accommodate small changes in the penicillin side chain which are not involved in penicillin binding to protein. T-cell clones also reacted both to free penicillin, in a processing-independent fashion, and to penicillin-modified albumin, in a processing dependent fashion (Brander et al., 1995). The requirement of penicillin modification of MHC-associated peptides and formation of a penicilloyl determinant for T-cell activation has been demonstrated by the stimulation of specific TCRs with designer peptides containing appropriate anchor residues (Padovan et al., 1997). Importantly, only peptides containing lysine residues in precise positions were able to stimulate TCRs (Padovan et al., 1997). The ability of penicillenyl or penicillamine conjugates to stimulate T-cells has not been investigated.

More recently, the involvement of dendritic cells in penicillin allergy has been evaluated. Immature dendritic cells from amoxicillin-hypersensitive patients, but not those from penicillin-naïve controls, were activated in response to amoxicillin, and were able to present processed peptides to autologous peripheral blood mononuclear cells and T-cells (Rodriguez-Pena et al., 2006). However, the mechanism behind this effect is not known at present, and will require further work to elucidate. Thus, while the primary focus on the mechanisms of penicillin hypersensitivity has been on the formation of the penicilloyl hapten, there is still the need to define the chemical basis of the generation

of the co-stimulatory signals involved.

1.3.8.3 Sulfamethoxazole

SMX is a sulfonamide which was largely phased out of clinical practice because of rare but serious cutaneous hypersensitivity reactions. In recent years it has been more widely prescribed in combination with TMP as treatment for *Pneumocystis carinii* pneumonia in patients with AIDS. Unfortunately, the incidence of hypersensitivity reactions is greatly increased in this population (Pirmohamed & Park, 2001).

Many studies exploring the mechanisms of drug hypersensitivity have focused on SMX, because it is known to cause hypersensitivity and much is known about its disposition in the body (see figure 1.1C). SMX is metabolised by CYP2C9 in human liver to a pro-reactive hydroxylamine metabolite SMX-NHOH (Cribb *et al.*, 1995; Gill *et al.*, 1996; Mitra *et al.*, 1996; van der Ven *et al.*, 1994). This readily reacts with molecular oxygen to generate SMX-NO (Cribb *et al.*, 1991; Naisbitt *et al.*, 1996), which is unstable and reacts both with SMX-NHOH to generate azo- and azoxy- dimers, and with protein (Manchanda *et al.*, 2002; Naisbitt *et al.*, 2002) and nonprotein thiols (Cribb *et al.*, 1991) to form both semimercaptal and sulfinamide adducts. Further oxidation can also generate nitro-sulfamethoxazole (SMX-NO$_2$) (Naisbitt *et al.*, 2002). Importantly, reduction of SMX-NO can occur either via interaction with nonprotein thiols (e.g., glutathione) or ascorbate, or enzymatically (Cribb *et al.*, 1995; Kurian *et al.*, 2004). Thus, the critical balance between metabolic activation and detoxification in a given cell system ultimately determines the level of exposure to the reactive metabolite.

Studies with animal models of SMX hypersensitivity have found that SMX-NO is a potent immunogen (Naisbitt *et al.*, 2002), and is capable of inducing hapten-specific T-cell responses similar to those generated by nitrohalobenzenes, i.e. both covalent binding and processing dependent (Farrell *et al.*, 2003). Interestingly, a study demonstrated that while administration of SMX was insufficient to induce a T-cell response, despite *in vivo* generation of SMX-NHOH, co-dosing with an adjuvant produced a T-cell response to SMX-NO (Naisbitt *et al.*, 2001). This demonstrates that *in vivo* generated reactive metabolites may form antigens through haptenation, although ad-

ditional activation of the innate immune system through danger signalling (Park *et al.*, 1998) is required to produce immunogenicity.

T-cells from hypersensitive patients have been isolated and cloned in order to explore the mechanisms by which SMX can act as an antigen in man. In contrast to animal studies, however, no T-cell clones have been found which respond to SMX-NO in a classical hapten-like fashion. Instead, in all patients studied so far, T-cell clones have been found which respond to SMX-NO in a binding-dependent but processing-independent fashion, presumably by binding to the preformed MHC-peptide complex (Schnyder *et al.*, 2000). This represents a vital distinction between immunogenicity in experimental animals, which is consistently observed, and hypersensitivity in a small subgroup of patients. However, the protein milieu in any given *in vitro* system will differ from that in the *in vivo* environment at the time of antigen encounter, so it may be premature to rule out the presence of T-cells responsive towards metabolite-modified endogenous proteins.

Unexpectedly, T-cell clones have been identified which also respond to SMX itself in the absence of covalent binding or antigen processing (Schnyder *et al.*, 1997; Zanni *et al.*, 1998). This T-cell activation is TCR dependent (Depta *et al.*, 2004) and MHC restricted, but does not appear to depend on the presence of specific peptides (Burkhart *et al.*, 2002), suggesting a reversible interaction between MHC-drug-TCR. Recently, studies with T-cells isolated from blister fluid in toxic epidermal necrolysis patients found a similar pattern of antigen specificity (Nassif *et al.*, 2004), suggesting that these T-cells may also be important effector cells in drug hypersensitivity.

The differences between the nature of the antigen in SMX-immunogenicity between humans and animals are as yet unexplained, but they suggest caution regarding the use of animal models to investigate what is an idiosyncratic reaction in man, and one that is therefore patient-specific. It is important to note that the ability of SMX to function as an antigen with T-cells from susceptible individuals does not necessarily prove that SMX per se was the original immunogen in those patients.

1.3.8.4 Carbamazepine

CBZ is a commonly used aromatic anticonvulsant, and like most drugs of this type, it has been associated with hypersensitivity reactions. These reactions often show a high degree of cross-reactivity between other, structurally unrelated, aromatic anticonvulsants (Knowles et al., 1999). This propensity for cross-reactivity has not been satisfactorily explained, and hopefully a more detailed understanding of the precise determinants of hypersensitivity will identify the reasons for this.

There is good evidence that, at least in some populations, the individual susceptibility to CBZ hypersensitivity is in part genetically controlled. Studies have looked for associations with polymorphisms in metabolic enzymes, such as epoxide hydrolase (Gaedigk et al., 1994; Green et al., 1995), with little success. However, certain haplotypes of immunological genes do appear to play a role. The presence of certain TNF-α polymorphisms (Pirmohamed et al., 2001) and specific HLA subtypes (Chung et al., 2004; Hung et al., 2006; Man et al., 2007) correlate with the presence of CBZ hypersensitivity, although the association is stronger for more severe cases. Interestingly, although one of these studies (Chung et al., 2004) found an extremely close linkage between HLA-B*1502 and Stevens-Johnson syndrome or TEN reactions to CBZ in a Han Chinese population (100% of hypersensitive patients; 3% of controls), this was not reproduced by two studies on Caucasian groups (Alfirevic et al., 2006; Lonjou et al., 2006), suggesting that ethnicity may be important, presumably as a marker for further unidentified genetic differences. Interestingly, the association with B*1502 was only observed with bullous reactions such as TEN and Stevens-Johnson syndrome; there was no association with maculopapular eruption or hypersensitivity syndrome (Hung et al., 2006). The overall conclusion to be drawn from these studies is that, while genetic factors can act as determinants of adverse reactions, our current understanding of both the interactions amongst the genome and the pathophysiology of the reaction itself mean that we should be very cautious before ascribing too much importance to individual genetic associations.

CBZ is composed of an iminostilbene moiety coupled via the nitrogen heteroatom to a carbamoyl side chain. CBZ undergoes complex oxidative metabolism (over 30

metabolites have been extracted from urine; Lertratanangkoon & Horning, 1982, see figure 1.2), and has been found to form several distinct reactive species. The most abundant oxidative metabolite excreted is the 10,11 epoxide, which is formed by the action of CYP3A4 and, to a lesser degree, CYP2C8 (Kerr et al., 1994). Although this metabolite is largely stable in plasma, recent studies have suggested that it actually forms glutathionyl and protein adducts (Bu et al., 2005). The hydrolysis product of this epoxide, generated by the action of epoxide hydrolase, 10,11-dihydroxy-CBZ, is another major excreted metabolite (Eichelbaum et al., 1985). This is also believed to be produced by two separate hydroxylation steps, with 10-OH-CBZ as an intermediate. Ring contraction of either CBZ-10,11-epoxide or 10,11-dihydroxy-CBZ produces 9-hydroxymethyl-10-carbamoyl acridan (Lertratanangkoon & Horning, 1982).

Ring hydroxylation, producing a 2,3-epoxide allows direct reactions with GSH, and presumably with protein (Madden et al., 1996), and hydrolysis to 2,3-dihydroxy-CBZ, 2-OH-CBZ and 3-OH-CBZ (Lertratanangkoon & Horning, 1982). 2-hydroxy-CBZ, which is also a direct product from CBZ (multiple CYPs, but especially CYP2E1; Pearce et al., 2002), can be further oxidised to form an iminoquinone, either following hydrolysis of the carbamoyl group to 2-OH-iminostilbene (Ju & Uetrecht, 1999) or directly from 2-OH-CBZ (Pearce et al., 2005). This iminoquinone readily reacts with sulfhydryl-containing nucleophiles such as GSH or NAC, and so it is likely to be able to form cysteinyl adducts on protein.

MPO-mediated chlorination of CBZ leads to ring contraction and the generation of 9-acridinecarboxaldehyde (Furst & Uetrecht, 1993), which can generate lysine adducts via schiff base formation (Furst et al., 1995). These are readily reversible adducts, although they can rearrange to amine adducts, which are more permanent. In addition, 9-acridinecarboxaldehyde can undergo loss of the carboxaldehyde moiety to form acridine, and this can further oxidise to acridone (Breton et al., 2005; Furst et al., 1995).

A final route of phase I metabolism involves hydrolysis of the carbamoyl group to leave iminostilbene (Csetenyi et al., 1973). This can either undergo ring contraction to the 9-acridinecarboxaldehyde derivative described earlier (Furst et al., 1995), or can

undergo oxidative metabolism as does CBZ itself. The formation of 2-OH-iminostilbene can clearly be seen as a precursor to the iminoquinone described earlier.

Little is known currently about the mechanisms by which CBZ activates the immune system. One recent study (Naisbitt *et al.*, 2003a) found that T-cells isolated from patients following hypersensitivity reactions responded to CBZ *ex vivo* by both proliferation and cell-directed killing. Furthermore, clones isolated from the same individuals were also found to proliferate following CBZ treatment, and this was apparently in the context of HLA-DR or DQ. Cytokine secretion was also observed, although there was a degree of heterogeneity seen between clones: all 10 clones secreted IFN-γ to varying degrees, whilst IL-4, 5 and 10 secretion was more variable. A range of structurally related and unrelated compounds were tested for their ability to cross-react with CBZ-'specific' clones. Of these, only the 10,11-epoxide and a 10,11-dihydro-CBZ were able to do so, and again there was significant heterogeneity between clones, with several only responding to CBZ itself.

It would be particularly interesting to develop these findings in order to gain a better understanding of both the mechanisms of CBZ presentation to T-cells, and the structural requirements which enable this.
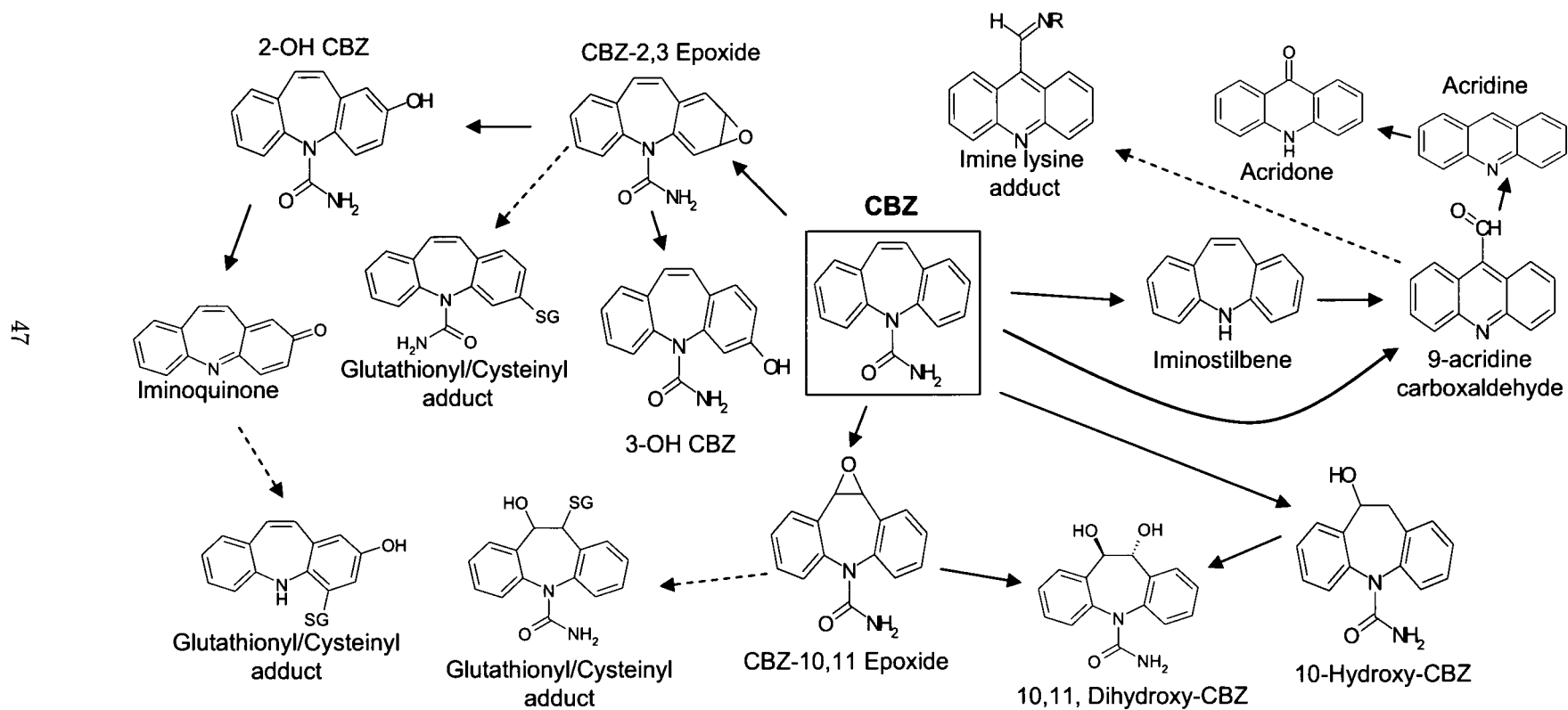
Figure 1.2: Known routes of oxidative CBZ metabolism. Dashed arrows represent formation of protein or GSH adducts.

1.4 Hypotheses and aims

The overall aim of this thesis is to investigate the role of drug metabolism in the activation of the immune system by drugs, both as a source of signal 1 and signal 2. In particular, this thesis will focus on three compounds, SMX, CBZ and TMP, as they are commonly associated with hypersensitivity reactions.

The work in this thesis is intended to test two independent hypotheses, as summarised in figure 1.3. The first hypothesis tested is that a reactive drug metabolite, in this case SMX-NO, can act to induce danger signalling in dendritic cells. To this end, the effect of SMX and SMX-NO on dendritic cells will be explored in Chapter 2, along with the functional consequences of these direct effects. The mechanisms by which the compounds induce their effects will be further investigated in Chapter 3, and the metabolic capacity of dendritic cells evaluated and compared to other relevant cell types. In order to accurately evaluate the metabolism of SMX it will be necessary to define the bioanalytical requirements for SMX-NHOH quantification, and these findings will be presented in chapter 4.

The second hypothesis to be explored is that drug-specific T-cells isolated from CBZ and TMP hypersensitive patients respond both to the stable parent drug, via reversible interactions, and to reactive drug metabolites, via hapten formation. Data presented in chapter 5 will describe investigations intended to explore the nature of the antigen recognised by CBZ-specific T-cell clones and evaluate the hypothesis that stable and reactive CBZ metabolites play a role in the activation of T-cells. Finally, chapter 6 will discuss similar investigations with T-cells from a patient both during and following severe TMP-induced hepatotoxicity.
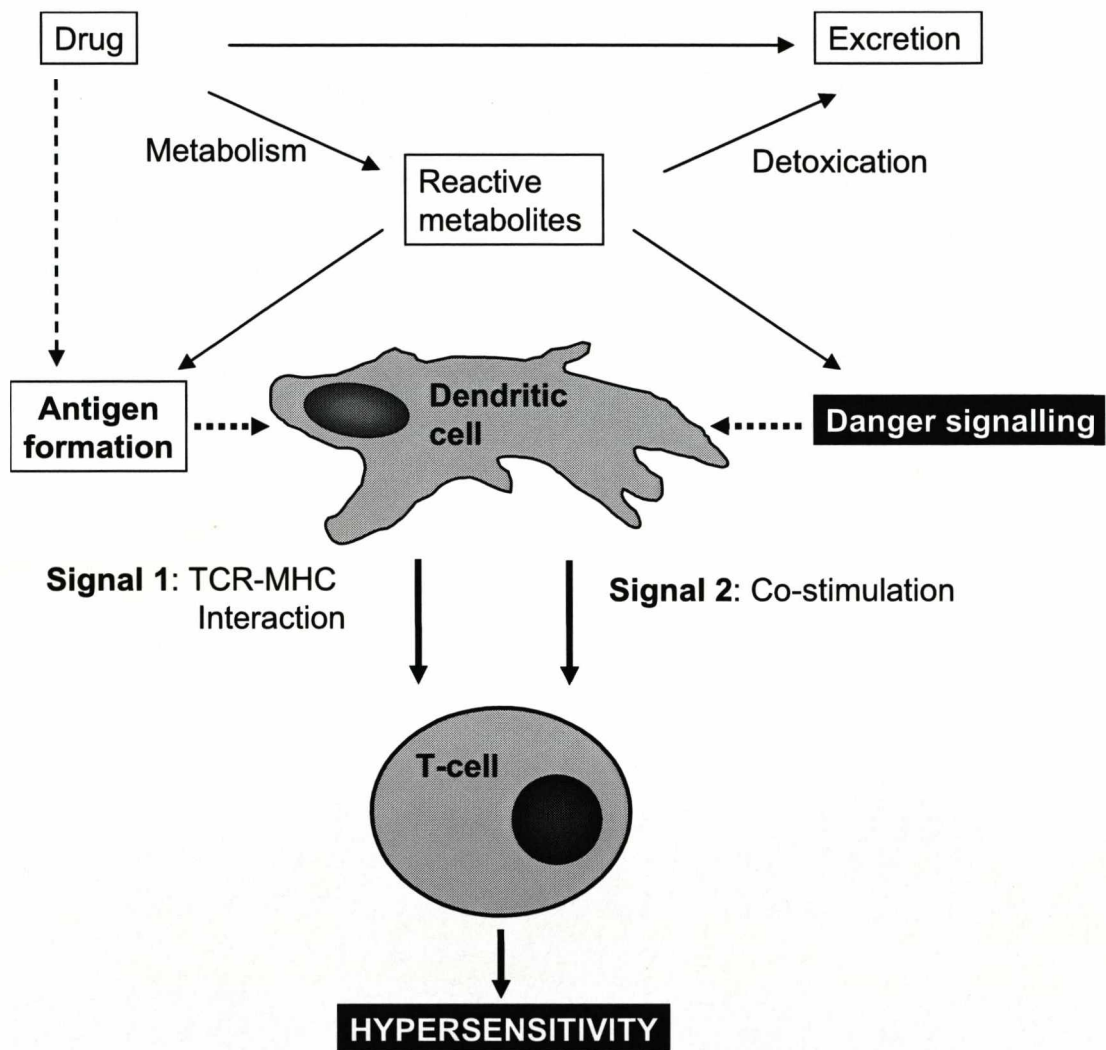
Figure 1.3: Different hypothetical routes by which a drug or drug metabolite can act to activate T-cells, and hence induce a hypersensitivity reaction

Chapter 2

Activation of Dendritic Cells by Sulfamethoxazole and Nitroso-Sulfamethoxazole

Contents

2.1 Introduction

SMX hypersensitivity reactions have been extensively studied. There are several reasons for this: first, SMX hypersensitivity is a significant clinical problem, particularly in the treatment of opportunistic infections in HIV+ patients; second, SMX is relatively straight-forward and well-understood in terms of its metabolism (although it must be said that there are still several uncertainties), and the known reactive metabolites can be synthesised chemically; and third, these metabolites are immunogenic *in vivo*, which has allowed the development of animal models.

Most mechanistic studies of SMX hypersensitivity have focused on T-cells isolated from patients either during or following their reaction. While these have given insights into the mechanism by which SMX is recognised by the immune system, they do not necessarily allow the mechanisms involved in the *induction* of the response to be explored.

For full induction of an immune response, the responding T-cell must receive two signals, as discussed previously (see 1.3.2).

The source of signal 1 in the induction of an immune response to SMX is often assumed (whether explicitly or not) to be related to the mechanism whereby activated and memory T-cells recognised SMX presented by APCs, but this is not necessarily the case (see the discussion in the previous chapter on avidity maturation; 1.3.1). We must therefore be careful not to assume that the antigen-specific T-cell repertoire present in patients either during or following a hypersensitivity reaction is perforce the same as that which was present during the induction phase.

Whilst the source of signal 1 is apparently well understood but still problematic, the source of signal 2, by contrast, is little understood. It has been postulated that the source of danger signals for the induction of hypersensitivity reactions is due to concurrent infection, either bacterial (Roth *et al.*, 2003) or viral (Hashimoto *et al.*, 2003; Pirmohamed *et al.*, 2002), but there is little direct evidence for either of these propositions. In particular, there is no apparent epidemiological link between bacterial infection and hypersensitivity, and while there is a clear association between viral infection and hypersensitivity, it does not appear to be essential, and there are al-

ternative explanations, including impaired detoxification mechanisms (Naisbitt *et al.*, 2000b; Trepanier *et al.*, 2004), depletion of regulatory cells (Eggena *et al.*, 2005), or the presence of activated T-cells and/or inflammatory cytokines (Gerber & Pichler, 2006). Furthermore, although certain viral infections are certainly associated with danger signalling, this is by no means universal: certain infections, including those associated with hypersensitivity, have actually been shown to *inhibit* danger signalling and dendritic cell maturation (Majumder *et al.*, 2005; Muthumani *et al.*, 2005; Niiya *et al.*, 2006).

A further proposed source of signal 2 in the induction of SMX hypersensitivity is toxicity produced either by the drug itself or by reactive metabolites. Some support for this has come from the finding that lymphocytes from SMX-hypersensitive patients are more susceptible to toxicity induced by SMX reactive metabolites (Carr *et al.*, 1993b; Rieder *et al.*, 1989). However, this has not been universally observed (Reilly *et al.*, 1999), and, if it is the case, the mechanisms are not known, although several possibilities exist, such as impaired detoxification, altered patterns of protein binding, and/or drug-specific cell-mediated killing. In any case, the known toxicity of SMX-metabolites and their *in vivo* immunogenicity suggest that they may be able to directly activate dendritic cells and hence generate signal 2 for the induction of SMX hypersensitivity.

Therefore, in this study the effects of SMX and SMX-NO on the phenotype of monocyte-derived dendritic cells was determined. Furthermore, two models of SMX-NO immunogenicity were investigated in order to explore the role of co-stimulatory signalling.

2.2 Materials and methods

2.2.1 Materials

Recombinant human cytokines (IL-2, IL-4, GM-CSF) were obtained from Peprotech (London, UK). FITC-labelled mouse anti-human CD11c, CD14, CD40, CD80, CD83, and CD86 antibodies were obtained from Serotec (Oxford, UK). Anti-mouse CD154 (MR1) was purchased from BD-Biosciences (Oxford, UK). Anti-human CD40 antibodies 82111 and 82102 were supplied by R&D systems (Abingdon, UK). [^{3}H]-methyl

thymidine was obtained from Moravek (California, USA). Lymphoprep was obtained
from Nycomed (Oxford, UK). All other reagents, chemicals and components were sup-
plied by Sigma-Aldrich (Gillingham, UK).

2.2.1.1 Culture medium

APC medium consisted of Roswell Park Memorial Institute (RPMI) medium, sup-
plemented with 10% fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N'-
2-ethanesulfonic acid (HEPES) buffer (25mM), L-glutamine (2mM), penicillin
(100U/ml) and streptomycin (100μg/ml).

T-cell medium consisted of RPMI with 10% human AB+ serum, HEPES buffer
(25mM), L-glutamine (2mM), penicillin (100U/ml), streptomycin (100μg/ml),
and transferrin (25μg/ml).

2.2.1.2 Synthesis of reactive SMX metabolites

The SMX metabolites SMX-NHOH and SMX-NO were synthesised as described pre-
viously (see figure 2.1; Naisbitt *et al.*, 1996). Nitro-sulfamethoxazole (SMX-NO$_2$)
was prepared by the reaction of 3-amino-5-methylisoxazole (20g; 203mmol) with 4-
nitrobenzenesulfonyl chloride (45g; 203mmol) in 75ml ice-cold pyridine. The reaction
mixture was stirred on ice overnight, and a brown precipitate was produced by the
addition of excess distilled water (dH$_2$O). This precipitate was filtered, washed with
further dH$_2$O to remove remaining pyridine, recrystallised from an ethyl acetate:toluene
mixture (1:3 v/v) and left overnight at 0°C. The final mixture was then collected by
filtration and analysed for purity by nuclear magnetic resonance (NMR).

SMX-NHOH was synthesised from SMX-NO$_2$ by reduction using sodium phos-
phinite as a hydrogen donor and a two-phase solvent system (tetrahydrofuran / wa-
ter) with a palladium / carbon catalyst. 5% palladium carbon catalyst (0.1g) was
added to a stirred solution containing sodium phosphinite (1.12g, 11mmol) and SMX-
NO$_2$ (1g, 3.53mmol) in dH$_2$O(10ml) / tetrahydrofuran (100ml). The reaction mix-
ture was monitored by thin-layer chromatography (TLC), using a mobile phase of
dichloromethane:ethyl acetate (65:35), until nearly all of the starting material had
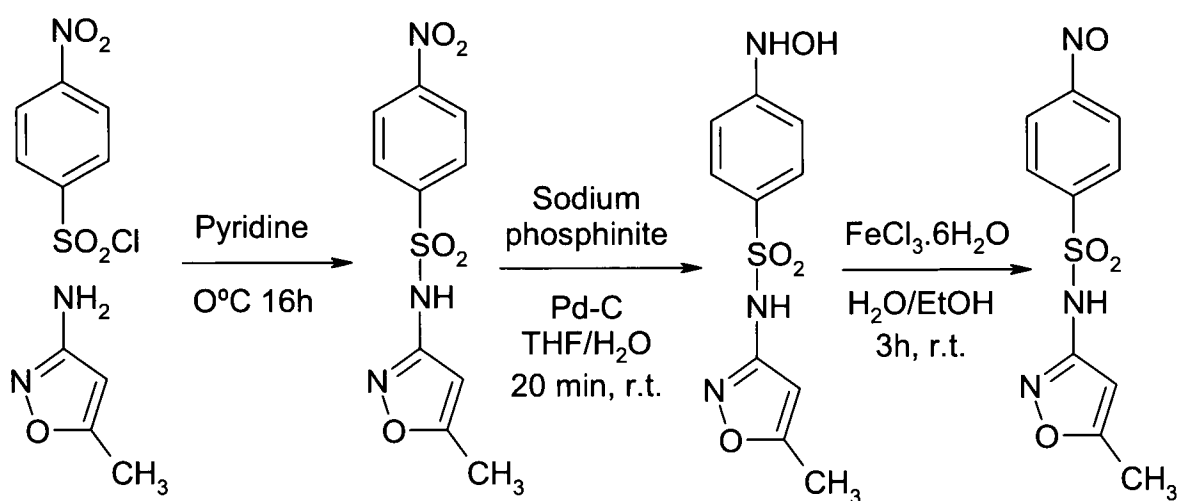
Figure 2.1: Synthesis of SMX-NHOH and SMX-NO

reacted. This typically took ~20 mins. The product was filtered under vacuum to remove excess catalyst, extracted with ether and dried with $MgSO_4$ to give a pale orange oily product. This was then further dried on a rotary evaporator (Rotovapor, Büchi, Switzerland) to give a pale yellow solid. This product was then recrystallised from chloroform, and the purity of the final product assessed by NMR and high-performance liquid chromatography (HPLC).

Oxidation of SMX-NHOH by iron(III) chloride was performed to produce SMX-NO. SMX-NHOH(200mg, 0.743mmol) in ethanol (30ml) was added to a stirred solution of iron(III) chloride hexahydrate (1.5g, 5.549mmol) in dH_2O(40ml) over a period of 10 mins. This mixture was stirred at room temperature for 3h, during which time a yellow solid precipitated within the reaction mixture. This solid was filtered and dried under vacuum and the purity was analysed by NMR and HPLC.

2.2.1.3 Chemical characterisation

Proton NMR spectra were obtained using a Bruker (400MHz) NMR spectrometer. Compounds were dissolved in deuterated dimethylsulfoxide (DMSO) and tetramethylsilane was used as an internal standard.

The compounds were also analysed by HPLC with both UV and mass spectrometry (MS) detection. Samples were eluted from a Prodigy 5 ODS 2 column (150 x 4.6mm; Phenomenex) using an isocratic mobile phase of glacial acetic acid (1% v:v) and acetonitrile (20% v:v) in dH_2O at a flow rate of 1 ml.min^{-1}. UV absorbance was monitored at 254nm. Split flow of eluate to the mass spectrometer was 50μl/min. Full scanning spectra and selected ion monitoring data were acquired with a Quattro II tandem quadropole instrument (Micromass, Manchester, UK). The source temperature was 70°C; the capillary voltage, 3.9x10^{-3} V and the standard cone voltage 30 V. Data were processed via Mass Lynx 2.0.

2.2.2 Isolation of peripheral blood mononuclear cells

PBMCs were isolated from heparinised venous blood of healthy human volunteers by centrifugation on a density gradient of Lymphoprep. 15ml blood was carefully layered

on 10ml of Lymphoprep and the tube was centrifuged at 750g for 25 min. PBMCs formed a distinct cloudy band between the plasma and the Lymphoprep layers, and could be aspirated with a sterile Pasteur pipette. The PBMCs were then washed twice in Hank's balanced salt solution (HBSS) to remove residual Lymphoprep, and resuspended in 10ml HBSS. Cell yield was assessed using an improved Neubauer haemocytometer (Sigma) under a Wilovert microscope (Will Wertzlar, Germany), and cell viability determined by trypan blue dye staining. For this, trypan blue ($10\mu l$, 0.2%, w/v) was added to a suspension of cells ($40\mu l$ in HBSS). $10\mu l$ of the stained sample was placed on a Neubauer haemocytometer and 200 cells were counted. % viability was calculated as:

$$100 \ x \ \frac{total \ number \ of \ viable \ cells}{total \ number \ of \ cells}$$

and was typically > 95%.

2.2.3 Dendritic cell generation

PBMCs were cultured at $2x10^6$/ml and 1ml/well in 24-well plates for 4 h followed by repeated washing to remove non-adherent cells. Adherent cells were then cultured at 37°C in an atmosphere of 5% CO_2 in 1ml APC medium, supplemented with interleukin (IL)-4 (800U/ml) and granulocyte-monocyte colony stimulating factor (GM-CSF) (800U/ml). Half of the medium was replaced with fresh complete media on days 1, 3 and 5, and immature dendritic cells were ready for use on day 6. Purity of dendritic cell populations and loss of monocyte phenotype was determined by flow cytometry with anti-human CD11c and CD14 antibodies using an EPICS-XL flow cytometer (Coulter Electronics, Luton, Beds., UK).

2.2.4 Drug treatment and analysis of cell surface markers

Phenotype analysis of drug-exposed dendritic cells was performed by flow cytometry. Immature dendritic cells were incubated with SMX, SMX-NO (both $0.5-4000\mu M$; made up in DMSO, final concentration <0.5%), LPS (*E. Coli* 0111; 1μg/ml) or DMSO-only control for 24 h, before being washed and incubated with a fluorescein isothiocyanate

(FITC)-labelled mouse anti-human CD40, CD80, CD83 or CD86 antibodies for 30 min at 4°C. Cells were washed repeatedly and fluorescence was measured by flow cytometry. Cells were gated according to forward and side scatter characteristics, and median FITC fluorescence determined. For some experiments polymyxin B (PMB) ($20\mu g/ml$) was pre-incubated with the drug for 30 min before addition to the cells.

2.2.5 Determination of sulfamethoxazole immunogenicity in the mouse

Balb/C strain mice (Charles River; n=4 per group) were administered SMX-NO (1 mg/kg) by subcutaneous injection into the nape of the neck on three consecutive days. Control animals received vehicle alone. A further group of mice were administered an anti-CD154 blocking antibody (MR1; $175\mu g$) via a single intraperitoneal injection 1 h prior to SMX-NO administration and subsequent subcutaneous doses ($25\mu g$) with each dose of SMX-NO. After 5 days, draining lymph node cells were harvested and incubated (1×10^6; total volume $200\mu l$) in dendritic cell medium with [^{3}H]-methyl thymidine ($2\mu Ci$) for 24 h to measure proliferation. Cells were extracted to filter plates using a cell harvester (Tomtuk, Finland), and incorporated radioactivity was determined with a 96-well β-counter (Perkin-Elmer, US). Results are recorded as counts per minute (cpm).

2.2.6 Induction of a primary T-cell response *in vitro* with lymphocytes from SMX naïve volunteers

Human naïve T-cells were primed with SMX-NO according to the technique of Engler *et al.* (2004). PBMCs were isolated from peripheral blood by density centrifugation as described earlier (2.2.2), and were frozen in 1ml FBS with 10% DMSO. Cells were defrosted and incubated at 2.5×10^6 cells/ml in 2ml T-cell medium with either $50\mu M$ SMX-NO or $250\mu M$ SMX. Some wells contained either agonistic anti-CD40 antibody (82111) or antagonistic anti-CD40 antibody (82102). On day 3 of culture, rhIL-2 (10U/ml) was added to the cultures, and on day 7 and weekly thereafter the cultures were stimulated with SMX-NO ($50\mu M$) and irradiated (4500 rad) autologous PBMCs from frozen stocks. Antigen specificity was determined weekly by stimulation of an aliquot of T-cells (1×10^6/ml) with SMX-NO (20–$60\mu M$) or SMX (50 - $500\mu M$)

and irradiated PBMCs (0.2×10^6/ml) as antigen presenting cells, and measurement of proliferation was determined by [^{3}H]-methyl thymidine incorporation. Proliferation was recorded as stimulation index (SI), calculated as,

$$\frac{cpm \ in \ drug - treated \ cultures}{cpm \ in \ cultures \ with \ DMSO \ alone}$$

Wells where the SI was reproducibly ≥ 2 with a given concentration of SMX or SMX-NO were designated positive.

2.2.7 Statistical analysis

Results are presented as mean $\pm$ standard error of the mean. Normality of data was determined by the Shapiro-Wilks test and equality of variance by the Levene test. Data which were normally distributed were compared by means of a t-test, whereas nonparametric data were compared with the Mann-Whitney test for unpaired data or the Wilcoxon signed rank test for paired data. P-values < 0.05 were considered to be significant. All statistical analyses were performed on SPSS 13.0.

2.3 Results

2.3.1 Analysis of purity of sulfamethoxazole metabolites

^{1}H NMR spectra were obtained for SMX, as a reference, and for all metabolites synthesised (all in d6-DMSO; 400MHz).

SMX δ11.42 (s, 1H, SO$_2$N$\underline{H}$), 7.47 (d, 2H, J=8.64, Ar$\underline{H}$), 6.59 (d, 2H, J=8.7, Ar$\underline{H}$), 6.08 (s, 1H, Ar$\underline{H}$), 6.08 (s, 2H, N$\underline{H}_2$), 2.29 (s, 3H, C$\underline{H}_3$)

SMX-NO$_2$ δ8.43 (d, 2H, J=8.73, Ar$\underline{H}$), 8.12 (d, 2H, J=8.75, Ar$\underline{H}$), 6.17 (s, 1H, Ar$\underline{H}$), 2.31 (s, 3H, C$\underline{H}_3$)

SMX-NHOH δ11.08 (s, 1H, SO$_2$N$\underline{H}$), 9.07 (s, 1H, N$\underline{H}$OH), 8.75 (s, 1H, NH$O\underline{H}$), 7.61 (d, 2H, J=8.72, Ar$\underline{H}$), 6.86 (d, 2H, J=8.8, Ar$\underline{H}$), 6.11 (s, 1H, Ar$\underline{H}$), 2.29 (s, 3H, C$\underline{H}_3$)

SMX-NO δ8.21 (d, 2H, J=8.47, Ar$\underline{H}$), 8.15 (d, 2H, J=8.5, Ar$\underline{H}$), 6.19 (s, 1H, Ar$\underline{H}$),

2.31 (s, 3H, C$\underline{H}_3$)

Purity of compounds was determined by both NMR and LC/MS analysis. In all cases, purity of compounds selected for further use was $\geq$99%, with the only detectable contaminant being parent SMX.

2.3.2   Analysis of purity of dendritic cell populations

Adherent PBMCs were washed and analysed, and found to be $\geq$95% monocytes (CD14+). Following 6 days culture *in vitro* with IL-4 and GM-CSF they were reanalysed and found to be $\geq$95% CD11c+/CD14- dendritic cells. This was accompanied by morphological changes associated with the acquisition of the dendritic cell phenotype, and up-regulation of MHC-II and CD83.

2.3.3   Effects of SMX/SMX-NO exposure on dendritic cell surface marker expression

Dendritic cells exposed to SMX (maximal effect: 250–500μM) and SMX-NO (maximal effect: 1μM) consistently displayed a concentration-dependent increase in CD40 expression (see figure 2.2), but no increase in CD80, CD83, or CD86 (see figure 2.3). This CD40-specific up-regulation was also seen with dendritic cell incubations with SMX and SMX-NO of up to 48 h (data not shown). In contrast, cells exposed to LPS showed an increase in expression of all the markers examined. Importantly, the response to SMX and SMX-NO was unaffected by preincubation with PMB (20μg/ml), whereas the response to LPS was completely eliminated (see figure 2.4). No effect was seen on the expression of surface markers on dendritic cells with PMB alone (data not shown).
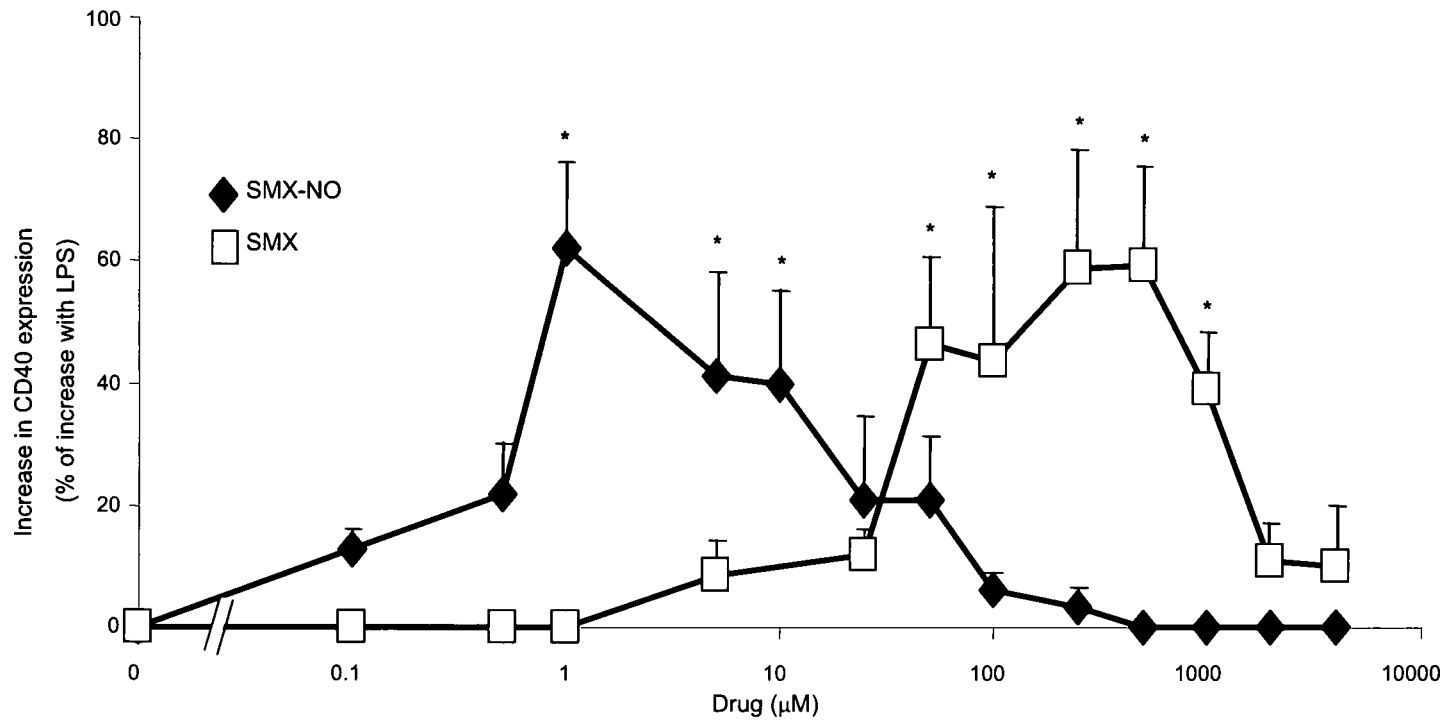
Figure 2.2: Dendritic cell CD40 up-regulation in response to SMX and SMX-NO. Extent of up-regulation of CD40 in monocyte-derived dendritic cells, as measured by flow cytometry and recorded as the % of the increase with LPS over control, induced by varying concentrations of SMX (n=8) and SMX-NO (n=6) over a 24 h incubation period. Data are presented as mean ± SEM. (*, Significantly different from untreated cells; p<0.05).

2.3.4 *In vivo* immunogenicity of SMX-NO

Subcutaneous dosing of Balb/C strain mice to SMX-NO was associated with a significant increase in *ex vivo* lymph node cell proliferation when compared with mice exposed to vehicle alone. However, concurrent dosing with an antagonistic anti-CD154 antibody completely eliminated the increased lymph node cell proliferation associated with SMX-NO treatment (see figure 2.5).

2.3.5 Primary induction of an immune response to SMX-NO *in vitro*

PBMCs isolated from SMX-naïve volunteers (n=5) were successfully sensitised *in vitro* to SMX-NO over the course of 4 weeks. When PBMCs from the same volunteers were co-cultured with an agonistic anti-CD40 antibody, a significantly enhanced SMX-NO specific response was seen (total positive wells: 11/20 vs. 3/20; average SI: 3.07 vs. 1.26; see figure 2.6) after 4 weeks. Furthermore, the addition of an antagonistic anti-CD40 antibody prevented the sensitisation in all cases (total positive wells 0/20 vs. 3/20).

However, in contrast to the results with SMX-NO, and to those previously published (Engler *et al.*, 2004), attempts to generate an *in vitro* primary response to SMX were unsuccessful, despite repeated attempts (data not shown).

2.4   Discussion

Little is known about whether drugs or drug metabolites may act to generate signals that lead to activation of dendritic cells. In order to address this, we studied the effects of SMX and SMX-NO on the surface expression of monocyte-derived dendritic cell co-stimulatory molecules, which are commonly used as markers of dendritic cell maturation. Dendritic cells were generated from healthy human drug-naïve volunteers, incubated with SMX or SMX-NO, and expression was determined by flow cytometry. With both compounds an increase in surface CD40, but not CD80, 83 or 86, expression was seen after 24 h (see figure 2.2). A specific effect on CD40 expression was also seen following a longer term drug exposure of up to 48 h (results not shown). However, the positive control LPS readily induced up-regulation of all surface markers examined, ex-
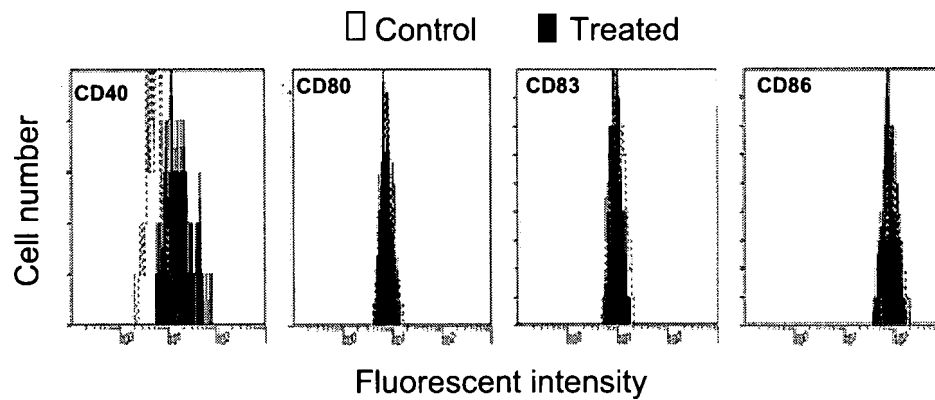
Figure 2.3: Selective dendritic cell up-regulation of CD40 by SMX-NO. Representative flow cytometry histograms showing CD40, but not CD80, 83, or 86 up-regulation following incubation with 5μM SMX-NO.
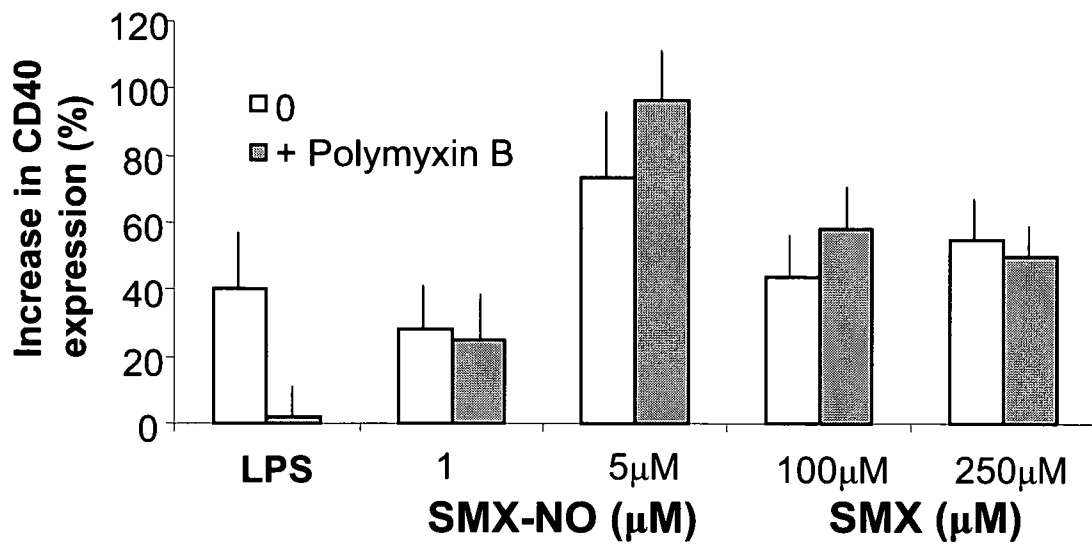
Figure 2.4: Polymyxin B inhibits dendritic cell activation by LPS, but not by SMX or SMX-NO
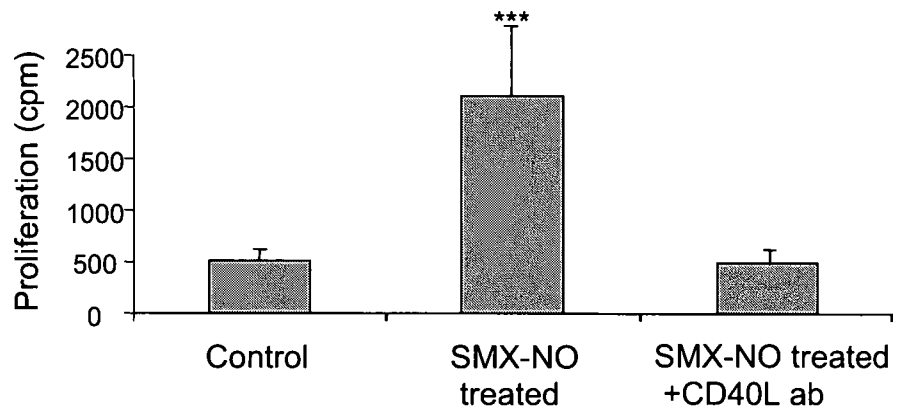
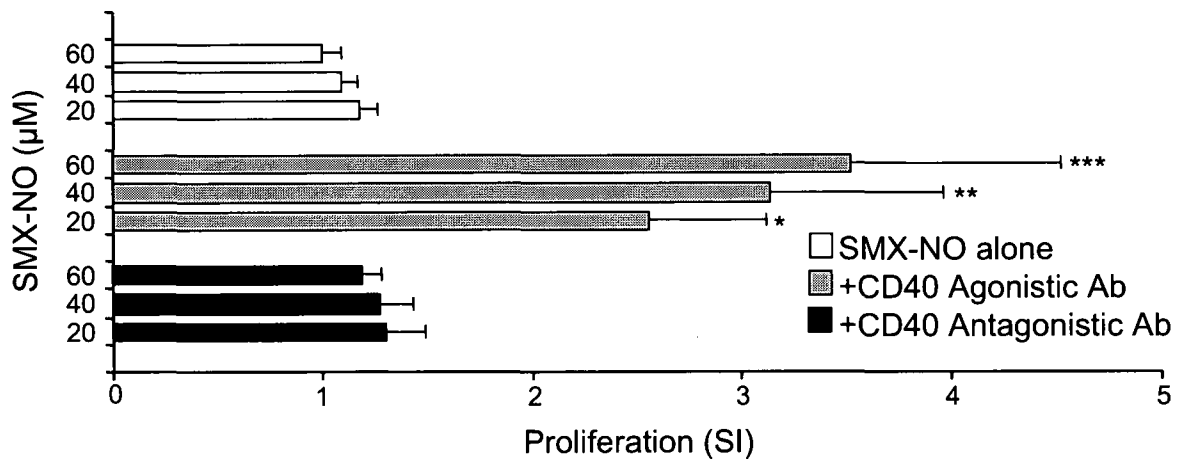Figure 2.5: CD40 blockade inhibits SMX-NO immunogenicity in mice. ***: significantly different to control p<0.001

Figure 2.6: CD40 ligation enhances *in vitro* primary stimulation of naïve PBMCs to SMX-NO. */**/***: significantly different to control p<0.05/0.01/0.001

cluding the possibility that the dendritic cells used were simply abnormal. The maximal response to both SMX and SMX-NO was similar, at ~70% of the response seen with 1μg/ml LPS, and the only difference observed between the dendritic cells' responses to SMX and SMX-NO was that the required molar concentration of SMX to induce maximal CD40 expression was ~250-fold higher. The concentrations of SMX which were maximally active are similar to those seen *in vivo* following high-dose therapy for *P. carinii* treatment (Stevens *et al.*, 1991), and the effective concentrations of SMX-NO were lower than those of SMX-NHOH *in vivo* (~3% of the ingested dose is excreted as SMX-NHOH; Cribb & Spielberg, 1992). However, as SMX-NO is too unstable to be successfully extracted from biological matrices, it is not known what proportion of SMX-NHOH is converted to SMX-NO at any one time, and so it is impossible to say with precision whether the concentrations used in this study are clinically relevant.

A selective up-regulation of CD40 against CD80/86 on dendritic cells in response to the hair-dye compound *p*-phenylenediamine (Coulter *et al.*, 2006) has been recently reported, although the implications of this are not yet fully understood. Dendritic cell regulation of CD40 and CD80/86 expression differs, and some of the upstream pathways are known. For instance, RelB deficient dendritic cells fail to upregulate CD40 in response to LPS, but upregulate CD80 and CD86 normally, and induce tolerance rather than effective antigen-specific priming (Martin *et al.*, 2003).

CD40 ligation by its natural ligand, CD154, leads to receptor trimerisation, which allows recruitment of TNF receptor-associated factors to the cytoplasmic tail (Pullen *et al.*, 1998). These adaptor proteins produce a signalling complex which induces down-stream events such as activation of the NF-κB and MAPK pathways (Ouaaz *et al.*, 2002), reviewed in Quezada *et al.* (2004). The overall effect of these signal trans-duction pathways is to enhance dendritic cell survival (Ludewig *et al.*, 1995), drive morphological changes, upregulate co-stimulatory markers, and induce cytokine release (Caux *et al.*, 1994).

So-called semimature dendritic cells have been associated with the induction of tolerogenic T-cell responses (Lutz & Schuler, 2002), either by induction of anergy (Sato *et al.*, 2002) or by preferential activation of CD4+CD25+ regulatory T-cells (Verginis

et al., 2005). However, this seems to be not so much a function of the dendritic cell than of the responding T-cell, as a recent study (Kleindienst *et al.*, 2005) found that while semimature dendritic cells preferentially activated tolerogenic CD4+ T-cells, they were perfectly capable of inducing an effective CD8+ T-cell response to antigen. A major problem with establishing the physiological and pathological function of semimature dendritic cells is the lack of a stringent definition for what actually constitutes 'semimature' in this sense: some papers use the term to refer only to dendritic cells which fail to secrete proinflammatory cytokines, others to refer to dendritic cells which actively release anti-inflammatory cytokines, and still others to those displaying an incomplete repertoire of surface markers. Furthermore, there is a wide discrepancy between the techniques used to generate semimature dendritic cells, which no doubt impacts heavily on their function.

Therefore, it is unclear whether our dendritic cells are genuinely semimature. It would be very interesting to investigate the effects of SMX and SMX metabolites on the pattern of cytokine release by dendritic cells, but this was not possible within this study due to time restraints. Importantly, one must also note that binding of the natural CD40 ligand CD154 to dendritic cells expressing high levels of the CD40 receptor stimulates further dendritic cell maturation (Caux *et al.*, 1994) and as such the observed highly specific effect of SMX and SMX metabolites on CD40 may, in a mixed cell population, ultimately result in fully matured dendritic cells. Again, this will require continuing investigation to completely elucidate.

There are several possible sources of dendritic cell for *in vitro* investigations. Of these, monocyte-derived dendritic cells are the most commonly used because they are both easily available in relatively high numbers, and they are an excellent surrogate for *in vivo* dendritic cells. Alternative sources are dendritic cells derived from CD34+ cells, either from umbilical cord (Romani *et al.*, 1994) or peripheral blood (Ohishi *et al.*, 2001), or from bone marrow (Bernhard *et al.*, 1995). Although some studies have found slight differences between these different sources of cells (Bracho *et al.*, 2003; Syme *et al.*, 2005), these have typically been minor, and have been phenotypic rather than functional changes. A comparison of CD14+ vs. CD34+ derived dendritic cells found

that they are both effective as a screening tool for contact sensitisers (Rustemeyer *et al.*, 2003).

It is possible that the effects which were observed on dendritic cells were in fact due to low-level contamination of SMX and SMX metabolite stocks by bacterial LPS. However, there are several reasons to believe this to be unlikely. First, the CD40-specific effects have not been previously described for any known microbial contaminant; second, the compounds were stored and used under sterile conditions, and endotoxin-free glass- and plasticware were continually used; third, the concentrations of SMX-NO at which the effects were observed were close in mass to the concentrations of LPS required for dendritic cell activation, indicating that any contamination of our SMX-NO stocks would have to constitute an implausibly large proportion of the stock; and fourth, and most convincingly, the addition of PMB, which binds to and inactivates LPS (Tsubery *et al.*, 2000), and completely eliminated all effects produced by LPS in our system, had no impact on the dendritic cell activation induced by SMX or SMX-NO. It is worth noting that most studies use PMB for inactivation of LPS at 10-25μg/ml (Nikulina *et al.*, 2004; Smiley *et al.*, 2001), which concurs with our use of PMB at 20μg/ml. A recent study has suggested that PMB has direct effects in inducing maturation of dendritic cells (Valentinis *et al.*, 2005), restricted to up-regulation of MHC-1 and -2, and CD86, with no effects seen on CD40 expression. However, we saw no similar effects in the absence of drug, and the inhibition of LPS-mediated activation suggested that the PMB would have been successful in blocking any potential LPS contamination.

Activation of antigen presenting cells through CD40 signalling is known to precipitate animal models of autoimmune disease (Garza *et al.*, 2000; Ichikawa *et al.*, 2002), while CD40 blockade can prevent the progression of diseases such as systemic lupus erythematosus, cardiac allograft arteriopathy, and spontaneous autoimmune diabetes (Davidson *et al.*, 2003; Nanji *et al.*, 2006; Polese *et al.*, 2002; Wang *et al.*, 2002). However, the importance of CD40 signalling in drug hypersensitivity has not been considered previously. We have therefore utilised two experimental approaches to evaluate the role of CD40 signalling in SMX metabolite immunogenicity.

First, we used the local lymph node assay. This is commonly used in industry to assess the sensitisation potential of novel drugs and chemicals (Gerberick et al., 2007; OECD, 2002). Compounds are applied either topically or subcutaneously and ex vivo lymph node cell proliferation is determined by [^{3}H]-methyl thymidine incorporation. Inhibition of CD40:CD154 signalling by systemic pre-treatment with a blocking anti-CD154 antibody completely eliminated the SMX-NO induced increase in proliferation, demonstrating that CD40 signalling is essential for in vivo SMX-NO immunogenicity. SMX itself was not used as previous work in our laboratories has found that it is not directly immunogenic in this assay.

Secondly, we used a recently published protocol for in vitro primary stimulation of T-cells to SMX and SMX-NO (Engler et al., 2004). This involves alternated stimulation of PBMCs from SMX-naïve individuals with IL-2 and either SMX or SMX-NO. Sensitisation was determined by proliferation assays on a weekly basis. The addition of a CD40 agonistic antibody significantly enhanced the response to SMX-NO, while a CD40 antagonistic antibody eliminated the response in all cases.

However, we were unable to generate a primary response to SMX, when rechallenged either with SMX or SMX-NO. It is not clear why this was unsuccessful, although it is worth bearing in mind that the original paper only successfully induced responses in 3/10 individuals. It is quite possible that only a small subset of individuals are susceptible to in vitro primary sensitisation by SMX, either due to genetic, immunological or metabolic factors, and that, simply by chance, none of the 5 individuals tested herein were of this group. If this is the case, then further investigations may produce interesting findings regarding the individual determinants of hypersensitivity. However, this is beyond the scope of this discussion.

These data indicate that CD40 plays an important role in the primary sensitisation of T-cells to SMX-NO in vitro. In addition, they support the thesis that this is a genuine primary sensitisation event, as opposed to a recall response with unknown original peptide specificity, as has been more recently proposed (Pichler, 2005).

In conclusion, these findings indicate that both SMX and the reactive SMX metabolite SMX-NO can induce CD40 up-regulation on dendritic cells, and that CD40 plays

an important role in the induction of a primary immune response to SMX-NO.

Chapter 3

Mechanisms of Dendritic Cell Activation by Sulfamethoxazole and Nitroso-Sulfamethoxazole

Contents

3.1   Introduction

Dendritic cell activation in response to 'danger signals', and co-stimulatory signals induced thereby, are requisite elements in the induction of an immune response. As discussed previously (see 1.3.2), in the absence of co-stimulatory signalling, novel antigens are tolerogenic, rather than immunogenic. The source of these signals in drug hypersensitivity is not known, although several hypotheses have been proposed. In the previous chapter (see chapter 2) it was found, for the first time, that both a drug and its reactive metabolite are capable of inducing partial dendritic cell maturation, and that signalling through this pathway is essential for the immunogenicity of the reactive metabolite.

Danger signals can be broadly divided into exogenous, i.e. derived from foreign pathogens, and endogenous signals, i.e. derived from cellular damage within the organism. Dendritic cell activation in response to endogenous signals has been well-documented, although the mechanisms by which this happens are not as well understood as the mechanisms by which dendritic cells activate in response to exogenous, pathogen-induced signals. While it is likely that many endogenous signals are also transduced by activation of TLRs, there have been several difficulties in identifying the precise TLR responsible—not least the risk of bacterial contamination of the endogenous signalling molecules (for instance, see Gao & Tsan, 2003).

The best understood mechanism by which endogenous cell damage produces danger signals is via the induction of cell death. Originally, it was claimed that only necrotic cell death led to the production of danger signals (Gallucci et al., 1999), presumably via the uncontrolled release of intracellular contents such as uric acid (Shi et al., 2003), heat-shock proteins (Asea et al., 2000) or adenosine triphosphate (Bours et al., 2006), but recent work has found that apoptotic cells may also activate dendritic cells, albeit via a TLR-independent pathway (Janssen et al., 2006). As apoptosis is a normal

function of homeostasis and development, it is not clear as yet whether this requires further discriminatory mechanisms to prevent unnecessary immune activation.

Compounds which produce cellular damage may also produce danger signals through the generation of reactive oxygen species and oxidative stress. Several lines of evidence led to this conclusion: firstly, direct application of either the reactive oxygen species donor hydrogen peroxide (Rutault et al., 1999) or the superoxide generating system xanthine-xanthine oxidase (Kantengwa et al., 2003) can induce dendritic cell activation; and secondly, the addition of anti-oxidants can prevent dendritic cell activation by toxic insults (Kantengwa et al., 2003)—including those generated by contact sensitisers (Bruchhausen et al., 2003). Interestingly, antioxidants have also been found to inhibit immune cell activation in response to other, apparently unrelated, stimuli, such as LPS (Matsue et al., 2003; Verhasselt et al., 1999), inflammatory cytokines (Tan et al., 2005) and bacterial CpG-containing DNA motifs (Yi et al., 1996), and the generation of reactive oxygen species has been identified during dendritic cell activation (Maemura et al., 2005; Matsue et al., 2003; Yamada et al., 2006), suggesting that reactive oxygen species may be a common pathway for dendritic cell activation.

It has been postulated that all danger signals share a common theme: the presence or unmasking of hydrophobic residues on macromolecules (Seong & Matzinger, 2004). In this light, it is interesting to reflect that denaturation of proteins through excessive covalent modification is likely to result in the exposure of internal hydrophobic residues, potentially forming a linkage between covalent modification and generation of endogenous danger signals. While this has never been precisely demonstrated, many directly protein-reactive compounds have been found to activate dendritic cells, including contact sensitisers such as dinitrohalobenzenes (Aiba et al., 1997; Manome et al., 1999), oxazolone (Verheyen et al., 2005), trimellitic anhydride (Toebak et al., 2006), and paraphenylenediamine (Coulter et al., 2006), as well as drugs such as β-lactam antibiotics (Rodriguez-Pena et al., 2006).

In this study, the mechanism by which both SMX and SMX-NO activate dendritic cells was investigated, and particular attention paid to the possibility that localised dendritic cell metabolism may allow the generation of SMX-NO from SMX in situ.

3.2 Materials and methods

3.2.1 Materials

Hydroxylamine and nitroso metabolites of SMX were synthesised as described previously (see 2.2.1.2), and determined by HPLC, mass spectrometry, and nuclear magnetic resonance to be >99% pure. Rabbit anti-SMX IgG antibody was a kind gift of Dr M.J. Rieder (Department of Medicine, University of Western Ontario, Canada). rhIL-4, and rhGM-CSF were obtained from PeproTech. FITC-labelled anti-CD40, CD80, CD83, and CD86 Abs, and the leucoperm fixation and permeabilisation kit were all obtained from Serotec. Lymphoprep was obtained from Nycomed. The Annexin-V^{FITC} kit was obtained from Bender MedSystems. All rhCYP-expressing supersomes were obtained from BD Biosciences. DNase I and diethyl pyrocarbonate-treated water were obtained from Ambion. RiboGreen RNA quantification kit was obtained from Molecular Probes. RNeasy 96 total RNA extraction kit and RLT lysis buffer were obtained from Qiagen. Oligo(dT)12-18 primer, 2'-deoxynucleoside 5'-triphosphate mix and the Superscript II reverse transcriptase kit were obtained from Invitrogen Life Technologies. Oligonucleotide fluorogenic probes and TaqMan Universal PCR Mastermix were obtained from Applied Biosystems. All other chemicals, components, and oligonucleotide primers were obtained from Sigma-Aldrich.

3.2.2 Dendritic cell generation

PBMCs and dendritic cells were generated as described previously (see 2.2.3) and analysed for purity as before.

3.2.3 Analysis of dendritic cell death following SMX/SMX-NO treatment

Immature dendritic cells were incubated with SMX or SMX-NO (0.01-1000μM) for 24 h. Cells were counted on an improved Neubauer haemocytometer to determine cell recovery. The populations were then analysed for apoptotic or necrotic cells using an annexin V-propidium iodide dual staining methodology (Vermes *et al.*, 1995). Briefly, cells were

stained with FITC-linked rhAnnexin V (1g/ml) for 10min before being washed and incubated with propidium iodide (PI) (35μM). The cells were then analysed for apoptotic and necrotic cell death by flow cytometry using an EPICS-XL flow cytometer (Coulter Electronics, Luton, Beds., UK). Cells were simultaneously analysed by forward scatter and side scatter characteristics, and a gated population defined to exclude cellular debris. Cells within this gated population were simultaneously assessed for FITC and PI staining. Cells were then divided into four groups according to staining characteristics: $FITC^-/PI^-$, viable; $FITC^+/PI^-$, early apoptotic; $FITC^-/PI^+$, necrotic; $FITC^+/PI^-$, late apoptotic, or secondary necrotic.

3.2.4 Determination of protein quantity

Protein levels in samples were determined by the method of Bradford (1976). A standard curve of 0.25-5.0μg bovine serum albumin (BSA) was prepared as per table 3.1 using a stock solution of 0.5mg/ml BSA in dH_2O. Samples were diluted as required and 10μl added to 10μl dH_2O in each well of a flat-bottomed 96-well plate. Bradford reagent was diluted 1:5 with dH_2O and 200μl added to each well immediately prior to spectrophotometric analysis (MRX microplate reader running Revelation version 3.04 software, Dynex Technologies, Billinghurst, W. Sussex) at 570nm. Blank wells were subtracted from all values, and linear standard curves were derived to allow quantification of sample protein concentrations.

Final quantity (μg)	Volume Stock (μl)	Volume dH_2O (μl)
0	0	20
0.25	0.5	19.5
0.5	1.0	19.0
1	2.0	18.0
2	4.0	16.0
3	6.0	14.0
4	8.0	12.0
5	10.0	10.0

Table 3.1: Dilution of BSA standards for Bradford assay

3.2.5 Analysis of glutathione depletion following SMX/SMX-NO treatment

Immature dendritic cells were incubated with SMX or SMX-NO (1-1000μM) for 24 h. The cells were then washed and lysed with 10mM HCl, and an aliquot taken for protein content determination as described above (3.2.4). Total glutathione and GSSG levels were determined by a microtitre plate assay according to the method of Vandeputte *et al.* (1994). Reduced glutathione GSH levels were determined by subtracting GSSG from total glutathione, and were expressed as nmols GSH/mg protein.

3.2.6 Determination of irreversible binding of SMX/SMX-NO to dendritic cells by flow cytometry

Antigen formation on dendritic cells was determined by a modification of Naisbitt *et al.* (1999). Immature dendritic cells were incubated with SMX (0.1-50μM), SMX-NO (50-4000μM) or vehicle for 24 h. Certain populations were fixed and permeabilized using a Leucoperm kit in order to identify intracellular binding. For this, cells were incubated in 100μl of Reagent A (fixation medium; formaldehyde) for 15 min at room temperature, followed by washing and resuspension in 100μl Reagent B (permeabilisation medium) for 30 min at room temperature. The cells were then stained using a rabbit anti-SMX IgG antibody (1:500 in HBSS, 40μl) for 30 min at 4°C, washed repeatedly to remove excess antibody, and incubated with FITC-conjugated goat anti-rabbit IgG (1:50 in HBSS, 40μl) for a further 30 min at 4°C, followed by further washes. Fluorescence was then measured by flow cytometry. Forward and side scatter characteristics were measured simultaneously, the cellular population of interest was gated with the forward scatter threshold being raised to exclude cellular debris, and the median FITC fluorescence was recorded.

3.2.7 Determination of irreversible binding of SMX/SMX-NO to dendritic cells by ELISA

Protein SMX adducts were identified using a previously described enzyme-linked immunosorbent assay (ELISA) protocol (Roychowdhury *et al.*, 2005). Dendritic cells were

incubated with SMX (500μM), SMX-NO (10μM) or vehicle for 0.1 or 24 h, washed repeatedly, resuspended in dH$_2$O and lysed by repeated freeze-thaw cycles. The samples were spun to remove insoluble cell debris at 10,000rpm for 5 min, protein content of supernatants was determined as above (see 3.2.4) and standardised to 250μg/ml. 100μl aliquots were then plated in triplicate on 96-well ELISA (BD Bioscience, Oxford) plates and left to adsorb for 16 h at 4°C. The wells were then washed repeatedly with casein-tris buffer (0.5% casein, 0.9% NaCl, 10mM tris-HCl, 0.01% thimerosal, pH 7.6), blocked for 2 h with casein-tris buffer, and incubated at 4°C with a rabbit anti-SMX antibody (1:500 in casein-tris buffer) for 24 h. The wells were then washed repeatedly with casein-tris buffer and incubated with a goat anti-rabbit alkaline phosphatase linked antibody (1:250) for 16 h. Finally, the wells were washed repeatedly and incubated for 1 h with alkaline phosphatase yellow liquid substrate. The optical density of the wells at 405nm was then determined by spectrophotometric analysis (MRX microplate reader running Revelation version 3.04 software, Dynex Technologies, Billinghurst, W. Sussex). For some experiments 1mM SMX was added to the primary antibody 1 h before addition to the wells to confirm hapten-inhibition of the primary antibody binding. The ELISA protocol does not require cells to be fixed before analysis and thus a possible interaction between SMX and formaldehyde can be excluded.

3.2.8 Design of primers and probes for qRT-PCR analysis of metabolic enzyme expression

Primers and probes for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis were designed for quantification of metabolic enzyme expression in selected cell types. Sequences were identified from AceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html), GnxWeb (GlaxoSmithKline) and Entrez Gene (http://www.ncbi.nlm.nih.gov/HomoloGene), and consensus sequences generated by aligning with LaserGene (DNAStar, Wisconsin, USA). Polymorphisms were identified with GnxWeb, intron-exon boundaries with GeneCruiser (GlaxoSmithKline) and repetitive sequences with RepeatMasker (ftp://ftp.genome.washington.edu/cgi-bin/RepeatMasker). The target complementary DNA (cDNA)

and exon were then entered into BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and Easy Blast 2 (GlaxoSmithKline) to identify any competing targets.

Potential single-exon primers and probes were designed from the chosen exon by Primer Express (Applied Biosystems, California, USA), and selected according to the following criteria:

For probes,

- G-C content between 30-80%

- Avoid runs of identical nucleotide

- Avoid guanine at 5' end

- Ensure C>G

- Tm between 68-70°C

For primers,

- Design as close to probe as possible

- G-C content between 30-80%

- Avoid runs of identical nucleotide

- Tm between 58-60°C

- The final five nucleotides at 3' end should have no more than 2 G/C bases

- Amplicons should be 50-150bp in length

The selected primer/probe sets were then inserted into BLAST and Easy Blast 2 again to confirm the lack of competing targets. The sensitivity and specificity of the primer/probe sets was determined experimentally by running a polymerase chain reaction (PCR) with genomic DNA and analysing the PCR product on an agarose gel

The primer/probe sets selected are listed below (see table 3.2).

Table 3.2: Primer/probe sets used for qRT-PCR analysis of metabolic enzyme expression

Gene	Forward primer	Reverse primer	Probe
CYP1A2	AGCACGCCCGCTGTGA	GGTGTCTTCTTCAGTTGATGGAGAA	5'-FAM-CATGTCCAGGCGCGGCTGC-TAMRA-3'
CYP1B1	CCAGCTTTGTGCCTGTCACTAT	GGGAATGTGGTAGCCCAAGA	5'-FAM-CTCATGCCACCACTGCCAACACCT-TAMRA-3'
CYP2A6	CTTCTGGTGGCCTTGCTGGT	AGCTGCAGGTAGTTTCCAATGAA	5'-FAM-AGCTGCCTCCGGGACCCACC-TAMRA-3'
CYP2B6	TCCCCGCCTCTGTAGACAAT	CTGGCTTGTAGCAGGTCTCTCA	5'-FAM-CTCTGACTCCCCGCAACTTCCT-TAMRA-3'
CYP2C8	TCTGAAGAATGCTAGCCCATCTG	TGGGAATATCCTTGATAAAAAAGA	5'-FAM-CTGCCGATCTGCTATCACCTGC-TAMRA-3'
CYP2C9	ATCTCACATTTTCCCTTCCCTGAA	GCAACTGTTACAGAGTATGGAGAATAGCA	5'-FAM-TCGACCTCCATTACGGAGAGTTTCCTATGTTTCA-TAMRA-3'
CYP2C19	GTCCCTGCAGCTCTCTTTCCT	GGGAAGGGAAAATGTGAGATGA	5'-FAM-TGGTCCAAATTTCACTATCTGTGATGCTTCTTCT-TAMRA-3'
CYP2D6	CACCATCCCGGCAGAGAA	TGAGAGCAGCTTCAATGATGAGA	5'-FAM-AGGTCAGCCACCACTATGCGCAGG-TAMRA-3'
CYP2E1	GTGCCATTTTGCAGCATTTTAA	CAATATGTATAGGGCTGAGGTCGAT	5'-FAM-TTGAAGCCTCTCGTTGACCCAAAGGA-TAMRA-3'
CYP3A4	TCTGGTGTTCTCAGGCACAGA	CAACCAGAAAAACCCGTTGTTC	5'-FAM-CGGTGCCATCCCTTGACTCAACCT-TAMRA-3'
CYP3A5	TTTATTGACTCCGTTGAAATCTCTGGTGTT	AAACCCATTGTTCTAAAGGTGGAT	5'-FAM-TGGTCTTCAAGAAAGCTGTGCCCCA-TAMRA-3'
CYP3A7	AAAGGCTGAGTCAAGGGATGAG	AGCTTTCTTAAAGAGCAAACCAGAA	5'-FAM-CCGTAAGTGGAGCCTGATTTCCCTAAGGA-TAMRA-3'
CYP4A11	GTAAATTGTGTGCTTAATGCAACAGT	GCCCCGAGCTCTGGAAGT	5'-FAM-CACCACCTGTTTCTTTGTTTGATCACCAATAAA-TAMRA-3'
MPO	TGCATTGAACCTGGCTTCCT	ATACCCCTCACTGCTGCACC	5'-FAM-CCTTACCTGGCCTCTAGGAGGCTTCCCT-TAMRA-3'
COX-2	ACATCTTCTGTAACAGAAGTCAGTACTCCT	TCCAAACTTAACAGCAACAGCAA	5'-FAM-AAGTCTTCACAAGTATGACTCCTTTCTCCGCAA-TAMRA-3'
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG	5'-FAM-ACCACAGTCCATGCCATCACTGCCA-TAMRA-3'

3.2.9 qRT-PCR analysis of metabolic enzyme expression

Expression of a panel of metabolic enzymes (CYP1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, 4A11, MPO, COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured by qRT-PCR. Firstly, total ribonucleic acid (RNA) was extracted from dendritic cells (n=10) generated as described above (2.2.3), PBMCs (n=10) generated as described above (2.2.2), and human hepatocytes (n=8) using a Qiagen RNeasy 96 kit. Genomic deoxyribonucleic acid (DNA) contamination was removed with DNAse treatment with 0.5U DNAse I in a thermocycler (Eppendorf, Cambridge), at 37°C for 10 min followed by 75°C for 5 min. Total RNA was quantified by fluorescent measurement with RiboGreen (excitation 480nm / emission 520nm). cDNA was synthesised with DNAse treated RNA as a template using Superscript II bulk mix according to the manufacturer's protocol. qRT-PCR was performed on the resulting cDNA in a 96-well optical reaction plate on an ABI Prism 7900 Sequence Detection System (Perkin-Elmer-Applied Biosystems). The reaction conditions were as follows: 2 min at 50°C, 15 min at 95°C, and 40 PCR cycles of 15 s at 95°C and 1 min at 60°C. Standard curves of human genomic DNA were set-up under identical conditions for quantitative purposes, and cycles to threshold (C_T) values used to derive absolute copy numbers, which were standardised by total RNA content.

3.2.10 *In vitro* metabolism of sulfamethoxazole

Cytochrome P450 (CYP)-expressing Supersomes (CYP1B1/2B6/2C8/2C9/2D6/3A4; 20pmols P450) were incubated in 50mM Tris buffer (pH 7.4) with SMX (800μM), MgCl (3.3mM), ascorbic acid (1mM), and NADPH (1mM) for 30 min. Alternatively, some incubations were set up with SMX (800μM), myeloperoxidase (MPO; 1.56U/ml) and 1mM ascorbic acid with H_2O_2 (200μM). Reactions were terminated and protein was precipitated by addition of 1ml ice-cold acetonitrile. The supernatant was analysed for SMX-NHOH formation by HPLC analysis as described below (4.2.4, condition 4). In certain experiments 1mM 1-aminobenzotriazole (ABT) was preincubated with the enzyme and cofactors for 1 h prior to addition of the drug and additional cofactors.

3.2.11 Time-dependent enzyme inhibition by 1-aminobenzotriazole

E. Coli bactosomes expressing CYP enzymes (25μg protein) were incubated with fluorescent substrates (ethoxyresorufin [50μM; CYP1A2], 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid [12.5mM; CYP2C9], 3-butyryl-7-methoxycoumarin [2.5mM; CYP2C19], 4-methylaminomethyl-7-methoxycoumarin [2.5mM; CYP2D6], 7-benzoquinoline [100μM; CYP3A4] and diethoxyfluorescein [2.5mM; CYP3A4]) and ABT (0-1000μM). ABT-free incubations were used as negative controls. Plates were incubated at 37°C for 5 min and 25μl of an NADPH-generating system (5.53mM glucose-6-phosphate; 0.44mM oxidised nicotinamide adenine dinucleotide phosphate (NADP); 1.2 U/ml glucose-6-phosphate dehydrogenase) was added to initiate the reaction. Fluorescence was measured at 5 min intervals for 60 min on a Cytofluor Series 4000 Plate Reader running Cytofluor v4.2 software (Applied Biosystems, Warrington). Initial blanks were subtracted from data, and inhibition was calculated as a % decrease from drug-free CYP activity.

3.2.12 Enzyme inhibition of dendritic cell activation

Immature dendritic cells were generated as described previously (2.2.3), incubated with ABT (1mM) or vehicle (DMSO; final concentration < 0.5%) for 1 h, and exposed to SMX (25-2000μM), SMX-NO (5-10μM), or LPS (*E.Coli* 0111; 1μg/ml) for 24 h. Cells were then washed and incubated with FITC-labelled mouse anti-human CD40, CD80, CD83 or CD86 antibodies for 30 min at 4°C. Cells were washed repeatedly and fluorescence was measured by flow cytometry.

3.2.13 Statistical analysis

Results are presented as mean ± standard error of the mean. Normality of data was determined by the Shapiro-Wilks test and equality of variance by the Levene test. Data which were normally distributed were compared by means of a t-test, whereas nonparametric data were compared with the Mann-Whitney test for unpaired data or the Wilcoxon signed rank test for paired data. P-values < 0.05 were considered to be significant. All statistical analyses were performed on SPSS 13.0.

3.3 Results

3.3.1 Dendritic cell death following SMX/SMX-NO exposure

Initially, cell populations were counted, and cell recovery was determined to be consistently > 95%. Proportions of necrotic and apoptotic cells were then determined using an annexin-V / propidium iodide staining methodology. There was no significant necrotic or apoptotic cell death when dendritic cells were incubated with SMX at any concentration below 4mM, at which point the limited solubility became a problem, whereas significant quantities of necrotic cells were observed with SMX-NO only at concentrations above 500μM (see figure 3.1). No increase in apoptotic cell death was observed at any concentration.

3.3.2 Depletion of glutathione in dendritic cells following SMX/SMX-NO exposure

GSSG levels were consistently at or below the level of detection, so total glutathione has been used as a surrogate for GSH concentrations. No significant GSH depletion was seen with SMX at any concentration studied (data not shown), whilst significant dendritic cell GSH depletion was seen with SMX-NO only at concentrations of 500μM or higher (see figure 3.2.)

3.3.3 Detection of SMX-adducts in dendritic cells following SMX/SMX-NO exposure

Incubation of dendritic cells for 24 h with SMX-NO (0.5-50μM), but not SMX (50-4000), resulted in the formation of surface adducts, as determined by flow cytometry (see figure 3.3.)

Dendritic cells were then permeabilized in order to identify total (i.e. both intracellular and surface) SMX adducts by flow cytometry. As before, significant adduct formation was seen in SMX-NO-treated dendritic cells (10μM). In addition, however, SMX-treated (50-4000μM) dendritic cells also displayed significant formation of SMX adducts at concentrations above 100μM (see figure 3.4.
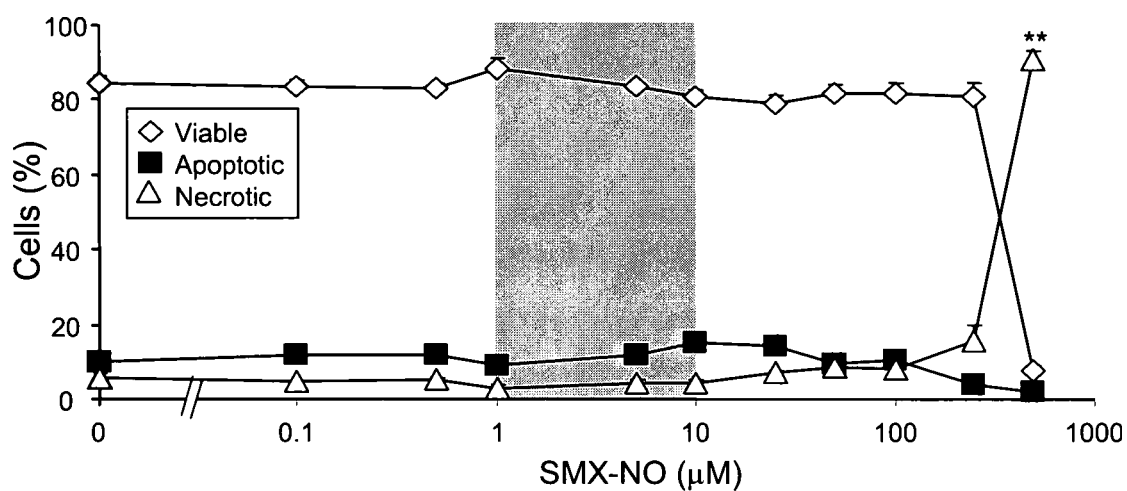
Figure 3.1: Apoptotic and necrotic dendritic cell death in response to increasing concentrations of SMX-NO. The gray region corresponds to the range of concentrations associated with dendritic cell activation. Data are presented as mean ± s.e.m. **: significantly different to control p<0.01
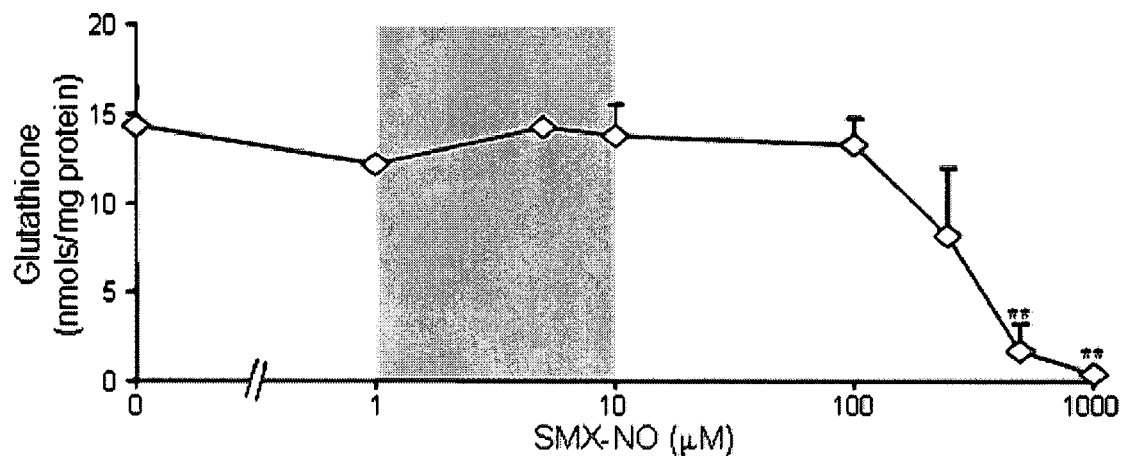
Figure 3.2: Depletion of GSH by increasing concentrations of SMX-NO. The gray region corresponds to the range of concentrations associated with dendritic cell activation. Data are presented as mean ± s.e.m. **: significantly different to control p<0.01
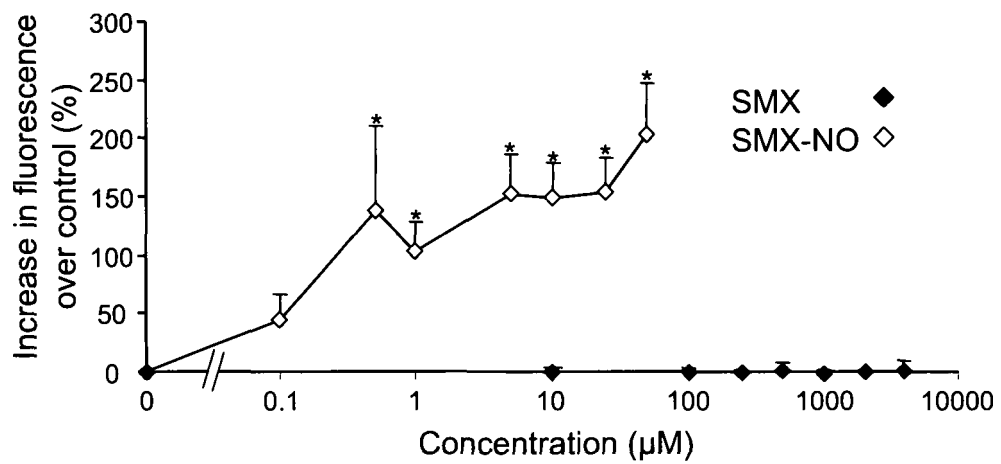
Figure 3.3: Cell surface covalent binding seen following incubation with SMX-NO, but not with SMX. Data are presented as mean ± s.e.m. *: significantly different to control p<0.05
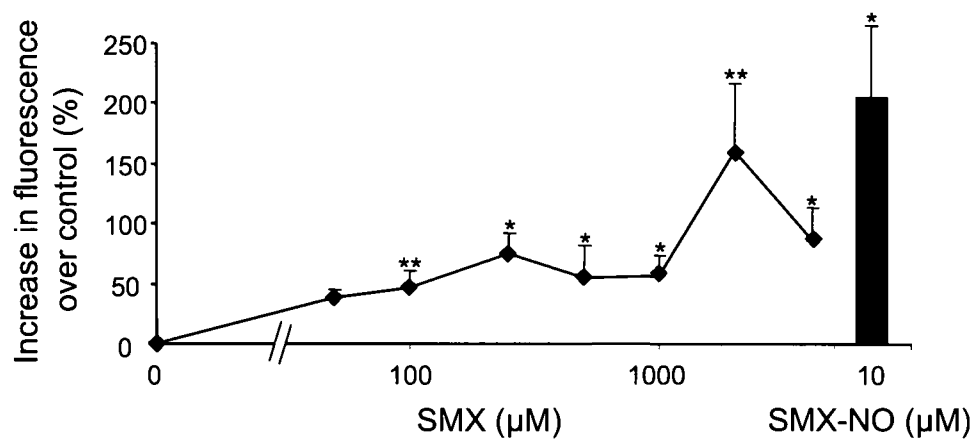
Figure 3.4: Total adduct formation seen following incubation with SMX-NO, but not with SMX, as determined by flow cytometry following permeabilisation. Data are presented as mean ± s.e.m. */**: significantly different to control p<0.05/0.01

Additionally, protein-SMX adducts could be identified using an ELISA methodology following either SMX-NO (100μM) incubation for 0.1 h or 24 h, and SMX (500μM) incubation for 24 h (see figure 3.5). Importantly, incubation of SMX (500μM) for 0.1 h did not led to apparent adduct formation. The addition of 1mM SMX to the primary antibody (to saturate antibody binding sites) prior to its addition to the wells completely abrogated any binding to adsorbed protein, demonstrating that the binding was SMX-specific (data not shown).

3.3.4 *In vitro* metabolism of sulfamethoxazole

Several metabolic enzymes were investigated for their ability to bio-activate SMX *in vitro*. The cytochrome P450s 1B1, 2B6, 2D6, and 3A4, and COX-2 were all incapable of catalysing SMX N-hydroxylation. In contrast, CYP2C8, CYP2C9 and MPO all catalysed the reaction, with relative activities CYP2C9>MPO>CYP2C8 (see table 3.3)

Table 3.3: Selected enzyme expression and SMX metabolism

Enzyme	Expression (copies/ng mRNA)			SMX-NHOH formation (nmols/ml)	
	Dendritic cells	PBMCs	Hepatocytes	- ABT	+ ABT
CYP1B1	591.1±82***	11.62±1.7	10.19±3.99	ND	ND
CYP2B6	0.04±0.02	5.98±0.83	67.23±18.2	ND	ND
CYP2C8	0.39±0.06	12.58±1.8	2465±381	0.308	ND
CYP2C9	0.30±0.20	12.48±1.2	7400±1600	0.780	ND
CYP2D6	0.93±0.11	6.79±1.3	39.10±13	ND	ND
CYP3A4	ND	0.45±0.21	3367±1060	ND	ND
MPO	9.73±3.2***	0.13±0.12	ND	0.394	ND
COX-2	0.08±0.03	23.11±2.6	1.75±1.28	ND	ND

*** : significantly different to expression in PBMCs/hepatocytes p<0.001; ND: Not detected.

3.3.5 Expression of metabolic enzymes in dendritic cells, hepatocytes, and PBMCs

qRT-PCR analysis of dendritic cell mRNA from 10 healthy donors was performed to quantify the expression of several metabolic enzymes, and this was compared to
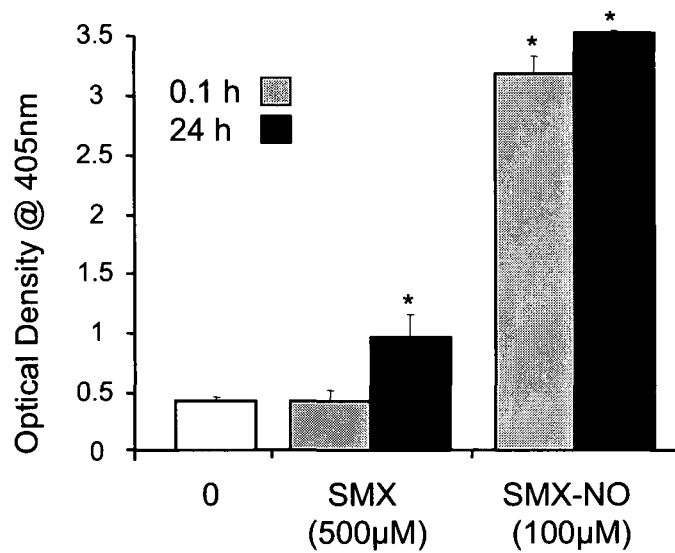
Figure 3.5: Protein-SMX adducts detectable in dendritic cell lysate following 0.1 or 24 h incubation with SMX-NO ($100\mu M$) or 24 h incubation with SMX ($500\mu M$). Data are presented as mean $\pm$ s.e.m. *: significantly different to control $p<0.05$

expression in both PBMCs from the same individuals and commercially available human hepatocytes from 8 donors (see table 3.3; figure 3.6).

As expected, CYP expression was high in human hepatocytes, and considerably lower in both PBMCs and dendritic cells. High levels of both CYP1B1 and MPO were detected in dendritic cells compared to both PBMCs (fold differences: 50; 60) and hepatocytes (fold differences: 80; no MPO detected in hepatocytes), but expression of other metabolic enzymes was considerably lower in dendritic cells when compared with either PBMCs (average fold difference: 90) or hepatocytes (average fold difference: 7800). GAPDH expression was comparable between cell types (dendritic cells: 84 copies/ng total RNA; PBMCss: 111 copies/ng total RNA; hepatocytes: 170 copies/ng total RNA).

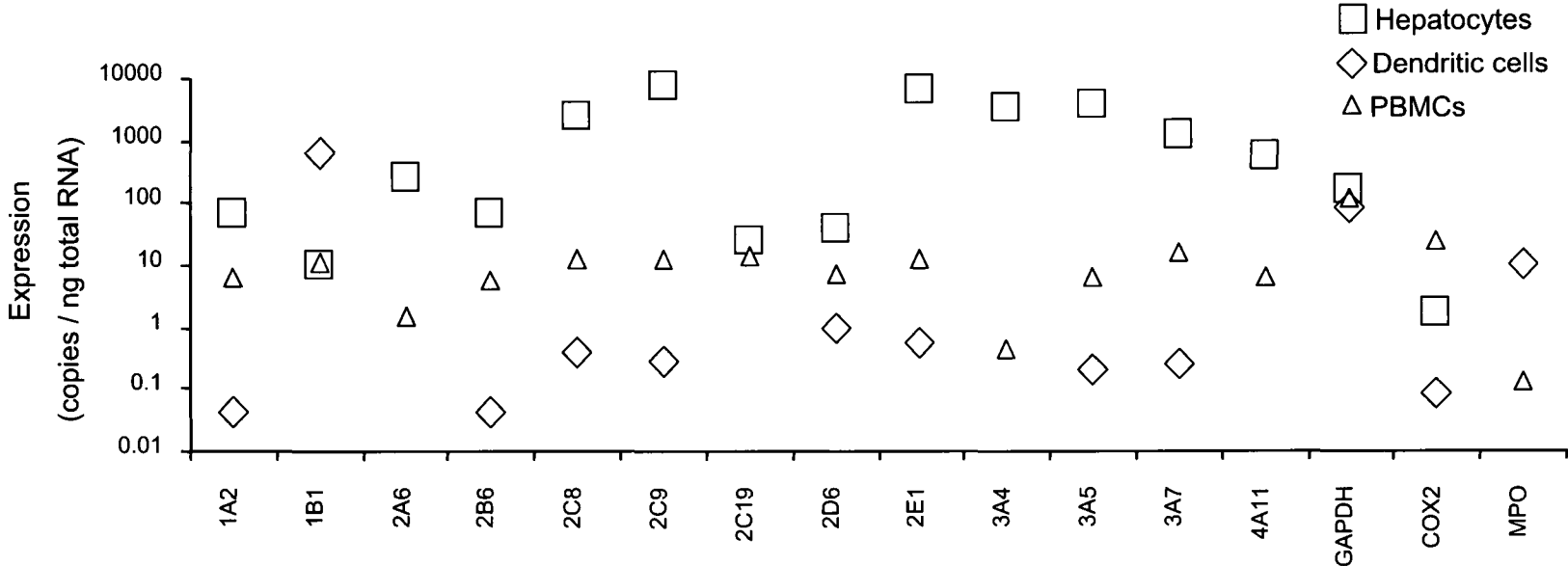Figure 3.6: Dendritic cells express MPO and CYP1B1, although other metabolic enzymes are expressed at low levels. Expression of metabolic enzymes in dendritic cells, PBMCs (both n=10) and human hepatocytes (n=8) was determined by quantitative real-time RT-PCR using cDNA library generated from RNA extracted from immature dendritic cells. The results are presented as mean ± s.e.m copies/ng total RNA.

3.3.6 Time-dependent inhibition of enzyme activity by 1-aminobenzotriazole

Inhibition of enzyme activity by ABT *in vitro* was studied both using a time-dependent inhibition assay with fluorescent probes and by direct inhibition of SMX-NHOH formation as determined by HPLC.

Time-dependent inhibition studies over 1 h demonstrated that 1mM 1-aminobenzotriazole (ABT) was a non-specific time-dependent inhibitor of all CYPs and peroxidases studied (inhibition after 1 h: 98.5-100%; see figure 3.7). Although there was a degree of selectivity (see figure 3.8), all enzymes were significantly inhibited by 1mM ABT following 1 h incubation.

In addition, SMX-NHOH formation catalysed by either MPO, CYP2C8 or CYP2C9 was completely inhibited by 1 h preincubation of the enzymes with 1mM ABT (see 3.3).

3.3.7 Inhibition of dendritic cell activation by 1-aminobenzotriazole

When ABT was incubated with dendritic cells 1 h prior to addition of drugs the response to SMX was completely abrogated, whereas the responses to both SMX-NO and the positive control LPS were unaffected (see figure 3.9).

3.4 Discussion

As discussed in the previous chapter 2, both SMX and its reactive metabolite SMX-NO have been found to induce dendritic cell maturation, as measured by an up-regulation of the co-stimulatory molecule CD40. The concentration of SMX required for maximal effect was found to be ~250-fold greater than that of SMX-NO required for a maximal response (see figure 2.2).

Therefore, it was important to determine whether metabolism by the dendritic cells was an obligatory step in the observed up-regulation of CD40 expression. It is
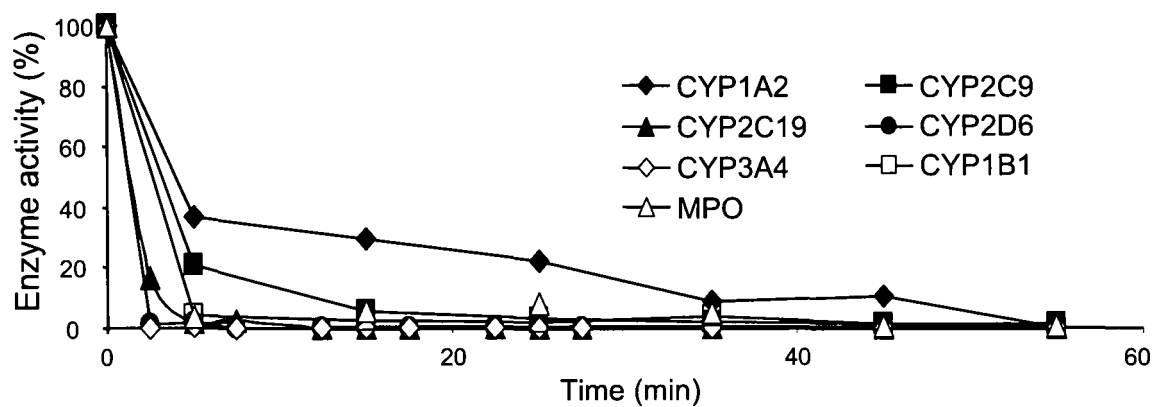
Figure 3.7: ABT acts as a potent inhibitor of a wide range of metabolic enzymes. Time-dependent inhibition of several metabolic enzymes was determined by inhibition by 1mM ABT of fluorogenic substrate turnover by expressed enzymes over 1 h. Results were calculated as the % inhibition at various time points.
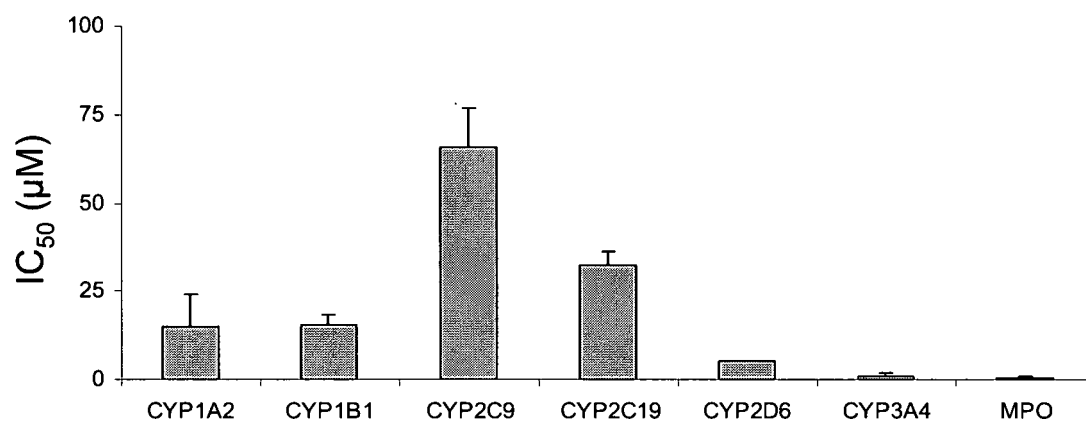
Figure 3.8: ABT shows a degree of enzyme selectivity. Time-dependent inhibition of several metabolic enzymes was determined by inhibition by 0-1000μM ABT of fluorogenic substrate turnover by expressed enzymes after 1 h incubation. Results were calculated as the IC_{50} of ABT for inhibition of specific enzymes.
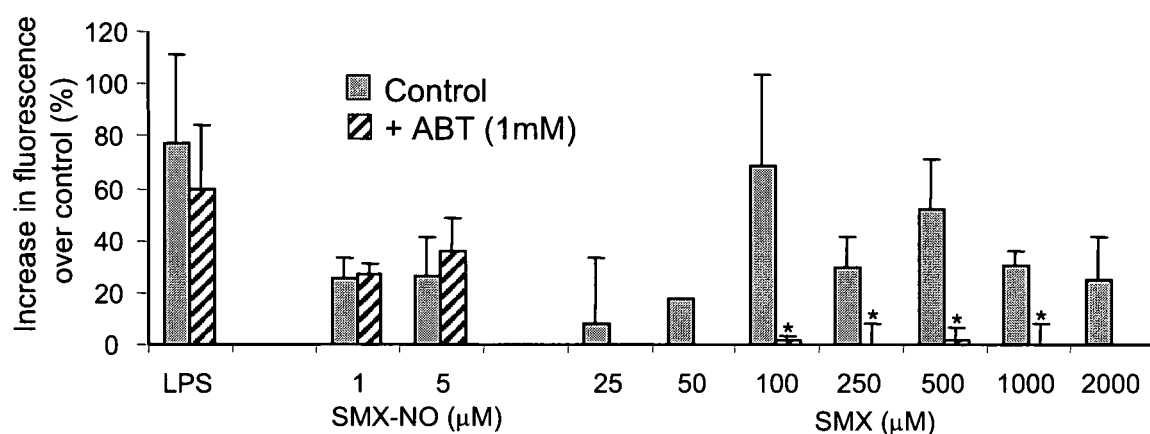
Figure 3.9: ABT inhibits CD40 up-regulation with SMX, but not SMX-NO or LPS. Monocyte-derived dendritic cells were pre-incubated with 1mM ABT or vehicle for 1 h, followed by incubation with SMX or SMX-NO for 24 h (n = 3). CD40 up-regulation was determined by flow cytometric analysis, and is presented as the % increase in median fluorescence over control. Data are presented as mean ± SEM. *, Significantly different from vehicle treated (p < 0.05).

possible that both the compounds in question could stimulate dendritic cell maturation directly. For instance, the increased CD40 expression may be due to direct interaction of SMX itself with receptors found on the surface of dendritic cells such as TLRs. A precedent for this already exists: the imidazoquinoline, imiquimod, has been shown to activate macrophages through TLR7 and thereby induce the production of cytokines (Hemmi et al., 2002). Alongside this direct activation by SMX, SMX-NO could induce maturation by toxicological pathways, for instance producing cell death, GSH depletion or covalent binding.

However, the evidence suggests that it is the intracellular metabolic activation of SMX in dendritic cells that is responsible for the increased CD40 expression. Using a highly specific anti-SMX antibody (Naisbitt et al., 1999; Manchanda et al., 2002; Roychowdhury et al., 2005), haptenated intracellular proteins were detectable by both flow cytometry and ELISA when SMX was incubated with dendritic cells at similar concentrations to those which stimulated increased CD40 expression (see figures 3.4 and 3.5). Importantly, the formation of haptenated proteins was time dependent and not observable immediately after incubation of SMX with cells (see figure 3.5). By contrast, SMX-NO bound rapidly to both intra- and extracellular proteins, consistent with previous work from several laboratories (Manchanda et al., 2002; Naisbitt et al., 1999, 2001, 2002; Reilly et al., 2000; Roychowdhury et al., 2005). A recent study (Roychowdhury et al., 2007) has looked for similar adduct formation in dendritic cells (albeit of a different source to those used in this study) exposed to SMX ($250\mu M$ for 3 h), dapsone and both of their hydroxylamine metabolites. While the authors' conclusions were that dapsone, but not SMX, was metabolised to form adducts, the primary data presented appears to suggest a small degree of binding, though considerably less than that found in this study. However, it is hard to draw comparisons with the present study considering the number of methodological differences: for instance, the concentration of drug, time of exposure, and source of cells all differed significantly.

Because analytical techniques are not sufficiently sensitive to detect dendritic cell-mediated SMX metabolism directly, a series of enzyme inhibitor studies were performed to substantiate a causal relationship between SMX metabolism by dendritic cells and

increased cell surface CD40 expression. ABT, a suicide inhibitor of haemoxygenases, which acts via alkylation of the enzyme-associated haem group by a benzyne radical (Ortiz De Montellado *et al.*, 1984), was shown to inhibit 1) activity of all recombinant metabolic enzymes studied over the course of 1 h (see figure 3.7); 2) metabolism of SMX to SMX-NHOH catalysed by CYP2C8, 2C9 and MPO (see table 3.3); and 3) SMX-induced, but not SMX-NO- or LPS-induced, increased CD40 expression on dendritic cells (see figure 3.9) . Taken together, our observations suggest that increased CD40 expression following exposure of dendritic cells to SMX is dependent on oxidative drug metabolism.

It is possible that the reactive species generated by metabolism of ABT (a highly reactive benzyne radical) binds to and inactivates SMX, and hence inhibits dendritic cell activation by SMX in a manner independent of its enzyme inhibitory activity. However, considering the near complete degree of enzyme inhibition following 1 h preincubation, it seems unlikely that there would still be sufficient enzymatic activity to produce enough benzyne to completely eliminate the response to SMX. It is worth bearing in mind that the range of active concentrations span a whole order of magnitude, and so at least 90% inactivation of SMX would be required at the highest active dose to produce the complete inhibition seen—at a 1:1 SMX-ABT reaction stoichiometry, this would require 90% of the ABT to be converted to benzyne, and all of that to bind to SMX.

There is some degree of variation between different studies with regard to the potency and selectivity of ABT, although much of the variation can be ascribed to differences in the incubation conditions—in particular, variations in the length of preincubation. One study, in which enzymes were not preincubated with ABT prior to the addition of the substrate, found considerable variation, with little or no inhibition of certain enzymes, including CYP2C9 (Emoto *et al.*, 2003). However, a later study by the same group (Emoto *et al.*, 2005) found considerably greater inhibitory potency and reduced selectivity following 30 min preincubation, in line with both the current work and other published studies (Balani *et al.*, 2002).

To explore further the role of metabolism in this process, expression of metabolic

enzymes in dendritic cells was determined, and contrasted with those seen in other cell types (PBMCs and hepatocytes). Dendritic cells expressed the majority of CYPs studied (see figure 3.6), albeit at a low level compared with both PBMCs and hepatocytes. The sole exception was CYP1B1, which was expressed at a significantly higher level than that seen in either alternative cell type. A previous study has looked at CYP expression in dendritic cells (Sieben et al., 1999), but the data presented herein are the first which have both quantified the expression and compared this to other cell types of known metabolic capacity. CYP1B1 is a polymorphic enzyme that plays an important role in the activation of carcinogens (Watanabe et al., 2000). However, our data suggests that it does not catalyse SMX metabolism (see table 3.3) and thus an association between CYP1B1 expression and SMX hypersensitivity seems unlikely. There is a degree of controversy surrounding the expression of MPO in dendritic cells, and it is possible that MPO is differentially expressed depending on the source and phenotype of the dendritic cells in question (Pickl et al., 1996; Scholz et al., 2004). In this study, it was apparent that dendritic cells generated from 10 donors expressed consistently high levels of MPO mRNA (see table 3.3), and that this was significantly higher than that in PBMCs (fold difference: 58.9; p<0.0001). CYP2C9 and MPO, consistent with previous literature (Cribb et al., 1990, 1995; Gill et al., 1996), but also CYP2C8, were found to be capable of SMX-NHOH generation (see table 3.3). All other enzymes studied were inactive. Based on this, and the CYP expression data, it seems plausible that MPO is, at least in part, responsible for the SMX metabolic activation observed in dendritic cells. Additionally, dendritic cells expressed low levels of COX-2; however, in contrast to early reports, COX-2 has recently been shown not to play a direct role in SMX oxidation (Vyas et al., 2006c). However, it is important to bear in mind that there are several enzymes which may be able to metabolise SMX for which we did not attempt to determine the expression levels in our cell types, not least FMO-3 (Vyas et al., 2006b) and several peroxidases, such as lactoperoxidase or thyroid peroxidase. Finally, it is well-known that the presence of the mRNA message for a given protein does not automatically allow the expression of the protein to be deduced. Further work is currently underway in order to identify the presence of MPO

protein in dendritic cells, as opposed to simply detecting the presence of MPO mRNA.

MPO has previously been linked to several idiosyncratic reactions as a result of its ability to locally bioactivate drugs within the immune system. In particular, several drugs which are associated with agranulocytosis, a deficiency of granulocytes within the blood, have been found to be metabolised to reactive species by MPO, including SMX (Cribb et al., 1990), clozapine (Williams et al., 1997), ticlopidine (Liu & Uetrecht, 2000), amodiaquine (Naisbitt et al., 1997), and indomethacin (Ju & Uetrecht, 1998). However, these are likely to be due to direct toxicity within the neutrophil rather than immunologically driven (Williams et al., 2000). It is known that MPO is typically stored in an inactive form within the cell, and is only activated upon cellular activation. It is possible therefore, that localised bioactivation and the resultant dendritic cell activation is dependent on a certain background level of cellular activation. Further work is underway in order to test this, and to explore the possibility that further activation of the cells enhances the degree of turnover within the system. It is not known whether MPO expressed by dendritic cells is released through degranulation, in the same manner as that produced by neutrophils. If so, it seems possible that SMX turnover in this system may have been initially produced extracellularly. When dendritic cells were treated with SMX, no surface adducts were detectable by flow cytometry, suggesting that SMX-NO haptenation was largely confined to the intracellular milieu, although the relative stability of SMX-NO would be expected to allow it to migrate across cell membranes. It might be instructive to examine the degree of modification of extracellular serum proteins in order to fully explore this issue.

There are several possible hypotheses by which reactive drug metabolites could induce increased CD40 expression on dendritic cells, which will be addressed sequentially. Firstly, release of cell contents from both necrotic (Barker et al., 2002; Sauter et al., 2000) and apoptotic (Janssen et al., 2006) cells stimulates dendritic cell activation. Indeed, in a recent study it was demonstrated that dendritic cell activation in response to contact sensitisers is dependent on low levels (~10-20%) of necrotic cell death (Hulette et al., 2005), although this has been challenged (Coulter et al., 2006). However, in the present work, there was no significant increase in necrotic or apoptotic cell death

observed with SMX at any concentration tested, and for SMX-NO only at concentrations that were ~500-fold higher than required for increased CD40 expression. This is comparable to the concentrations of SMX-NO found to be toxic *in vitro* to a variety of immune-derived cell types by previous investigators (Lavergne *et al.*, 2006; Naisbitt *et al.*, 2002).

Secondly, depletion of glutathione has also been shown to be associated with dendritic cell maturation, particularly increased expression of co-stimulatory receptors (Verhasselt *et al.*, 1999; Yamada *et al.*, 2006). However, depletion of glutathione was only seen at concentrations of SMX-NO ~200-fold higher than required for increased CD40 expression, and was not seen at all with SMX. Additionally, the level of oxidised glutathione was below the limit of detection at all times, and no increase in oxidised glutathione was observed.

Thirdly, it is possible that the association is due to the generation of reactive oxygen species. This hypothesis was not explicitly tested, although previous studies have found that low concentrations of SMX-NHOH produce detectable quantities of reactive oxygen species (Vyas *et al.*, 2005). However, considering the evidence that reactive oxygen species generation is a requisite event in the activation of dendritic cells (Matsue *et al.*, 2003), it would be very hard to be certain that any reactive oxygen species identified in dendritic cells exposed to either SMX or SMX-NO were causative rather than secondary to the dendritic cell activation. For this reason, and due to time constraints, it was decided not to investigate this possibility further.

Finally, covalent binding to intracellular macromolecules could potentially generate danger signals for the induction of dendritic cell maturation. As discussed above, SMX adducts were detected by means of different immunochemical methodologies (flow cytometry, ELISA) and that this binding occurred with concentrations of SMX and SMX-NO that were associated with dendritic cell activation. Again, this is in line with findings from previous investigators, which found that covalent binding can be detected with significantly lower concentrations of SMX-NO than that which produces toxicity (Manchanda *et al.*, 2002; Naisbitt *et al.*, 2002).

In conclusion, these data reveal that activation of dendritic cells by SMX is sec-

ondary to local bioactivation of SMX to SMX-NO, at least in part through MPO, and that both compounds appear to transduce their effects through covalent binding to cellular macromolecules. The site and nature of SMX metabolite modification, and indeed whether specific proteins are modified, has yet to be deciphered.

Chapter 4

Non-enzymatic Generation of Hydroxy-Sulfamethoxazole in Microsomal and Peroxide Incubations

Contents

4.1 Introduction

The antimicrobial SMX is associated with a relatively high risk of immune-mediated hypersensitivity reactions (see 1.3.8.3; 2.1). This is believed to be, although defini-

tive proof is lacking, due to the enzymatic generation of the hydroxylamine metabolite (SMX-NHOH), and subsequent auto-oxidation to a protein-reactive nitroso species SMX-NO (Cribb *et al.*, 1991). The cellular distribution (Naisbitt *et al.*, 1999), toxicity (Lavergne *et al.*, 2006) and immunogenicity (Naisbitt *et al.*, 2001) of SMX metabolites have been well-studied.

SMX-NHOH generation has been found both *in vitro* (Cribb & Spielberg, 1990; Cribb *et al.*, 1995) and *in vivo* (Cribb & Spielberg, 1992; Gill *et al.*, 1996). Several enzymes have been demonstrated to bioactivate SMX to SMX-NHOH, including CYP2C9 (Cribb *et al.*, 1995) and MPO (Cribb *et al.*, 1990, see also 3.3.4). Although it was believed that COX could also activate SMX, a recent study has claimed that this is actually secondary to hydrogen peroxide (H_2O_2) in the reaction mixture (Vyas *et al.*, 2006c). Importantly, however, most studies in this area use co-chromatography with an authentic standard using relatively simple HPLC conditions as a means of identifying SMX-NHOH formation.

In this study we identified a novel non-enzymatically formed hydroxylated SMX derivative, investigated the mechanism of formation, and determined the minimum bioanalytical requirements for accurate SMX-NHOH identification.

4.2 Materials and methods

4.2.1 Materials

SMX-NHOH was synthesised as described previously (2.2.1.2), and its purity was confirmed by NMR and LC/MS to be >99%. Unless specified otherwise, all other reagents were purchased from Sigma-Aldrich Ltd. (Gillingham, UK).

4.2.2 Microsomal oxidation of sulfamethoxazole

Human liver microsomes were incubated in tris buffer (50mM; pH 7.4) with SMX (800μM), ascorbate (1mM), MgCl (3.3mM) and NADPH (1mM) at 37°C. Additional reactions were set up with either desferrioxamine (25mM) or mannitol (0.5M). Control incubations contained SMX in the absence of either ascorbate or H_2O_2, or with boiled microsomes (30 min at 95°C). Following 1 h incubations the metabolites were

extracted, separated by HPLC using one of the conditions below, identified by MS-MS, and quantified by UV detection.

4.2.3 H_2O_2 oxidation of sulfamethoxazole

SMX ($800\mu M$), ascorbate (1mM), and H_2O_2 (1mM) in tris buffer (50mM; pH 8.00) were incubated at 37°C for 1 h. Additional incubations contained either desferrioxamine (25mM), mannitol (0.5M), or $FeSO_4$ (100mM) and ethylenediamine tetraacetic acid (EDTA) (100mM). Control incubations contained SMX in the absence of either ascorbate or H_2O_2. Metabolites were extracted and separated by HPLC using one of the conditions given below, identified by MS-MS, and quantified by UV detection.

4.2.4 HPLC conditions

Different protocols were used to attempt to separate the hydroxylated species from an authentic SMX-NHOH standard, both to try to identify suitable conditions for analysis and to determine if any previous studies may have failed to adequately separate the two compounds. These conditions are listed below, and numbered sequentially.

1. As described in Gill *et al.* (1996), samples were eluted from a Prodigy 5 ODS 2 column (150 x 4.6mm; Phenomenex) using an isocratic mobile phase of glacial acetic acid (1% v:v) and acetonitrile (20% v:v) in dH_2O at a flow rate of 1 ml.min^{-1}.

2. As described in Vyas *et al.* (2006c), samples were eluted from a Nova-pak $4\mu m$ C-18 column (150 x 3.9mm; Waters) using a isocratic mobile phase of acetonitrile (18.95% v:v), glacial acetic acid (1% v:v), and triethylamine (0.05% v:v) in dH_2O at a flow rate of 1 ml.min^{-1}.

3. As described in Cribb & Spielberg (1990), samples were eluted from an Ultrasphere C18 ODS $5\mu m$ column (150 x 4.6mm; Beckman Instruments) using an isocratic mobile phase of acetonitrile (25% v:v), glacial acetic acid (1% v:v) and triethylamine (0.05% v:v) in dH_2O at a flow rate of 1 ml.min^{-1}.

Furthermore, an additional method was developed in order to ensure complete resolution of the hydroxylated SMX derivatives:

4 Samples were loaded onto an Ultrasphere C18 ODS 5μm column (250 x 4.6mm; Beckman Instruments) using 10% acetonitrile in 50mM formic acid and washed for 5 minutes at a flow rate of 1ml.min^{-1}. The acetonitrile content was then increased linearly from 10-70% over 20 min and from 70-100% over 2 min. The column was re-equilibrated with the starting buffer for 10 min prior to any further runs.

4.2.5   UV detection

A BioTek 560 UV detector (Sci-Tek, Bucks, UK) was used to determine UV absorbance at 254μm. Metabolite quantification was performed by comparison to an internal standard of 10μM sulfadimethoxine.

4.2.6   Mass spectrometric analysis

A Deca-XP^{Plus} ion-trap mass spectrometer (ThermoFinnigan, Hemel Hempsted, UK) was used to detect SMX and its metabolites via negative ion electrospray ionisation. Data dependant MS-MS experiments provided collision-induced dissociation spectra of the fragment ions of the compounds of interest.

4.3   Results and Discussion

4.3.1   Identification of the novel SMX-OH derivative

Although standard microsomal incubations of SMX with the continuous presence of ascorbate produced a single peak corresponding to a hydroxylated metabolite ([M-H]$^-$ m/z:268), this peak was also present, albeit reduced, in control incubations without NADPH. Further investigations found that although the peak present in these incubations coeluted with an SMX-NHOH authentic standard (see figure 4.1A) under previously used HPLC conditions (condition 1; see 4.2.4), it had a distinct fragmentation pattern under MS-MS (see figure 4.2 for all fragment allocations), lacking the

characteristic neutral loss of 17 seen with SMX-NHOH. In all other respects it resembled SMX-NHOH. Unlike the previously identified metabolite 5-OH SMX (Vree *et al.*, 1994), the neutral loss of 96 (loss of the methylisoxazole ring) was unchanged from the parent SMX molecule, demonstrating that the hydroxylation must be on the benzene ring, although the precise location was not determinable. Changes to the chromatography conditions (condition 4) produced good separation of the novel compound (hereafter referred to as SMX-OH) from an SMX-NHOH standard (see figure 4.1B). The continued presence of SMX-OH in microsomal incubations both without NADPH and in incubations using boiled microsomes demonstrates that it is generated non-enzymatically.
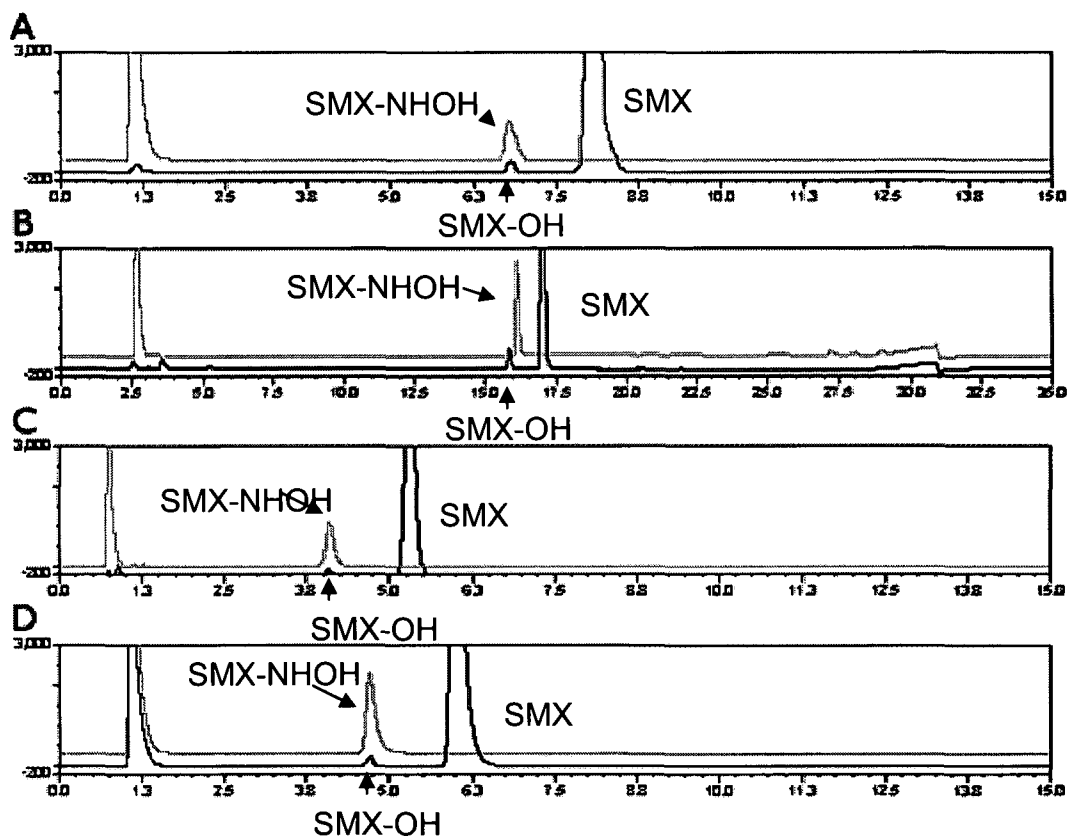
Figure 4.1: (A) SMX-OH generated by addition of ascorbate following termination of a microsomal incubation (grey trace) co-elutes with an SMX-NHOH authentic standard (black trace) under previously used HPLC conditions (condition 1); (B) SMX-OH generated by addition of ascorbate following termination of microsomal incubation (grey trace) separated effectively from a SMX-NHOH standard (black trace) by novel chromatographic conditions (condition 4); (C,D) SMX-OH (grey trace) and SMX-NHOH (black trace) display chromatographic similarities under two previously used HPLC conditions (conditions 2 and 3). All traces are representative of 3 independent experiments.

Figure 4.2: HPLC-MS-MS spectra of SMX, SMX-NHOH and SMX-OH with diagnostic fragment ions

4.3.2 Investigation of the mechanism of SMX-OH formation

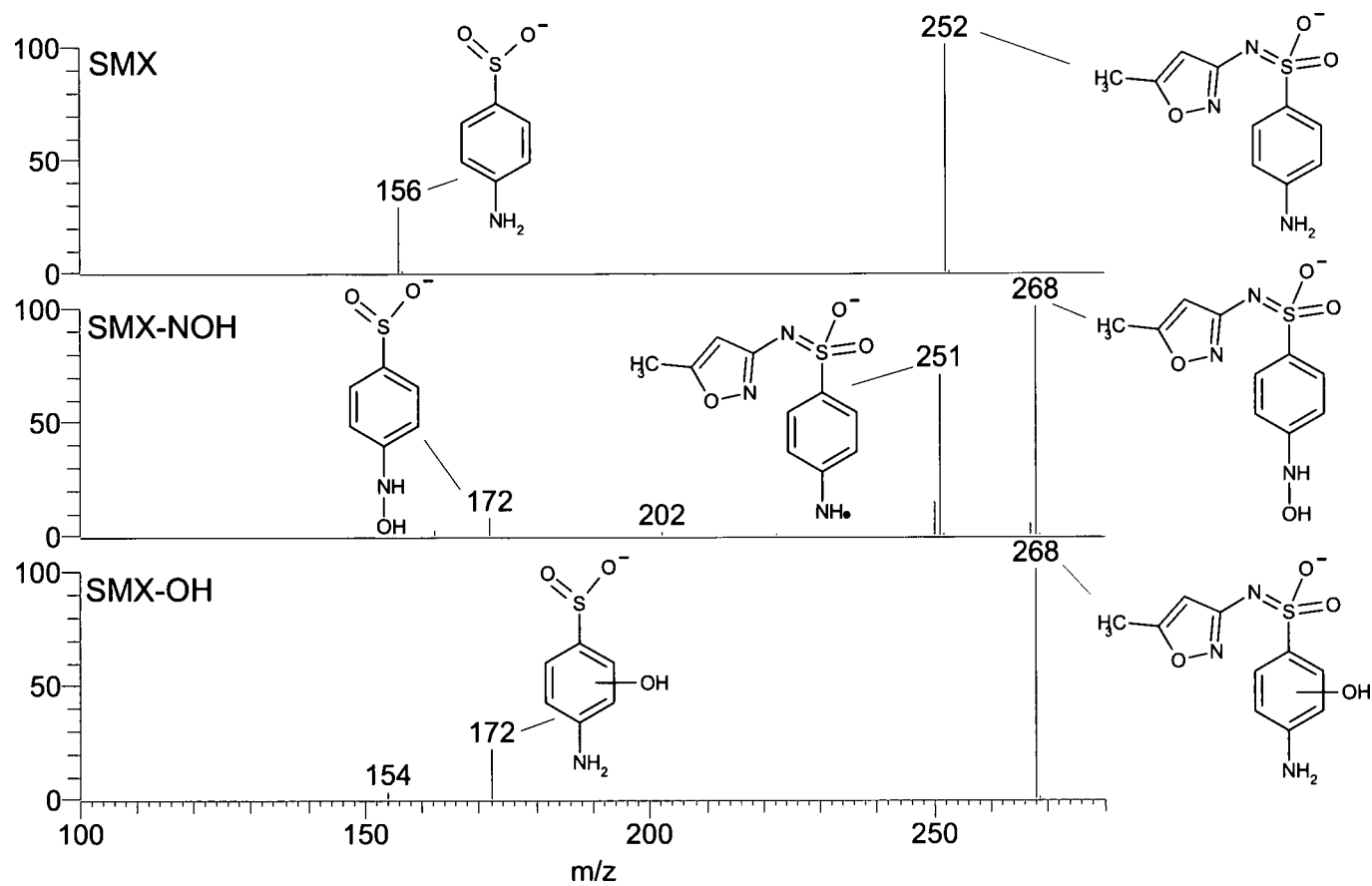Further investigations found that an incubation of SMX with H_2O_2 and ascorbate in tris buffer generated a single hydroxylated metabolite with an MS-MS fragmentation pattern identical to SMX-OH. Contrary to previous reports (Vyas *et al.*, 2006c), when SMX was incubated with H_2O_2 and ascorbate, SMX-NHOH was not detected. This non-enzymatic reaction was further investigated. In initial experiments, the kinetics of SMX-OH formation were investigated in an SMX/H_2O_2/ascorbate incubation. As appreciable quantities were detected within 0.1 h, it was not possible to determine the minimum required time for formation. Maximal quantities of SMX-OH were found following 1.5 h, which stayed stable for at least 4 h (see figure 4.3). Incubations with either H_2O_2 or microsomes only generated SMX-OH in the presence of ascorbate. Furthermore, the addition of the iron chelator desferrioxamine completely inhibited the generation of SMX-OH, whereas the hydroxyl radical scavenger mannitol had no effect, even at high concentrations. Addition of $FeSO_4$ and EDTA to the peroxide reaction increased the amount of SMX-OH formed over 1 h (see table 4.1). These data are consistent with the synthesis being via an Fe^{2+}/ascorbate/O_2 oxidising complex, as first described by Udenfriend *et al.* (1954), rather than via hydroxyl radical generation from Fenton reactions. Although there have been conflicting reports regarding the role of hydroxyl radicals in this reaction (Hamilton, 1962; Ito *et al.*, 1993), the lack of inhibition following mannitol addition suggested no role for radical involvement here.

Table 4.1: Generation of SMX-OH under varying conditions.

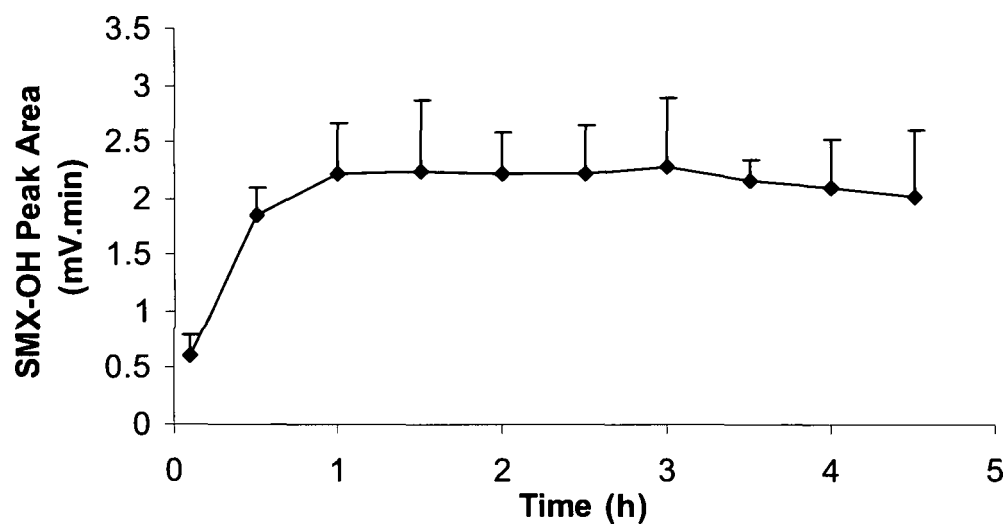|	Condition	SMX-OH generation[*]
1	SMX (800μM)	0
2	1 + H_2O_2 (100μM)	0
3	2 + ascorbate (100μM)	0.262
4	3 + Fe^{2+} (100mM) + EDTA (100mM)	1.258
5	3 + mannitol (0.5M)	0.283
6	3 + desferrioxamine (25mM)	0.053

[*] Calculated as ratio of SMX-OH: sulfadimethoxine peak area

Figure 4.3: Time-course of SMX-OH formation in an SMX/H_2O_2/ascorbate incubation

4.3.3 Validation of previously used analytical techniques

With a very few exceptions (Cribb & Spielberg, 1990; Cribb *et al.*, 1990), all previous studies to have quantified SMX-NHOH formation in microsomal or peroxidase systems, or in clinical samples, have used HPLC separation with either UV or MS detection, and identified SMX-NHOH by co-chromatography with an absolute standard. We tested three sets of previously-used conditions, designated 1, 2 and 3, for their ability to separate SMX-OH from SMX-NHOH, but all three systems were not suitable (see figure 4.1). Furthermore, the use of UV detection is clearly insufficient for accurate metabolite verification, given the chromatographic similarity of SMX-NHOH to SMX-OH. The minimum requirements for accurate identification and quantification should be confirmed separation from SMX-OH, and either MS-MS fragmentation analysis or conversion to $SMX-NO_2$ by addition of excess base (Rieder *et al.*, 1988). While there is no question that the major oxidative metabolite in microsomal incubations is SMX-NHOH, previous studies have insufficiently separated this from the non-enzymatically generated SMX-OH, and as a result may not have accurately identified or quantified the generation of SMX-NHOH. These data have major implications for designing future analyses of SMX metabolite formation.

Chapter 5

Activation of T-cells by Carbamazepine and Stable Carbamazepine Metabolites

Contents

5.1 Introduction

CBZ is a commonly used anti-epileptic which has been associated with the induction of hypersensitivity reactions. These typically involve rash, eosinophilia, fever and systemic organ involvement, most commonly hepatitis. Several lines of evidence indicate that these reactions are immune-mediated, particularly the initial sensitisation period from first exposure, followed by rapid relapse of symptoms on rechallenge. Furthermore, several investigators have found T-cell involvement, including T-cell proliferation *ex vivo* when exposed to CBZ (Naisbitt *et al.*, 2003a), and the presence of T-cell infiltration in the dermis and epidermis in CBZ-hypersensitive patients with TEN and erythema multiforme (Friedmann *et al.*, 1994). T-cells from both peripheral blood and skin of patients with CBZ-induced TEN express the skin-homing marker cutaneous lymphocyte antigen, and this returns to normal as the clinical situation improves (Leyva *et al.*, 2000).

T-cell cloning is a powerful technique for generating clones of T-cells, i.e. populations of T-cells which have been grown *in vitro* from a single precursor cell. As all members of these clones share an identical T-cell receptor, these populations are ideal tools for exploring antigen-specificity and distinguishing genuine intraclonal cross-reactivity from mere interclonal diversity. This technique has been used to investigate hypersensitivity reactions to several drugs, in particular SMX (Schnyder *et al.*, 1997, 2000), penicillins (Brander *et al.*, 1995; Padovan *et al.*, 1997) and trinitrohalobenzenes (von Bonin *et al.*, 1992; Ortmann *et al.*, 1992), and has been instrumental in distinguishing T-cell reactions to hapten-modified peptides from reactions to the unmodified parent drug (Zanni *et al.*, 1998).

CBZ, structurally N-carbamoyl-iminostilbene, undergoes a complex pattern of metabolism within the body, including formation of several structurally distinct reactive species.

The major oxidative metabolite is CBZ-10,11-epoxide, the formation of which is catalysed by CYP3A4, with minor involvement of CYP2C8 (Kerr *et al.*, 1994). The 10,11-epoxide was for some time believed to be non-protein reactive as it is stable in plasma, although recent studies have actually identified glutathionyl and protein conjugates derived from it (Bu *et al.*, 2005). This metabolite is readily hydrolysed *in vivo* by epoxide hydrolase to 10,11-dihydrodiol-CBZ. In addition, several reactive species have also been identified, including CBZ-2,3-epoxide (Madden *et al.*, 1996), CBZ-iminoquinone (Pearce *et al.*, 2005), and 9-acridine carboxaldehyde (Furst *et al.*, 1995). For a more detailed treatment of this issue, see 1.3.8.4 and figure 1.2.

The structurally related CBZ analog oxcarbazepine (oxCBZ), used clinically for epilepsy and mood disorders, is keto-substituted on the unsaturated 10-carbon of the iminostilbene structure. This alters its main route of metabolism such that the major oxidative metabolite is 10-OH-CBZ, which is also believed to be the active component (Theisohn & Heimann, 1982). Previous studies have investigated the possibility that oxCBZ may be tolerated in patients hypersensitive to CBZ (Pirmohamed *et al.*, 1991; Troost *et al.*, 1996; Zakrzewska & Ivanyi, 1988), although clinical studies are rare (Beran, 1993; Misra *et al.*, 2003). One of the key issues regarding clinical cross-reactivity is that both oxCBZ and CBZ produce 10-OH-CBZ, and therefore if any of the initial T-cell response is directed towards this stable metabolite, clinical cross-reactivity seems almost inevitable.

One of the main unanswered questions regarding the aetiology of drug hypersensitivity is the ultimate nature of the antigen which T-cells respond to, both in the primary sensitisation event and in the secondary effector response. It has long been believed that small molecules (molecular weight <1000Da) become immunogenic by coupling to a carrier macromolecule, most likely protein, a proposal known as the hapten hypothesis (Park *et al.*, 1998). However, more recent studies have suggested that drug-specific T-cells may actually be activated by the soluble drug itself, presumably via a reversible three-way interaction between the MHC, drug and TCR (the p-i concept; Schnyder *et al.*, 1997; Zanni *et al.*, 1998). This has been demonstrated with several important drugs with relatively high incidences of immune-mediated adverse

reactions, including SMX (Schnyder *et al.*, 2000), lamotrigine (Naisbitt *et al.*, 2003b), lidocaine (Zanni *et al.*, 1997), and phenindione (Naisbitt *et al.*, 2005a). Importantly, however, for SMX this was not the only mechanism by which T-cell clones could be stimulated by the drug—the protein-reactive metabolite SMX-NO could also activate T-cells when bound directly to the MHC molecule. It is quite possible that the only reason that this finding is not seen with a wider range of drugs is because few drugs have protein-reactive metabolites as easy to work with as SMX-NO. However, it is important to be clear that these findings are only able to inform us about the antigens which can activate T-cells *following* an adverse event—they do not, and indeed, cannot, inform us about the antigens involved in the initial sensitisation event. For one, there is good evidence that the antigenic repertoire changes both during and after an immune reaction, both via shifts in the population frequencies of responsive clones (Amrani *et al.*, 2000), and through the alteration of antigen-specificity of the TCR on a given clone (McMahan & Fink, 2000; Serra *et al.*, 2002). Caution is therefore required when drawing conclusions from studies on T-cells isolated post-event.

In this chapter, studies intended to explore the specificity of lymphocytes isolated from CBZ or oxCBZ-hypersensitive patients towards both CBZ and oxCBZ and a range of stable, circulating metabolites of the two compounds will be described. Furthermore, the mechanism by which CBZ is recognised by T-cell clones will be discussed, in order to give some insights into the nature of the antigen involved in the clinical response.

5.2 Materials and Methods

5.2.1 Materials

Lymphoprep was obtained from Nycomed (Oxford, UK). Recombinant human IL-2 was obtained from Peprotech (London, UK). Anti-CD4, Anti-CD8, anti-CD3, and anti-Vβ antibodies were all obtained from Serotec (Oxford, UK). [^{3}H]-methyl thymidine was obtained from Moravek (California, USA). 10-OH-CBZ, 2-OH-CBZ, 3-OH-CBZ, CBZ-10,11-*trans*-diol, and CBZ-10,11-*cis*-diol were obtained from Novartis (Basel, Switzerland). All other reagents, chemicals and components were supplied by Sigma-Aldrich (Gillingham, UK).

114

N-acetyl-iminostilbene was prepared by reacting iminostilbene (2.0g, 10.4mmol) with acetic anhydride (15ml) at 100°C for 5 h. After cooling to room temperature, the reaction mixture was added to an ice/water mix (150ml) and stirred for 30 min. The crude product was extracted into ethyl acetate and the extracts stripped once with water. The organic phase was dried over Na_2SO_4 and solvents were removed *in vacuo*. The crude material was re-crystallised from ethyl acetate/n-hexane to give the desired N-acetyl-iminostilbene as a white solid (yield, 1.98g, 81%; ^{1}H-NMR spectrum, δ (ppm) (d$_6$-acetone): 7.23-7.47 (m, 8H, aromatic); 6.90-6.99 (AB qt, 2H, $\underline{H}$-C aromatic); 1.86 (s, 3H, $\underline{H}_3$-CO); ^{13}C-NMR spectrum, δ (ppm) (d$_6$-acetone): 170.82 ($\underline{C}$O); 140.55 ($\underline{C}^{13}$); 140.31 ($\underline{C}^{15}$; aromatic). Accurate mass calculated: 236.10753, found: 236.10760. Melting point: 110-112°C. This synthesis was performed by Dr Elizabeth Bowkett (Dept of Chemistry, University of Liverpool).

5.2.1.1 Culture medium

T-cell and APC culture media were prepared as described previously (see 2.2.1.1).

5.2.1.2 Isolation of PBMCs

PBMCs were isolated and viability was analysed as described previously (see 2.2.2).

5.2.2 Clinical details

Blood was obtained and lymphocytes isolated from 12 patients hypersensitive to CBZ and 1 patient hypersensitive to oxCBZ. Approval for the study was obtained from Liverpool local research ethics committee, and informed consent was obtained. The clinical details of the patients are shown in table 5.1.

2 groups of volunteers were also recruited: individuals who had been exposed to CBZ without overt hypersensitivity, and drug naïve individuals (n=5 for both groups).

5.2.3 Determination of lymphocyte proliferation

Lymphocyte proliferation was measured by means of the lymphocyte transformation test (LTT), as described previously by Nyfeler & Pichler (1997). Freshly isolated

Table 5.1: Clinical details of CBZ hypersensitive patients

	Age (y)	Sex	Days to reaction	Details of reaction	Months since reaction	Rechallenge
1*	70	M	21	Widespread maculopapular eruption with tissue eosinophilia	28	No
2	66	F	42	Maculopapular rash, fever, eosinophilia, abnormal LFTs	84	No
3	33	M	21	Exfoliative dermatitis, facial swelling, fever and eosinophilia	180	No
4	67	F	6	Widespread erythematous rash, lymphocytosis and eosinophilia	180	No
5	31	M	28	Erythematous rash, jaundiced, hepatomegaly	160	No
6	33	M	21	Widespread severe skin rash	21	No
7	27	M	21	Widespread rash with fever, history of allergy to multiple drugs	36	No
8	40	F	21	Widespread maculopapular erythematous rash	6	No
9	55	F	21	Widespread maculopapular rash, similar reaction to lamotrigine	12	No
10	65	F	Rechallenge	Desquamating rash with fever on inadvertent rechallenge, history of rash on initial exposure	<1	Positive
11	28	F	Rechallenge	Widespread maculopapular rash with fever/eosinophilia after 1 dose, history of allergy to antibiotics	2	Positive
12	31	M	12	Widespread severe maculopapular rash with fever	229	No
13	67	F	22	Generalised maculopapular rash	23	No

* Number assigned to patient throughout chapter (patient 13 sensitive to oxCBZ).

PBMCs (1.5×10^5; total volume 0.2ml) were incubated with CBZ, oxCBZ, CBZ-10,11-epoxide, 10-OH-CBZ, 2-OH-CBZ, 3-OH-CBZ, CBZ-10,11-trans-diol and CBZ-10,11-cis-diol, iminostilbene (all 10–50μg/ml; for structures of all of the above, see figure 5.1) and tetanus toxin (10μg/ml) as a positive control in 96-well U-bottomed tissue culture plates for 6 days (37°C; 5% CO_2). Proliferation was measured by the addition of [^{3}H] thymidine (0.5μCi) for the final 16 h of the incubation). Cells were harvested, and counted as cpm on a β-liquid scintillation and luminescence counter (PerkinElmer Life Sciences, Boston, MA). Proliferative responses were calculated as SI calculated as described previously (see 2.2.6).

5.2.4 Generation of EBV-transformed B-lymphoblastic cell lines

Epstein-Barr virus (EBV)-transformed B-lymphoblastic cell lines (B-LCLs) were generated from the CBZ-allergic patients by transformation of freshly isolated PBMCs with supernatant from the EBV-producing cell line B9-58. B9-58 cells were maintained in APC medium, and the supernatant was 0.2μm filtered to remove remaining cells prior to use. For transformation, 2×10^6 PBMCs were incubated in 5ml B9-58 supernatant, supplemented with cyclosporin A (1μg/ml) to prevent T-cell mediated suppression of B-cell infection. EBV-transformed B-cell cultures were maintained in APC medium.

5.2.5 T-cell cloning

T-cell cloning involves three major stages: firstly, PBMCs are bulk cultured with the antigen in question in order to enrich their initially low precursor frequency, so as to make generation of antigen-specific T-cell clones more feasible. Secondly, T-cells are serially diluted from enriched bulk cultures so as to allow the setting up of incubations containing, on average, a single T-cell. Finally, these incubations are allowed to grow in the presence of IL-2, and are tested for antigen specificity by antigen challenge and assessment of proliferation, and tested for clonality by analysis of Vβ expression.

CBZ

oxCBZ

CBZ-10,11-epoxide

10-OH-CBZ

CBZ-10,11-cis-diol

CBZ-10,11-trans-diol

2-OH-CBZ

3-OH-CBZ

Iminostilbene

N-acetyl-iminostilbene
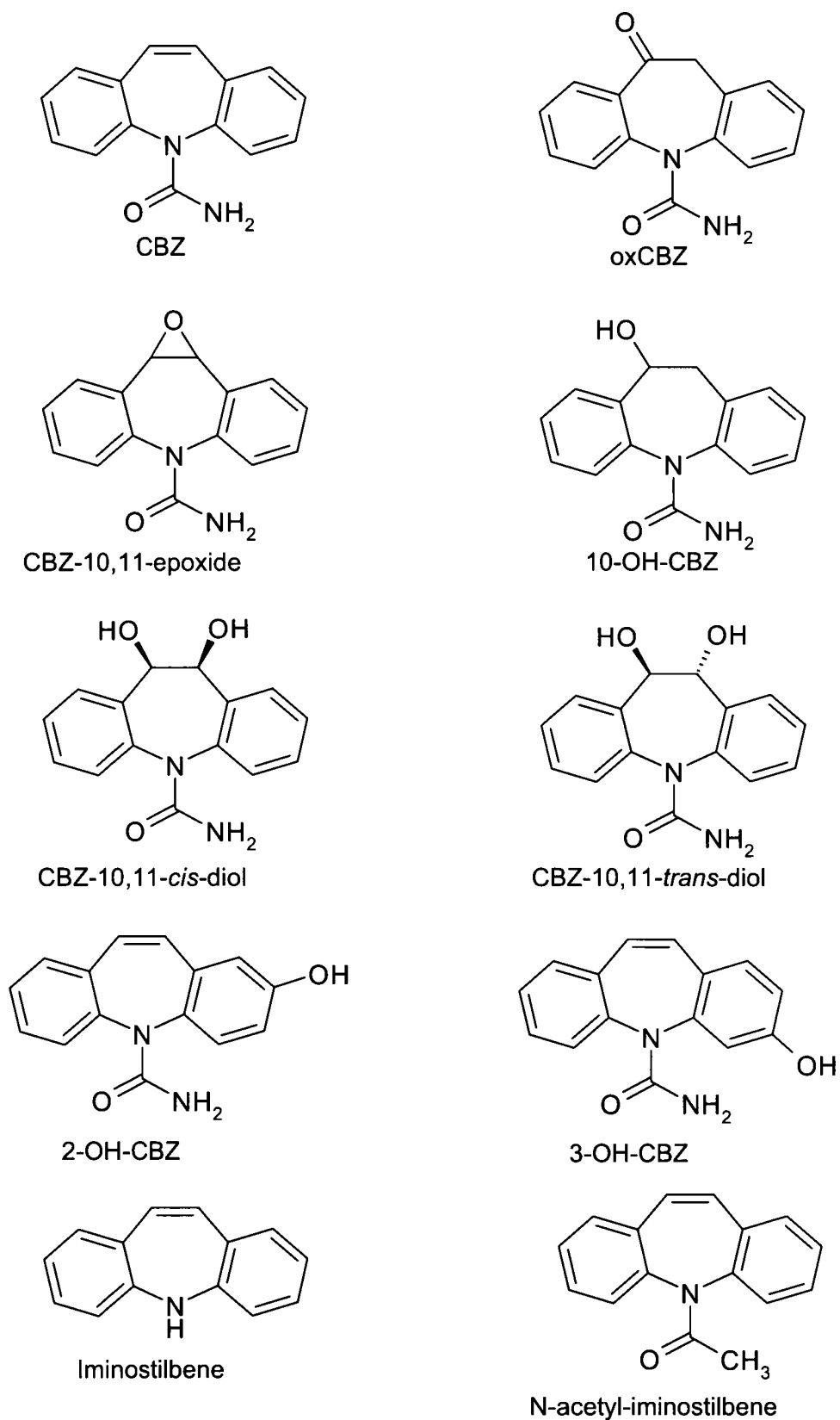
Figure 5.1: CBZ analogs used for LTT

5.2.5.1 Generation of T-cell clones

For the generation of T-cell clones, freshly prepared PBMCs were incubated as bulk cultures with CBZ or CBZ metabolites (10–100μg/ml) in 0.5ml T-cell medium in 48-well tissue culture plates. Cultures were supplemented with IL-2 (60U/ml) on days 6 and 9 to maintain drug-specific proliferation. On day 14, T-cell clones were prepared by serial dilution (Schnyder et al., 2000). T-cell bulk cultures were serially diluted so as to leave 0.3, 1 or 3 cells to be incubated in 96-well round-bottomed tissue culture plates in 100μl T-cell medium, supplemented with 5×10^4 allogenic irradiated (3000 rad) lymphocytes, 5μg/ml phytohaemagglutinin and 60U/ml IL-2. T-cells were expanded in T-cell medium with 60U/ml IL-2 and restimulated every 14 days with irradiated (3000 rad) allogenic lymphocytes and 5μg/ml phytohaemagglutinin.

5.2.5.2 Specificity of T-cell clones

CBZ-specific T-cell clones were identified by proliferation assay. 0.5×10^5 T-cells were incubated with autologous irradiated (4500 rad) B-LCLs (0.1×10^5) and CBZ (10, 25μg/ml) in a final volume of 200μl in a 96-well round-bottomed tissue culture plate. After 48 h, [^{3}H]-thymidine was added (0.5μCi), and 16 h later proliferation was determined as described earlier. T-cell lines with a positive proliferative response (as determined by SI > 2.5) were taken to be drug-specific and maintained in IL-2 containing medium.

5.2.5.3 Phenotypic analysis of T-cell clones

Clonality of T-cell lines was determined by Vβ analysis. T-cells were incubated with a panel of FITC-labelled anti-Vβ antibodies, washed repeatedly, and Vβ expression was determined by flow cytometry by comparison to isotype matched controls. CD4/8 phenotype was determined by dual staining with FITC-labelled anti-CD4 and PE-labelled anti-CD8 antibodies followed by flow cytometry.

5.2.6 Role of metabolism in T-cell activation by CBZ

Three variations of the standard proliferation assay were used in order to explore the role of CBZ metabolism in T-cell activation.

5.2.6.1 T-cell and B-cell pulsing with CBZ prior to proliferation assays

The role of covalent binding in T-cell activation with CBZ was explored by pulsing of T-cells or APCs with CBZ prior to combining the two cell types, as previously described (Schnyder *et al.*, 1997, 2000). These experiments involved the incubation of CBZ ($25\mu g$/ml) with either T-cells (0.5×10^5) or irradiated (4500rad) B-LCLs (0.1×10^5) for 2 h, followed by repeated washes to remove non-covalently bound drug, and incubation in fresh medium (0.2ml) with additional irradiated (4500rad) B-LCLs or T-cells respectively, and proliferation determined by thymidine incorporation as described previously.

5.2.6.2 Enzyme inhibition by aminobenzotriazole in proliferation assay

The role of localised metabolism in T-cell activation by CBZ was explored by the addition of ABT, a mechanism based inhibitor of haemoxygenases (Ortiz De Montellado *et al.*, 1984), to the proliferation assay. 1mM ABT was pre-incubated with T-cells and irradiated (4500rad) B-LCLs 1 h prior to the addition of CBZ for a standard proliferation assay.

5.2.6.3 Addition of glutathione to proliferation assay

T-cells and irradiated (4500rad) B-LCLs were incubated with GSH (2mM) for 35 min prior to the addition of CBZ for a standard proliferation assay. GSH irreversibly binds to CBZ reactive metabolites and hence inhibits covalent modification of protein (Ju & Uetrecht, 1999; Madden *et al.*, 1996).

5.2.7 Role of antigen processing in T-cell activation by CBZ

A potential requirement for antigen processing in T-cell activation by CBZ was determined as described below.

5.2.7.1 CBZ presentation by fixed EBV-transformed B-cells

Chemical fixation of B-LCLs by glutaraldehyde was performed to evaluate the possibility of antigen processing in T-cell activation by CBZ. B-LCLs (2×10^6 in 0.5ml

HBSS) were fixed by the addition of glutaraldehyde (0.05%) for 30 sec at room temperature. The reaction was terminated by the addition of glycine (0.2M) for 45 sec. Fixed B-LCLs were washed repeatedly, and used in proliferation assays as described previously. Glutaraldehyde inhibits antigen processing but does not alter MHC expression or presentation of preprocessed antigens (Shimonkevitz *et al.*, 1983; Zanni *et al.*, 1997).

5.2.7.2 Determination of minimum time requirement for T-cell/B-cell exposure to CBZ

To determine the minimum time requirement for T-cell activation by CBZ, T-cells and B-LCLs were incubated with CBZ (25μg/ml) in 1ml T-cell medium for 0.1, 1, 2, 4 and 16 h, followed by repeated washing, resuspending in fresh T-cell medium, and incubation for the remainder of 48h prior to the addition of $[^3H]$-thymidine and determination of proliferation as before.

5.2.7.3 Evaluation of time-course of T-cell activation by CBZ

The kinetics of T-cell activation by CBZ was determined by analysis of TCR internalisation. TCR-internalisation is one of the first detectable stages in T-cell activation, and is believed to act to prevent excessive T-cell activation and activation-induced cell death. T-cells (0.5×10^5) were incubated with B-LCL (0.25×10^5) in a final volume of 0.2ml with CBZ (10, 25μg/ml). After 1, 4 or 16 h, the cells were harvested, washed, and stained for CD3 expression with a FITC-labelled anti-CD3 antibody. Fluorescence was then determined by flow cytometry as described previously, and values were calculated as a % decrease from the baseline expression..

5.2.8 Statistical analysis

Results are presented as mean ± standard error of the mean. Normality of data was determined by the Shapiro-Wilks test and equality of variance by the Levene test. Data which were normally distributed were compared by means of a t-test, whereas nonparametric data were compared with the Mann-Whitney test for unpaired data or

the Wilcoxon signed rank test for paired data. P-values < 0.05 were considered to be significant. All statistical analyses were performed on SPSS 13.0.

5.3 Results

5.3.1 Lymphocyte proliferation in response to CBZ and CBZ metabolites

PBMCs from 11/12 CBZ-hypersensitive patients proliferated *in vitro* when exposed to CBZ (5–50μg/ml; see figure 5.2 and table 5.2). CBZ concentrations of 75μg/ml and above did not induce proliferation, presumably via direct toxic effects on lymphocyte function.

Additionally PBMCs from 1/1 oxCBZ patient proliferated when exposed to oxCBZ. PBMCs from CBZ-hypersensitive patients (5/5 tested) were also stimulated *in vitro* by the major CBZ oxidative metabolite CBZ-10,11 epoxide, and by oxCBZ and its major oxidative metabolite 10-OH-CBZ, but not by other oxidative CBZ metabolites (2-OH-CBZ, 3-OH-CBZ, CBZ-10,11-*cis*-diol, CBZ-10,11-*trans*-diol, or iminostilbene). Figure 5.3 shows proliferation of PBMCs from 6 hypersensitive patients in response to all positive compounds. In addition, all 5 hypersensitive individuals tested responded weakly to the stable CBZ-analog, N-acetyl-iminostilbene (see figure 5.4).

No increased proliferation was seen by either drug-naïve (SI: 1.1±0.2) or drug-tolerant (SI:1.2±0.4) individuals in response to CBZ, although they all responded to the positive control tetanus toxin (data not shown).

5.3.2 Generation and characterisation of CBZ and CBZ-metabolite specific T-cell clones

More than 1000 T-cell clones isolated from 6 CBZ-hypersensitive patients were tested for CBZ-reactivity, and over 100 CBZ-reactive clones were thereby detected (control incubations, 4981.5±5846.4 cpm; CBZ [25 μg/mL], 17721.4±15964.9 cpm; patients 1, 2, 3, 4, 6 and 7). The majority of these clones were CD4+, with 11 being either CD8+ (n=7) or CD4+CD8+ dual positive (n=4), and all clones expressed a single $\alpha\beta$ TCR,
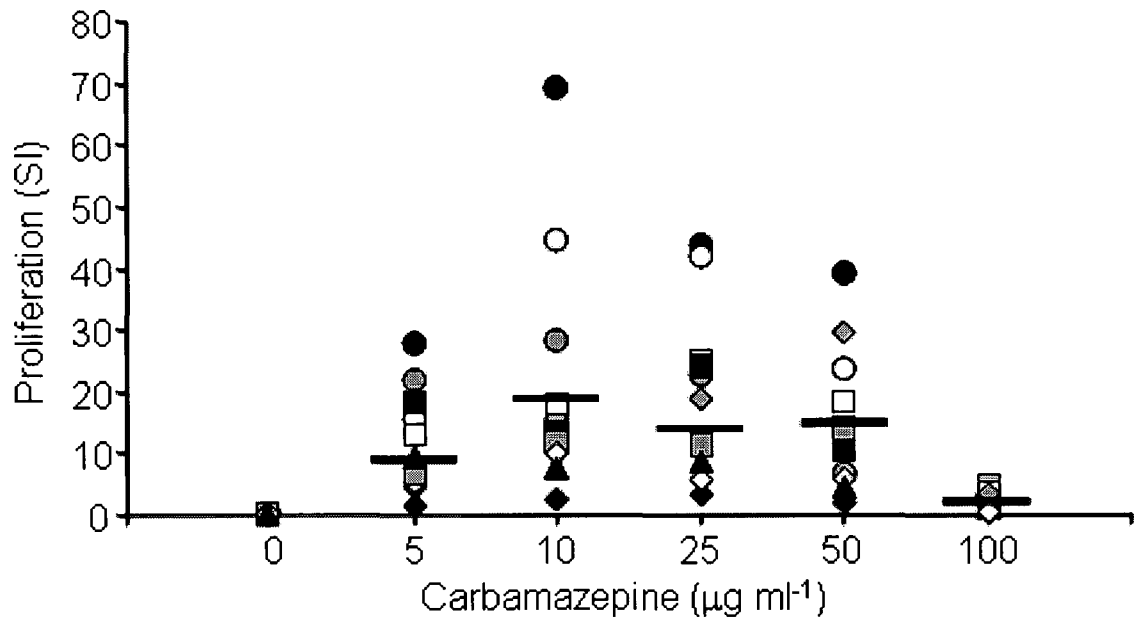
Figure 5.2: Proliferation of lymphocytes from 10 CBZ hypersensitive patients. Drug specific proliferation was determined by LTT. Lymphocytes were incubated with CBZ for 5 days, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean SI of triplicate cultures. Bold bars indicate mean SI.

Table 5.2: CBZ-specific proliferation of lymphocytes from hypersensitive patients

	cpm in control	Carbamazepine (μg/ml)				
		5	10	25	50	100
1	584	5.2*	7.5	9.8	3.3	1.0
2	289	8.5	44.3	42.1	22.8	2.1
3	1003	3.1	14.3	18.7	29.8	5.1
4	1443	6.0	12.5	10.5	13.5	2.1
5	1078	2.8	8.4	6.4	4.9	0.5
6	1321	1.5	2.3	3.0	1.6	0.3
7	687	8.1	7.1	7.6	4.2	1.5
8	399	9.2	17.6	23.4	18.9	4.2
9	881	11.5	13.8	24.7	9.9	1.2
10	670	22.5	69.4	42.3	38.7	3.2
11	1004	16.4	28.6	23.2	6.4	1.4
12	1294	1.1	0.9	1.2	1.0	1.1
13	920	2.8	4.8	5.3	4.2	1.7
Mean		7.6	17.8	16.8	12.2	2.0

* : Data presented as SI: stimulation index.
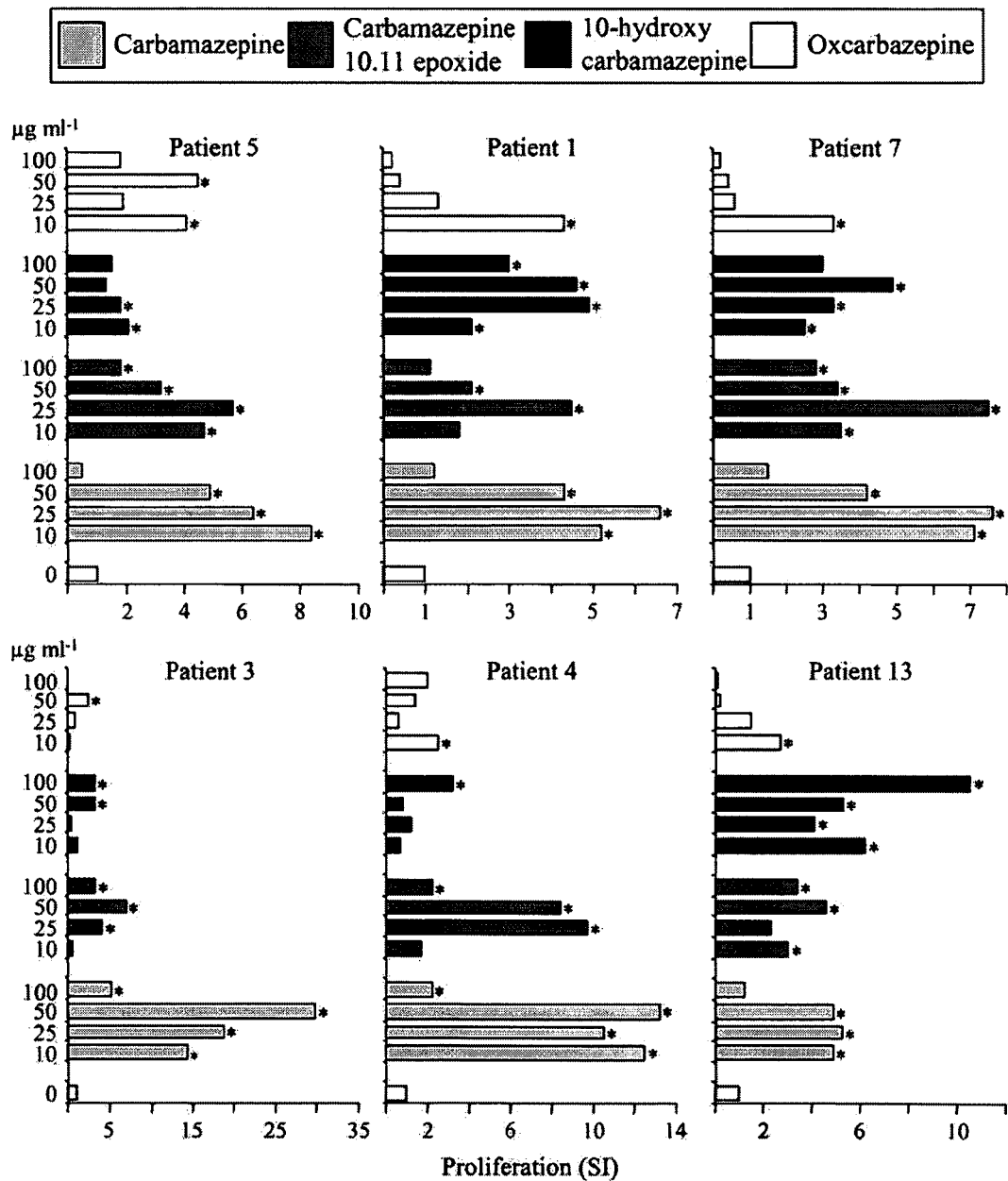
Figure 5.3: Proliferation of lymphocytes from hypersensitive patients stimulated with CBZ, oxCBZ and major oxidative metabolites. Drug specific proliferation was determined by incubating lymphocytes with CBZ for 5 days, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean SI of triplicate cultures. *: $p < 0.05$ compared to control incubations
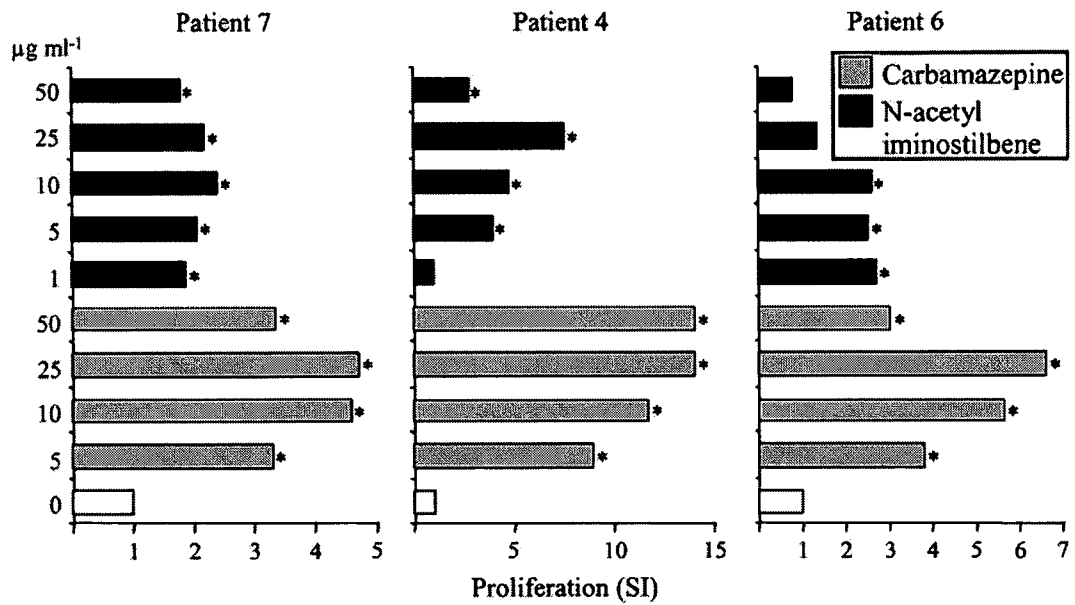
Figure 5.4: Proliferation of lymphocytes from hypersensitive patients stimulated with CBZ or N-acetyl iminostilbene. Drug specific proliferation was determined by incubating lymphocytes with CBZ for 5 days, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean SI of triplicate cultures. *: p < 0.05 compared to control incubations

although there was no clear pattern of expression. All clones responded to CBZ in a dose-dependent manner, with maximal responses at concentrations between 5–50μg/ml. As with PBMC cultures, concentrations above 75μg/ml inhibited proliferation. Figure 5.5 shows proliferation data from 8 representative clones.

5.3.3 Role of metabolism in T-cell activation by CBZ

Three modifications of the basic proliferation assay were used to explore the role of CBZ metabolism and covalent binding in T-cell activation, which we will discuss sequentially.

Firstly, either T-cell clones or irradiated B-LCLs were incubated with CBZ for 2 h prior to the addition of irradiated B-LCLs or T-cells respectively. In no case was T-cell proliferation observed following pulsing of cells (see figure 5.6), indicating that the simultaneous presence of CBZ, T-cells and B-LCLs was an absolute prerequisite for T-cell activation. This indicates that covalent binding of CBZ to either T-cells or B-LCLs cannot substitute for, and cannot be the essential result of, the role of free CBZ in CBZ-induced T-cell activation.

Secondly, ABT was used as a nonspecific inhibitor of oxidative metabolism. ABT inhibits a wide range of haemoxygenases, including all CYPs and myeloperoxidase (Ortiz De Montellado et al., 1984, see 3.3.6). The addition of ABT to T-cell/B-LCL cultures 1 h prior to the addition of CBZ had no effect on the proliferative response induced, whereas ABT alone had no effect (see figure 5.7).

Thirdly, GSH was added to the cells 35 min prior to the addition of CBZ. GSH is known to bind to and detoxify all known CBZ-reactive metabolites (Bu et al., 2005; Ju & Uetrecht, 1999; Madden et al., 1996; Pearce et al., 2005), and so the failure of this pre-treatment to reduce T-cell activation compared to control incubations (see figure 5.8) was taken to be further evidence that CBZ-reactive metabolites do not play an important role in this in vitro CBZ-induced T-cell activation.

5.3.4 Role of antigen processing in T-cell activation by CBZ

In addition, several approaches were undertaken in order to explore the possibility that antigen processing by APCs is an essential step in the activation of T-cells by CBZ.
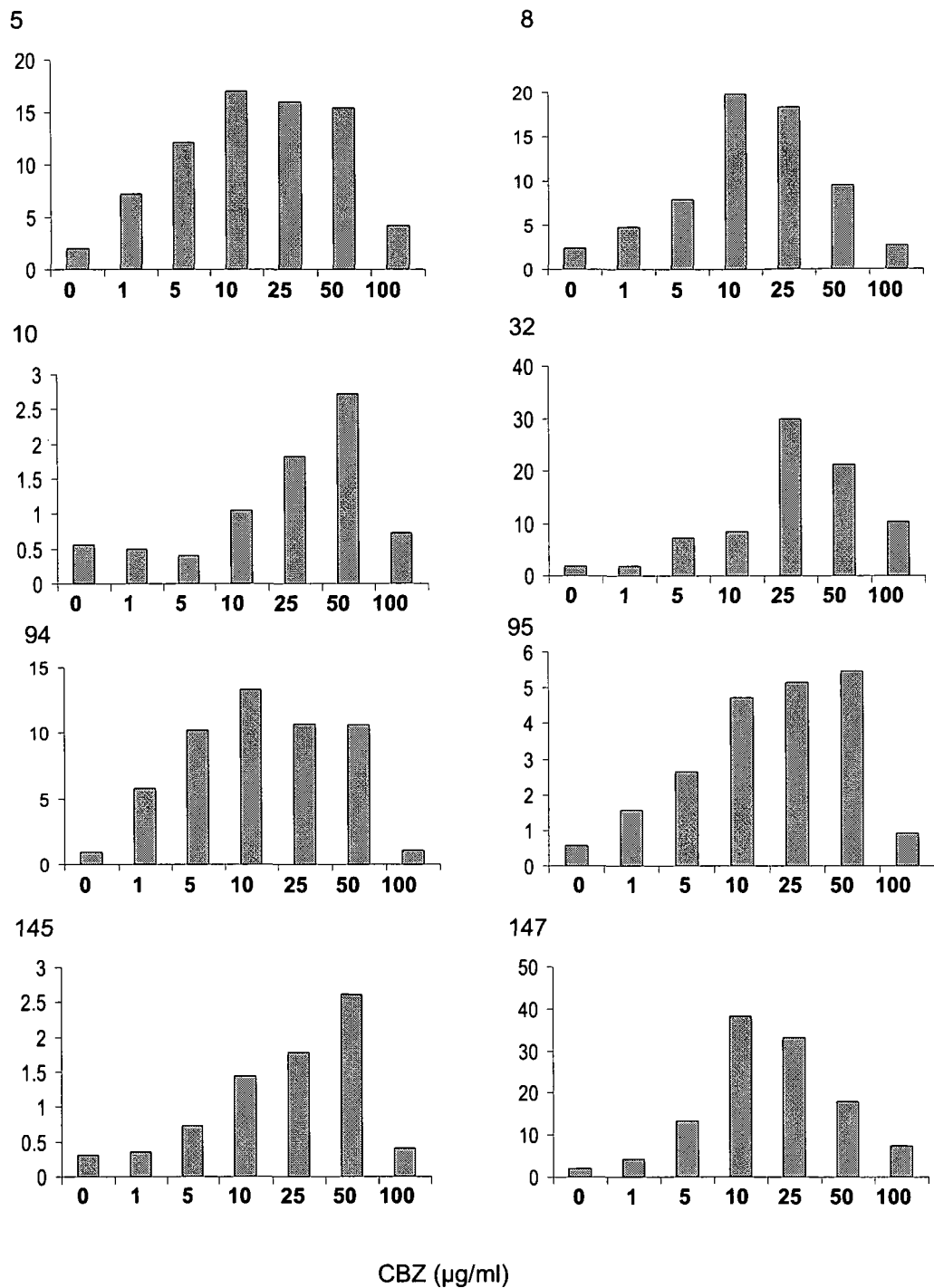
Figure 5.5: Proliferation of 8 representative T-cell clones isolated from CBZ hypersensitive patients stimulated with CBZ. Drug specific proliferation was determined by incubating T-cell clones with CBZ and B-LCLs as APC for 48 h, followed by the addition of [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures.
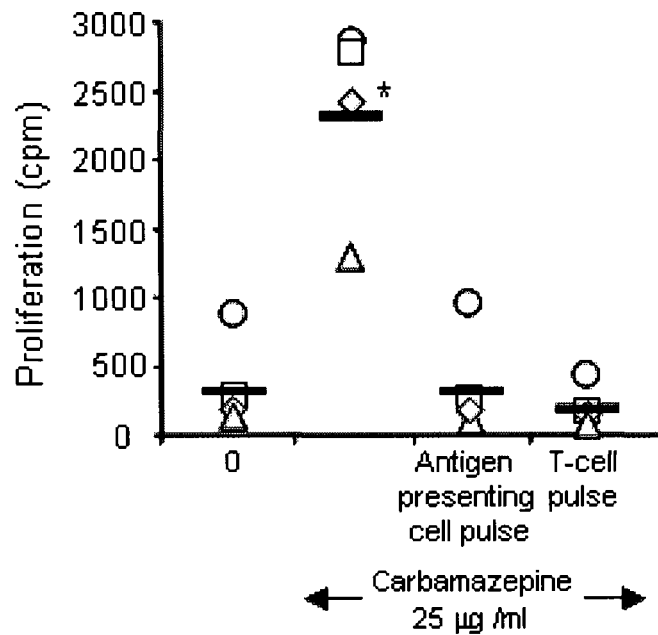
Figure 5.6: CBZ-specific T-cell clones do not proliferate in the presence of B-LCLs or T-cells pulsed with CBZ for 2 h. Drug specific proliferation was determined by incubating lymphocytes with CBZ for 48 h, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures. *: p < 0.05 compared to control or pulsed incubations

50000

40000

30000

20000

10000

0

0   + ABT   + ABT

CBZ
25 µg/ml
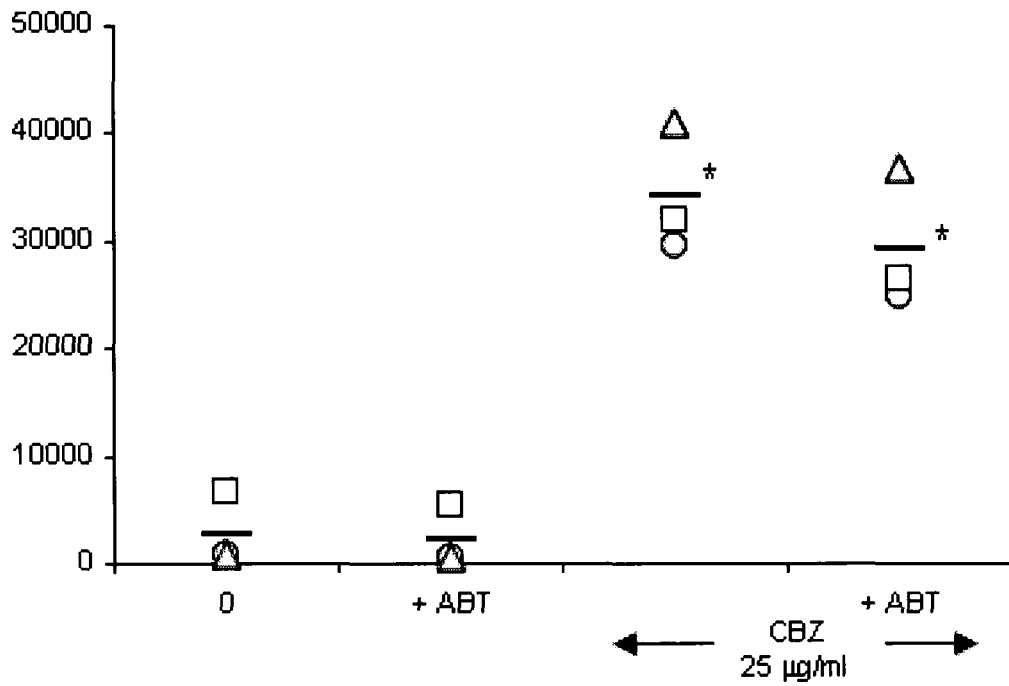
Figure 5.7: The addition of 1mM ABT does not affect the T-cell proliferative response induced by CBZ. Drug-specific proliferation was determined by incubating lymphocytes with CBZ for 48 h, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures. *: $p < 0.05$ compared to control incubations

Figure 5.8: The addition of 2mM GSH does not affect the T-cell proliferative response induced by CBZ. Drug-specific proliferation was determined by incubating lymphocytes with CBZ for 48 h, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures. *: $p < 0.05$ compared to control incubations
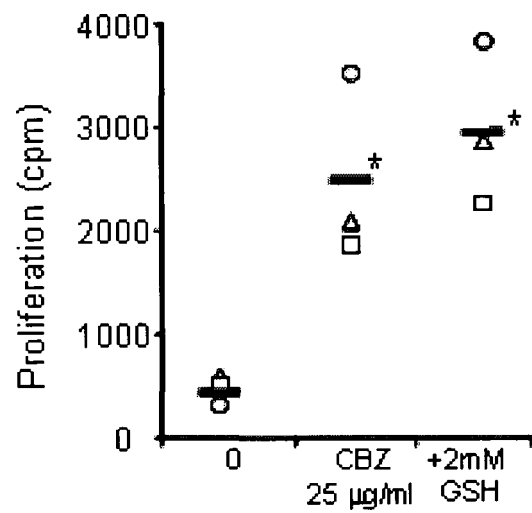
Firstly, B-LCLs were chemically fixed with glutaraldehyde prior to addition of CBZ and T-cells. This is known to inactivate the internal antigen processing and presentation machinery, although it does not eliminate MHC expression. In previous studies (Schnyder et al., 1997) it was sufficient to eliminate processing of protein antigens, but did not prevent presentation of pre-processed protein antigens, nor did it prevent the presentation of small molecules in a metabolism- and processing-independent fashion. In this study, it was found that glutaraldehyde fixation was insufficient to prevent CBZ presentation (see figure 5.9), suggesting that CBZ is presented directly on the MHC molecule, without being processed as part of a peptide antigen.

Secondly, the minimum time requirement for effective T-cell activation was determined by incubating T-cells, B-LCLs and CBZ for various periods (0.1, 1, 4, 16 h), followed by washing away soluble drug and resuspending cells in fresh medium for the remainder of 48 h in culture. T-cell clones did appear to be heterogenous in response, but in 4/5 cases activation was observed with as little as 1 h exposure, and in 2/5 cases only 5 mins. In the remaining clone, 4 h exposure was sufficient for activation (see figure 5.10.

Thirdly, an early marker of T-cell activation, TCR internalisation, was used as a surrogate of T-cell activation in order to identify the earliest point that T-cell activation can be detected following incubation with B-LCLs and CBZ. Significant TCR internalisation was detectable in all clones within 1 h of T-cell exposure to CBZ (see figure 5.11).

Since antigens which require processing takes a minimum of 4-6 h exposure to induce T-cell activation (Panina-Bordignon et al., 1991; Zanni et al., 1998), and since they cannot be presented by fixed APCs (Schnyder et al., 1997), these data demonstrate that, for the clones under study, CBZ is presented in a processing-independent fashion.

5.4 Discussion

Many of the adverse reactions to CBZ are believed to be immunological in nature, although convincing final evidence is still lacking. In this study, PBMCs and T-cell clones from patients with apparent CBZ hypersensitivity were studied both in order
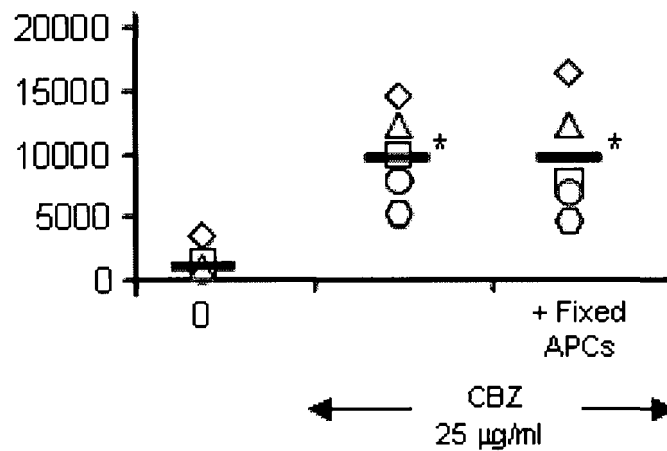
Figure 5.9: Fixation of B-LCLs does not affect the T-cell proliferative response induced by CBZ. Drug-specific proliferation was determined by incubating lymphocytes with CBZ and fixed B-LCLs for 48 h, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures. *: p < 0.05 compared to control incubations
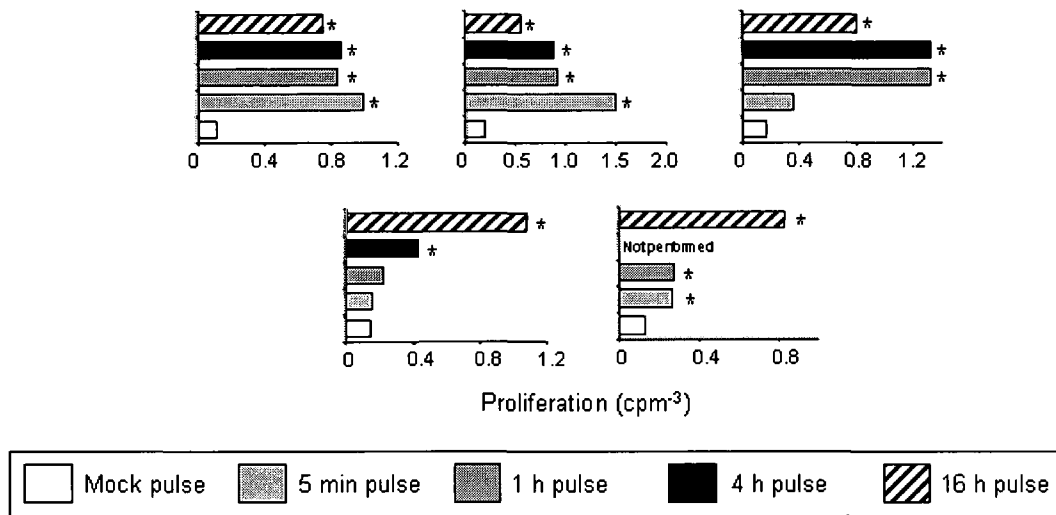
Figure 5.10: Determination of the minimum required period of exposure to CBZ for activation of CBZ-specific T-cell clones. T-cells and B-LCLs were incubated with CBZ (25μg/ml) for 0.1-16 h, prior to washing and incubated in drug-free medium for the remainder of 48 h. Proliferation as determined by measuring [^{3}H]-thymidine incorporation. *: $p < 0.05$ compared to control incubations
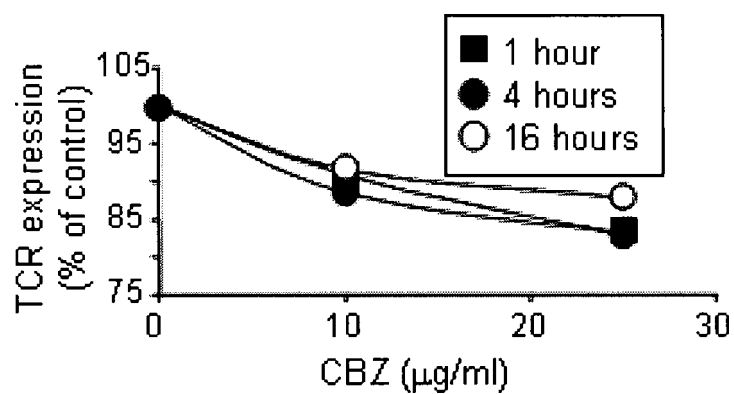
Figure 5.11: Kinetics of T-cell receptor internalisation following CBZ exposure. T-cells were exposed to CBZ (10, 25μg/ml) for varying periods (1, 4, 16 h), followed by staining with FITC-labelled anti-CD3 antibody and fluorescence determination by flow cytometry. Data show one representative clone.

to explore both the cross-reactivity patterns to a panel of stable and reactive CBZ metabolites and analogs, and in an attempt to identify the mechanism by which CBZ is presented to CBZ-specific T-cells, in particular, whether localised drug metabolism or antigen processing are essential parts of the response.

PBMCs from CBZ-hypersensitive patients have been shown previously to proliferate in the presence of CBZ (Mauri-Hellweg *et al.*, 1995; Naisbitt *et al.*, 2003a; Zakrzewska & Ivanyi, 1988), although little work has been done to investigate the potential for CBZ metabolites to also act as antigens. CBZ is primarily metabolised by oxidative reactions, largely through the formation of the stable 10,11-epoxide and subsequent hydrolysis products. In addition, oxidation of the benzene rings both to a 2,3-epoxide and 2-OH products has been associated with the formation of reactive species. The technique used to investigate PBMC reactivity to CBZ, the LTT, has been previously used as a aid to diagnosis, or in order to differentiate the culprit drug in cases of polypharmacy (Pichler & Tilch, 2004). In one major study (Nyfeler & Pichler, 1997), over 900 past patients who had had an LTT performed during their treatment were retrospectively analysed, and the accuracy of the test was determined. Sensitivity and specificity values of 74% and 85% were obtained, although the preponderance of β-lactam antibiotics amongst the culprit drugs makes it hard to extrapolate too far from these findings. Other studies have found wide variation in sensitivity, which may reflect differences in the drugs studied, the clinical characteristics required for enrolment, methodological differences, or the technical skill of the individuals performing the test.

In this study, 11/12 CBZ-hypersensitive patients and 1/1 oxCBZ patient were found to be positive in an LTT, whereas none of the 10 control individuals demonstrated increased proliferation. Importantly, the concentrations associated with PBMC proliferation were comparable with those expected *in vivo* in patients receiving therapeutic doses (5-10μg/ml; Tomson *et al.*, 1980). The response was surprisingly stable over time, with two of the individuals who responded most vigorously having had their initial reaction some 15 years previously.

Studies with T-cell clones have previously been used to elucidate the mechanism by which T-cells are stimulated by antigens. In particular, the use of T-cell clones rather

than whole blood lymphocytes allows the investigator to reduce the complexity which a polyclonal response necessarily produces, and hence allows greater insight into the fundamental mechanisms in play.

Studies with T-cell clones from SMX-hypersensitive patients have suggested that they can respond to SMX in a metabolism- and processing-independent fashion (Schnyder *et al.*, 1997; Zanni *et al.*, 1998), and similar results also have been found with several other drugs (Naisbitt *et al.*, 2003b, 2005a; Zanni *et al.*, 1997). In order to investigate the possibility that CBZ acts on T-cells in a similar fashion, various functional assays were used to explore the mechanisms of antigen processing and presentation.

Previous studies have shown that pulsing APCs with protein-reactive compounds can allow T-cell activation when T-cells are added following washing of the APCs (Naisbitt *et al.*, 2001; Schnyder *et al.*, 2000; Zanni *et al.*, 1998). However, non-protein reactive compounds appear to require the continual presence of the soluble drug, suggesting that covalent binding to the cells, as may potentially occur through the action of enzymes expressed by either of the cell types present, is not an essential or sufficient step (Burkhart *et al.*, 2002; Schnyder *et al.*, 1997, 2000) for a low molecular weight compound to act as an antigen. In these studies, 2 h pulsing of either T-cells or APCs by CBZ, followed by incubation with the other cell type in fresh medium, was always insufficient for T-cell stimulation. It remains possible that 2 h is not a long enough period of time for exposure, and that longer pulses might have given different results. However, this seems unlikely in light of some of the later results, which found that pulsing combined T-cells and APCs with CBZ for as little as 0.1h was sufficient to activate the T-cells. These data appear to suggest that CBZ-specific T-cell clones respond to CBZ in a covalent-binding-independent fashion.

The broad-spectrum metabolic enzyme inhibitor ABT was added to proliferation assays in order to further explore the role of oxidative metabolism in T-cell activation by CBZ. ABT is a suicide inhibitor of haemoxygenases which functions by alkylating the haem group via a benzyne radical (Ortiz De Montellado *et al.*, 1984). Data presented previously showed that ABT inhibited all CYPs and peroxidases studied (see 3.3.6), yet it had no effect on the T-cell proliferative response when preincubated with APCs

137

for 1 h prior to the addition of T-cells and CBZ. Again, this offers evidence that the T-cell response to CBZ is truly to the parent drug and not to metabolites formed *in situ*.

The addition of GSH would also be expected to inhibit T-cell responses to CBZ-derived protein reactive metabolites, as indeed it does with SMX-NO specific T-cells (Farrell *et al.*, 2003; Burkhart *et al.*, 2001). All reactive metabolites derived from CBZ which have been characterised are detoxified by the formation of conjugates with GSH (Bu *et al.*, 2005; Ju & Uetrecht, 1999; Madden *et al.*, 1996; Pearce *et al.*, 2005), and so the fact that prior incubation of APCs with GSH was unable to prevent, or even reduce, CBZ-specific T-cell activation must be seen as further evidence that CBZ-specific T-cells respond directly to the parent drug without any requirement for metabolism and covalent binding.

Further studies were undertaken in order to explore the requirements for antigen processing. In the first instance, glutaraldehyde-fixed B-LCLs were used as APCs as they are known to be incapable of antigen processing (Schnyder *et al.*, 1997; Shimonkevitz *et al.*, 1983). In all of the clones tested, fixed APCs were still able to present CBZ to T-cells, suggesting that antigen-processing is not a necessary step in antigen presentation. Glutaraldehyde fixation involves the formation of multiple cross-linkages between free amino groups on protein, and it has been suggested that fixation could therefore allow CBZ-protein covalent adducts via a glutaraldehyde linker binding to the terminal amine group of CBZ. While this is a concern, there are some reasons to be cautious about this critique. Firstly, the fixation process involves quenching with excess glycine prior to addition of the drug. Though this is unlikely to completely eliminate the possibility of covalent interactions, it will greatly reduce the number of potential binding sites. Furthermore, since the processing machinery is inactivated, immunologically relevant adducts will have to be very specifically placed, presumably either on or very close to the peptide within the MHC cleft, further limiting the potential number of binding sites. Secondly, the structure of the postulated CBZ-glutaraldehyde-protein adduct will be quite different from any probable non-covalent interaction of CBZ with protein. Further work on CBZ-reactive T-cells (Wu *et al.*, 2006) found that several of

the clones investigated herein were extremely specific with regards to their structural requirements for binding, with some failing to cross-react with any structural analog tested. It is possible that these clones do in fact cross-react with CBZ-glutaraldehyde-protein adducts despite their apparent stringency, but it seems implausible. However, this criticism does carry some force, and so further experiments were undertaken to explore the kinetics of T-cell activation in order to better understand the process of antigen presentation within this *in vitro* system.

Firstly, the minimum period of T-cell exposure to CBZ was assessed by use of a combined pulse methodology. There was some degree of heterogeneity between clones, but in no case was more than 4 h exposure required for a full proliferative response, and in some cases the time required was as low as 0.1 h. It was not clear why the heterogeneity was observed, but repeated experiments demonstrated that it was true clonal variation rather than experimental error. It may be related to the quantity of receptors which need to be triggered in order to induce a full response, as both this and the density of receptors may vary between clones (Depta *et al.*, 2004).

Secondly, the kinetics of TCR internalisation in response to CBZ stimulation was studied. TCR internalisation for all clones was detectable within 1 h of CBZ exposure, and maximal within 4 h. This further suggests that the T-cell response to CBZ is too rapid to allow for the process of antigen processing, which is believed to take a minimum of 4-6 h (Panina-Bordignon *et al.*, 1991; Zanni *et al.*, 1998).

Taken together, these data all support the hypothesis that CBZ is presented to T-cell clones in a metabolism- and processing-independent fashion, as has been shown previously for several drugs, and in keeping with the p-i concept.

While some studies have explored PBMC proliferation in response to some stable CBZ metabolites or analogs (CBZ-10,11-epoxide, oxCBZ; Naisbitt *et al.*, 2003a; Zakrzewska & Ivanyi, 1988), no previous study has investigated a wide range of known CBZ and oxCBZ metabolites or structural analogs. Of the compounds studied here (see figure 5.1), only CBZ, oxCBZ, CBZ-10,11-epoxide, 10-OH-CBZ and N-acetyl-iminostilbene were capable of inducing a PBMC proliferative response *in vitro*. Figure 5.12 summarises these findings. It is worth bearing in mind that these find-

Table 5.3: Non-covalent molecular interactions

Type	Energy[*]	Relationship between strength and distance
Ion-ion	20–40	$1/d$
Ion-dipole	8–20	$1/d^2$
Dipole-dipole	4–20	$1/d^3$
Hydrogen	5–30	$1/d^4$
Van der Waals forces	0.5–5	$1/d^5$–$1/d^8$
Hydrophobic interactions	3.4[†]	

[*] : kJ/mol

[†] : per methylene group

ings relate solely to proliferation of mixed PBMC cultures, and so cannot inform us about whether these results indicate different responding populations or genuine cross-reactivity amongst individual clones. For a fuller study, involving analysis of cross-reactivity within clones, please see Wu *et al.* (2006), in which genuine cross-reactivity of T-cell clones to several patterns of CBZ structural variations was identified.
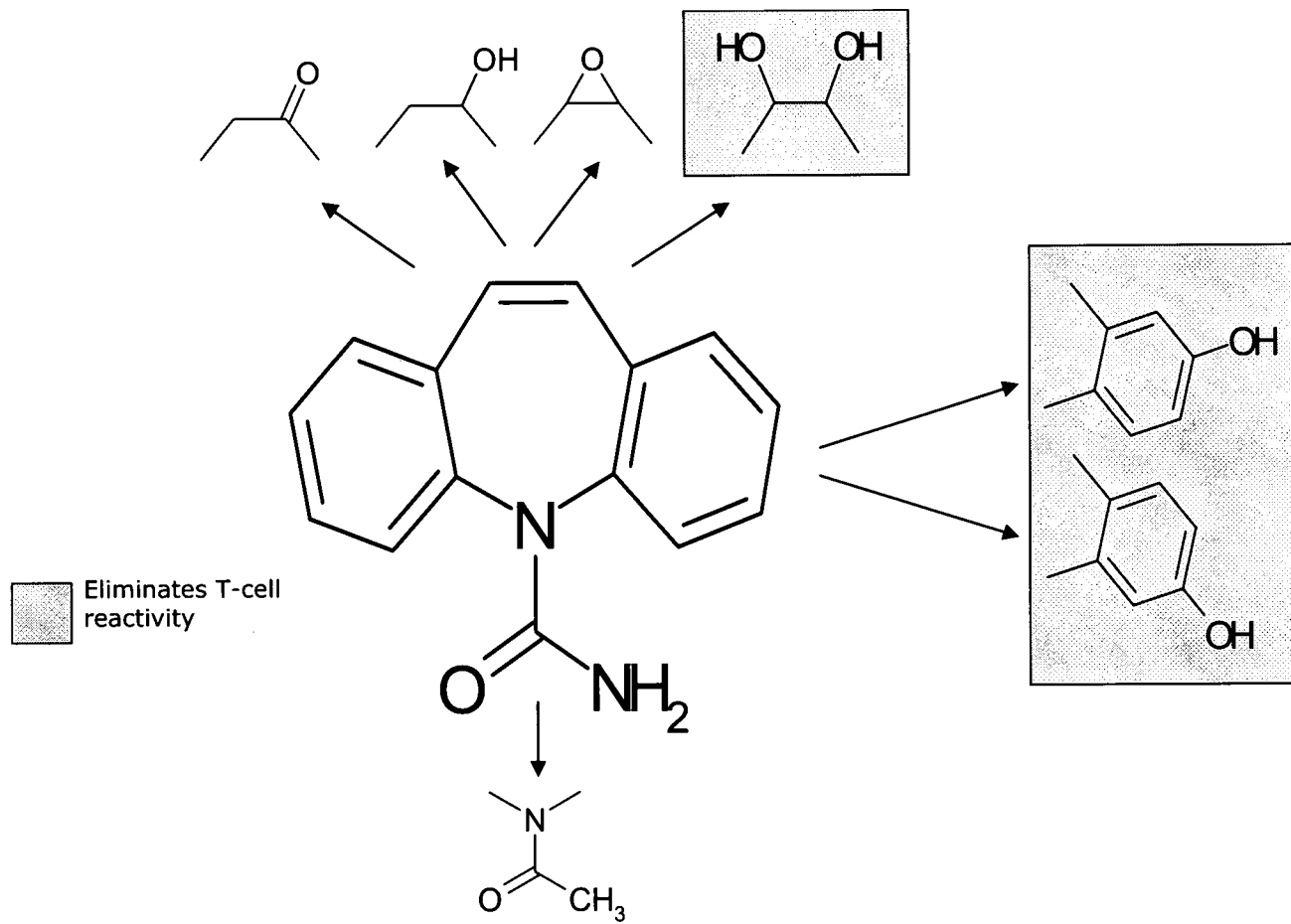
Figure 5.12: Structural requirements for CBZ activation of PBMCs

For a full understanding of the implications of these findings, it is briefly worth recapping the non-covalent interactions which may be involved in CBZ binding to TCR/MHC. The interactions which are known to mediate non-covalent molecular binding are summarised in 5.3. The strongest non-covalent interactions which are commonly involved in small-molecule binding to proteins are ionic interactions, but these are unlikely for CBZ binding, as the only potentially ionisable atom of the CBZ molecule, the primary amine of the carbamoyl moiety, is extremely hard to ionise (a weak acid with a pKa of 14; Scheytt *et al.*, 2005). Weaker, but more likely to be involved with CBZ binding, are hydrogen bonds, of which CBZ contains one acceptor, the oxygen of the carbamoyl group, and one donor, the amine. It is possible therefore, that these are involved in the binding of CBZ to the TCR/MHC complex, a suggestion supported by the finding that iminostilbene was completely ineffective with regard to PBMC stimulation. However, since replacement of the carbamoyl side chain with an acetyl moiety (effectively switching the primary amine with a methyl group), restored some (but not all) of the activity of the compound, it is also apparent that this is not essential in its entirety. Dipole-dipole and dipole-induced-dipole interactions also require this side chain, as there are no other dipole moments within the remainder of the molecule. Interactions through this part of the molecule will therefore need to be through either Van der Waals forces (otherwise known as induced-dipole-induced-dipole interactions or London dispersion forces) as a consequence of transient dipoles, or through hydrophobic interactions, in which reordering of molecules to exclude water becomes thermodynamically more stable. While individually weak, these are potentially quite powerful in summation, and CBZ binding is likely to rely heavily on them, as the tricyclic core of the molecule is a large hydrophobic region (log P of whole molecule, 2.45; Physprop online database, Syracuse Research Corporation, http://www.syrres.com/esc/physprop.htm). Therefore, it is interesting to note that modifications of these regions can have major impacts on the ability of the compound to be recognised by CBZ-specific T-cells. In particular, the MHC-drug-TCR interaction appears able to accommodate some degree of modification of the unsaturated 10,11 bond, although both *cis* and *trans* diol modifications were inactive. This may

be due to changes in the electron density of the region, which may affect the ability of the compound to form hydrophobic interactions with the MHC or TCR, or to direct steric hindrance at the site of binding, or indirect steric hindrance through realignment of the aromatic regions, both of which which would prevent the close alignment required for multiple Van der Waals forces. Hydroxylation of the aromatic rings led to complete inactivity. Work is underway to attempt to synthesise CBZ analogs with halogen-substitutions on the rings, which should allow insights into whether this lack of activity is due to steric alterations or modified electrochemical properties, and in turn should allow predictions to be made as to the bonds of importance involved in non-covalent interactions within the TCR-CBZ-MHC complex.

An important consideration when interpreting the data presented here is that it is not necessarily the case that T-cell clones isolated *ex vivo* are a good model for T-cells *in vivo*. For one thing, the extended period of enrichment *in vitro* prior to serial dilution may introduce an artificial stimuli which could induce artifactual T-cell specificities to develop (see, for instance, Engler *et al.*, 2004). Additionally, it is known that the process of avidity maturation (see 1.3.1 for details) leads to shifts in the pattern of T-cell specificity over the course of a reaction, and so potentially could lead to significant differences between the antigen-specificity of the initial T-cell population, and the antigen-specificity of the T-cell population at the time of PBMC isolation. However, seen simply as an exercise in exploring the possibilities of TCR binding, these studies demonstrate that the postulated molecular weight cut-off for antigenicity is not necessarily the case. A stronger case can be made for this being analogous to the pathological situation when one recalls the studies by Nassif *et al.* (2004), in which considerable cytotoxicity was observed within 4 h when T-cells isolated directly from blister fluid were incubated with target cells and SMX. In this study, essentially similar findings were obtained from T-cells at the time of the reaction without *ex vivo* stimulation or enrichment. However, further work is certainly required in order to test the suitability of *ex vivo* enriched T-cell clones post-reaction as models for effector T-cells during a drug hypersensitivity reaction.

A further potential criticism of this current work is that the it does not encompass

the whole potential range of CBZ-derived antigens. Remember that SMX is presented in a similar fashion (Schnyder *et al.*, 1997), and without further information, one would be tempted to assume that reactive metabolites play no role in SMX antigenicity (to leave aside the issue of immunogenicity for a moment). Of course, further studies have found that SMX-NO is also antigenic for T-cells derived from hypersensitive patients (Schnyder *et al.*, 2000), leaving open the question of the ultimate antigen in hypersensitivity. To extend the analogy, it may be the case that while CBZ can be presented directly, as it appears to be doing so here, CBZ reactive metabolites may also be able to act as antigens. However, it has not been possible as yet to fully explore this, although some attempts have been made. It has been claimed that the addition of microsomes to an LTT with CBZ led to proliferation and cytokine release which was not detectable in the absence of microsomes (Merk, 2005), although it has not been possible to identify the primary data to support this claim. Additionally, it has been found that PBMCs from hypersensitive patients proliferate in response to cell-free supernatant containing CBZ which has been incubated with PBMCs (and, it is claimed, has therefore been metabolised), but not to CBZ alone or to the washed cells thus exposed (Acharya *et al.*, 2001; Strickland *et al.*, 1996). However other studies (this chapter; Naisbitt *et al.*, 2003a) have found that PBMCs from CBZ-hypersensitive patients readily respond to the parent drug alone without this initial step, and so these findings are of uncertain importance. A further major study explored the possibility that either CBZ-metabolites generated by a microsomal system *in situ* or CBZ-iminoquinone could activate T-cells, but was unable to demonstrate this (Wu, 2006). In any event, there is a strong case to be made for the extracellular application of reactive metabolite being sufficiently different from the intracellular generation of reactive metabolite in terms of the ultimate antigen as to make the exercise unviable. A more promising approach would be to use APCs which are sufficiently metabolically active to generate intracellular antigen. As dendritic cells are known to be metabolically active, at least regarding MPO (see chapter 3), a previous study from our group did attempt to investigate them as APCs in CBZ proliferation assays (Wu, 2006). However, they were unable to identify any differences between them and B-LCLs with regards to the mechanism of presentation; CBZ

was presented in a mechanism- and processing-independent fashion by both cell types. However, as dendritic cells appear to primarily express MPO rather than CYPs, this does not allow us to rule out the possibility that CYP-competent APCs (for instance, keratinocytes) could present CBZ in a metabolism-dependent fashion.

In conclusion therefore, it is important to bear in mind that whilst these studies do have limitations—not least, the important distinction between antigenicity, which these studies can address, and immunogenicity, which they cannot—they do also allow an insight into the mechanisms of T-cell activation, and show that the intricacies of TCR-specificity and mechanisms of presentation can be elucidated.

Chapter 6

T-cell activation by

Trimethoprim

Contents

6.1 Introduction

TMP is an antibiotic, most commonly used in conjunction with SMX in the formulation co-trimoxazole. This combination therapy has been associated with the induction of apparently immune-mediated adverse reactions, including delayed-type hypersensitivity reactions with skin rash and internal organ involvement. However, since both the components of the formulation are known to induce hypersensitivity reactions, it is often hard to determine the precise culprit drug in any given reaction. Most work on the subject has focused on the SMX component, although one study found ~20% of co-trimoxazole hypersensitive individuals responded to rechallenge with TMP alone (Bonfanti et al., 2000), and a recent case study reported a fatal case of TEN upon inadvertent rechallenge with TMP alone (Mortimer et al., 2005).

The structure of TMP consists of a trimethoxybenzene ring connected via a methyl group to a diamine-substituted pyrimidine structure. It is excreted largely unchanged (50-60%) in urine, but several metabolites have also been detected. Of these, the most prominent stable oxidative metabolites are two demethylated species, 3'-OH-TMP and 4'-OH-TMP, a hydroxylated derivative, α-OH-TMP, and two N-oxides, 1-NO-TMP and 3-NO-TMP (van 't Klooster et al., 1992). In addition, it has been found that TMP can form a reactive iminoquinone methide species following oxidation by a MPO/H_2O_2 oxidising complex (Lai et al., 1999). It has been postulated that this occurs through N-chlorination of the para-amino group, although complete proof for this is lacking. See figure 6.1 for a summary of these known metabolites. The initial study which identified the iminoquinone methide species found NAC adducts via both the α-methyl group and the 4-position on the pyrimidine ring, while a later study (Dieckhaus et al., 2005), found GSH adducts only through the pyrimidine ring. This may be due to the increased pH at which the reaction was performed, or may be due to intrinsic differences between the reactivity of the two model nucleophiles.

The nature of the ultimate antigen responsible for the activation of T-cells in TMP hypersensitivity has not previously been explored. In this study, the immune response in a patient who had experienced severe hypersensitivity with hepatic involvement following TMP administration was explored by means of ex vivo rechallenge of PBMCs
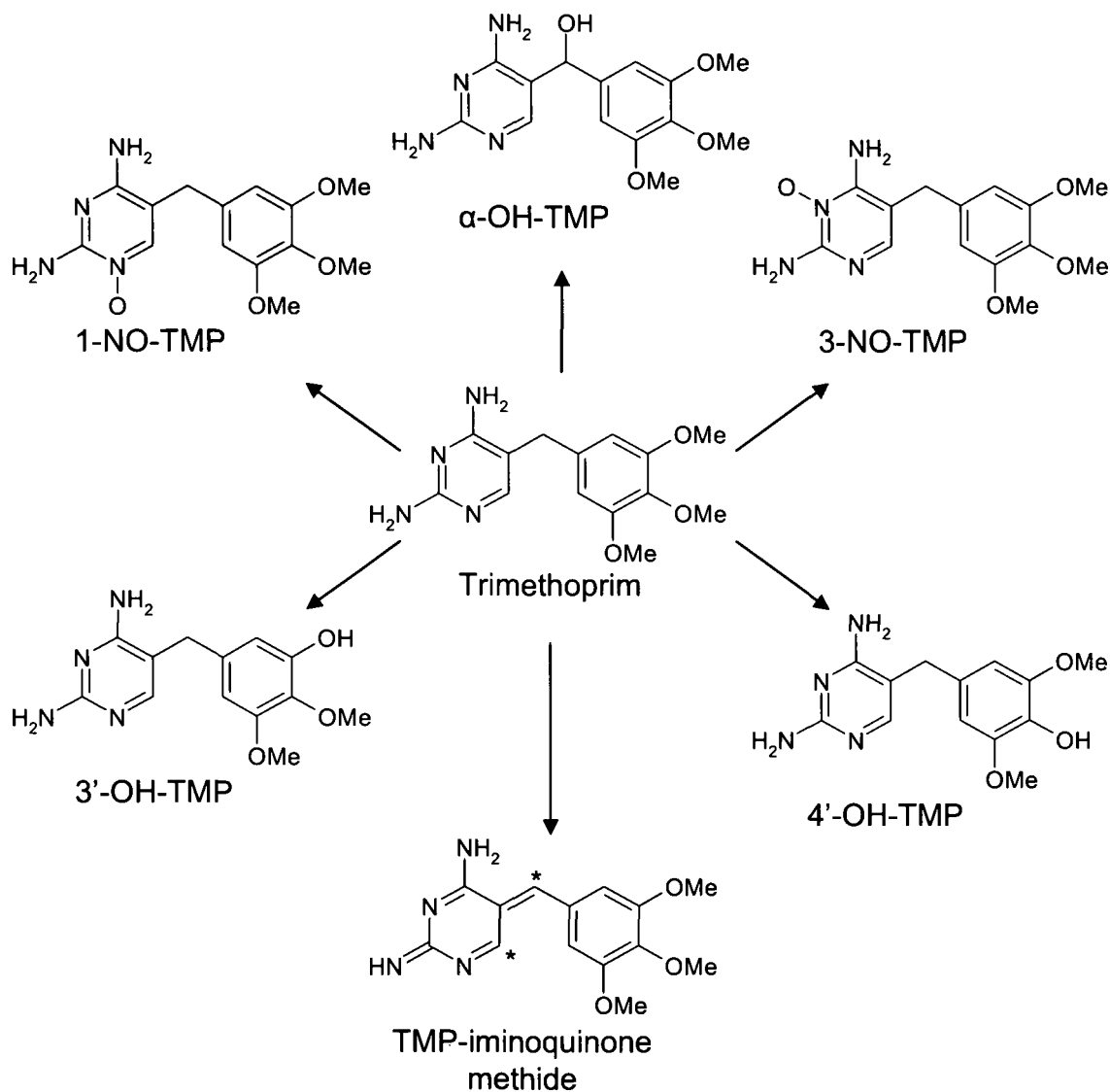
Figure 6.1: Known routes of oxidative TMP metabolism. *: sites of NAC adduct formation

and T-cell clones, in order to explore the requirements for T-cell activation by TMP.

6.2 Materials and Methods

6.2.1 Materials

Lymphoprep was obtained from Nycomed (Oxford, UK). Recombinant human IL-2 was obtained from Peprotech (London, UK). Anti-CD4, Anti-CD8, anti-CD3, anti-Vβ and anti-chemokine receptor antibodies were all obtained from Serotec (Oxford, UK). [^{3}H]-methyl thymidine was obtained from Moravek (California, USA). All other reagents, chemicals and components were supplied by Sigma-Aldrich (Gillingham, UK).

6.2.1.1 Culture medium

T-cell and APC culture media were prepared as described previously (see 2.2.1.1).

6.2.1.2 Isolation of PBMCs

PBMCs were isolated and viability was analysed as described previously (see 2.2.2).

6.2.2 Clinical Details

A 20-year old female was prescribed TMP at 300mg b.d. for acne. 2 weeks after the initial treatment, she developed a maculopapular rash, starting in the legs and spreading to affect the trunk and upper limbs. The TMP was stopped 1 week later (3 weeks from onset of treatment) as the rash was worsening.

She was admitted to a local hospital in the 4th week after onset of treatment with fever, desquamating rash and pruritis. Initial haematology results on admission were white cell count of 24.9×10^6/ml (reference range: 4-11) with eosinophilia of 13% (0-7); and liver function test results as in table 6.1. A blood film performed at the time of initial admission showed atypical lymphocytes with eosinophilia.

She was later transferred to Queen Elizabeth Hospital in Birmingham when her liver tests continued to deteriorate, with aspartate transaminase >6000. Clinical assessment in Birmingham showed ongoing exfoliating rash with facial swelling. Liver function tests on admission in Birmingham were as given in table 6.1.

Table 6.1: Liver function test results

Test	Reference range	On admission	On referral	On discharge
Bilirubin (μM)	2-14	13	283	177
AST (IU/L)	10-34	399	3407	116
ALP (IU/L)	44-147	183	599	358
INR	0.9-1.2	NP	2.4	1

Abbreviations in this table, ALP: alkaline phosphatase, AST: aspartate transaminase, INR: international normalised ratio, IU: international unit, NP: not performed.

Treatment was largely symptomatic, alongside N-acetylcysteine given as a 1mg/kg infusion over 24 h. Steroids were not given, and a liver biopsy sample was not obtained. Eosinophilia peaked 6 days after admission to Birmingham, despite all other haematological tests indicating decreases, and the patient was discharged 8 days after Birmingham admission, with liver function test results as given in 6.1. Blood test results were normal on a 2 month follow-up.

Blood samples for this study were taken upon referral to the Queen Elizabeth Hospital and 3 months after recovery.

6.2.3 Determination of lymphocyte proliferation

Lymphocyte proliferation was measured by means of the LTT, as described previously (Nyfeler & Pichler, 1997, see 5.2.3;). Freshly isolated PBMCs (1.5 x 10^5; total volume 0.2ml) were incubated with TMP (0.1-500μg/ml; either with or without 2mM NaOCl), SMX (1-1000μM), SMX-NO (0.1-50μM) or tetanus toxin (10μg/ml) as a positive control in 96-well U-bottomed tissue culture plates for 6 days (37°C; 5% CO_2). Proliferation was measured by the addition of [^{3}H] thymidine (0.5μCi) for the final 16 h of the incubation. Cells were harvested, and counted as cpm on a β-liquid scintillation and luminescence counter (PerkinElmer Life Sciences, Boston, MA). Proliferative responses were calculated as SI as described previously (see 2.2.6).

In addition, LTTs were performed with two separate modifications: firstly, GSH (1mM) was added to the incubations 30 min prior to addition of the drugs; and secondly, PBMCs (0.5x10^5 or 1.5x10^5/incubation) were either modified with a TMP iminoquinone methide generating system (1-50μg/ml TMP and 2mM NaOCl) or TMP alone (1-50μg/ml), or were left untreated, then were irradiated (3000 rad) and added

to a standard LTT with further PBMCs in fresh media.

6.2.4 Generation of EBV-transformed B-lymphoblastic cell lines

EBV-transformed B-LCLs were generated from the TMP-allergic patient by transformation of freshly isolated PBMCs with supernatant from the EBV-producing cell line B9-58. B9-58 cells were maintained in APC medium, and the supernatant was $0.2\mu m$ filtered to remove remaining cells prior to use. For transformation, 2×10^6 PBMCs were incubated in 5ml B9-58 supernatant, supplemented with cyclosporin A ($1\mu g/ml$) to prevent T-cell mediated suppression of B-cell infection. EBV-transformed B-cell cultures were maintained in APC medium.

6.2.5 Generation of T-cell clones

For the generation of T-cell clones, freshly prepared PBMCs were incubated with TMP ($10\text{--}100\mu g/ml$) in 0.5ml T-cell medium in 48-well tissue culture plates. Cultures were supplemented with IL-2 ($60U/ml$) on days 6 and 9 to maintain drug-specific proliferation. On day 14, T-cell clones were prepared by serial dilution (Schnyder *et al.*, 2000). 0.3, 1 or 3 cells were incubated in 96-well round-bottomed tissue culture plates in $100\mu l$ T-cell medium, supplemented with 5×10^4 allogenic irradiated (3000 rad) lymphocytes, $5\mu g/ml$ phytohaemagglutinin and $60U/ml$ IL-2. T-cells were expanded in T-cell medium with $60U/ml$ IL-2 and restimulated every 14 days with irradiated (3000 rad) allogenic lymphocytes and $5\mu g/ml$ phytohaemagglutinin.

6.2.6 Specificity of T-cell clones

TMP-specific T-cell clones were identified by proliferation assay. 0.5×10^5 T-cells were incubated with autologous irradiated (4500 rad) B-LCLs (0.1×10^5) and TMP (10, $25\mu g/ml$) in a final volume of $200\mu l$ in a 96-well round-bottomed tissue culture plate. After 48 h, [^{3}H]-thymidine was added ($0.5\mu Ci$), and 16 h later proliferation was determined as described earlier. T-cell lines with a positive proliferative response (as determined by SI > 2.5) were taken to be drug-specific and maintained in IL-2 containing medium.

6.2.7 Phenotypic analysis of TMP-specific T-cell clones

Monoclonality of T-cell lines was determined by $V\beta$ analysis. T-cells were incubated with a panel of 25 FITC-labelled anti-$V\beta$ antibodies which detect more than 75% of all known $V\beta$ families, washed repeatedly, and $V\beta$ expression was determined by flow cytometry by comparison to isotype matched controls.

CD4/8 phenotype was determined by dual staining with FITC-labelled anti-CD4 and PE-labelled anti-CD8 antibodies followed by flow cytometry.

6.2.7.1 Chemokine receptor expression on TMP-specific T-cell clones

Chemokine receptor expression was determined by comparison of fluorescence as determined by flow cytometry with a panel of FITC-labelled anti-chemokine receptor (CXCR3, CCR4, CXCR4, CCR5, CXCR6, CCR8) antibodies versus FITC-labelled isotype controls.

6.2.7.2 Cytotoxicity of TMP-specific T-cell clones

TMP-induced T-cell mediated cytotoxicity was investigated through use of a 4-hour [^{51}Cr]-release assay. To measure immune-mediated killing, autologous B-LCLs (1×10^6) were labelled with ^{51}Cr (50μCi) for 1 h. B-LCLs were washed repeatedly to remove free ^{51}Cr and incubated (2.5×10^3; total volume 0.2mL) with T cells at effector:target ratios of 3:1, 10:1, and 30:1 in the presence and absence of TMP (10, 25μg/ml) for 4 h. Spontaneous release was determined by parallel incubations without TMP, and maximal release was determined by repeated freeze-thaw cycles to lyse remaining cells. Specific lysis was therefore calculated as

$$100\% \times \frac{experimental\ release\ -\ spontaneous\ release}{maximal\ release\ -\ spontaneous\ release}$$

Direct toxicity of TMP was excluded by incubating TMP (50μg/ml) with [^{51}Cr]-loaded B-LCLs (2.5×10^3) for 4 h in the absence of T cells.

6.2.8   Role of metabolism and processing in the activation of T-cell clones

Three modifications of the standard proliferation assay were used to explore the role of metabolism, covalent binding, and processing in the activation of TMP-specific T-cell clones.

6.2.8.1   Additional pulse with TMP-modified B-LCLs

The ability of TMP-covalently-modified cells to act as sources of antigens was explored by means of an additional pulsing methodology. In this, B-LCLs were exposed to a TMP-iminoquinone methide generating system ($100\mu g/ml$ TMP, 2mM NaOCl; from Lai et al., 1999), irradiated (4500 rad), and added to fresh T-cells and irradiated (4500 rad) B-LCLs. Proliferation was determined 48 h later as for a standard proliferation assay.

6.2.8.2   T-cell and B-cell pulsing with TMP prior to proliferation assays

The role of covalent binding in T-cell activation with TMP was explored by pulsing of APCs with TMP prior to combining them with T-cells, as previously described (Schnyder et al., 1997, 2000). These experiments involved the incubation of TMP ($25\mu g/ml$) with irradiated (4500rad) B-LCLs (0.1×10^5) for 2 h, followed by repeated washes to remove non-covalently bound drug, and incubation in fresh medium (0.2ml) with additional T-cells and proliferation determined by thymidine incorporation as described previously.

6.2.8.3   Addition of glutathione to proliferation assay

T-cells and irradiated (4500rad) B-LCLs were incubated with GSH (2mM) for 35 min prior to the addition of TMP for a standard proliferation assay. GSH irreversibly binds to TMP iminoquinone methide and hence can inhibit covalent modification of protein (Dieckhaus et al., 2005).

6.2.8.4 TMP presentation by fixed EBV-transformed B-LCLs

Chemical fixation of B-LCLs by glutaraldehyde was performed to evaluate the possibility of antigen processing in T-cell activation by TMP. B-LCLs ($2x10^6$ in 0.5ml HBSS) were fixed by the addition of glutaraldehyde (0.05%) for 30 sec at room temperature. The reaction was terminated by the addition of glycine (0.2M) for 45 sec. Fixed B-LCLs were washed repeatedly, and used in proliferation assays as described previously. Glutaraldehyde inhibits antigen processing but does not alter MHC expression or presentation of preprocessed antigens (Shimonkevitz et al., 1983; Zanni et al., 1997).

6.2.9 Statistical analysis

Results are presented as mean ± standard error of the mean. Normality of data was determined by the Shapiro-Wilks test and equality of variance by the Levene test. Data which were normally distributed were compared by means of a t-test, whereas nonparametric data were compared with the Mann-Whitney test for unpaired data or the Wilcoxon signed rank test for paired data. P-values < 0.05 were considered to be significant. All statistical analyses were performed on SPSS 13.0.

6.3 Results

6.3.1 Lymphocyte transformation test

Lymphocytes from the TMP-hypersensitive patient proliferated in response to either TMP (maximal response 10–25μg/ml; cpm of control incubations, 1222.7±652.5, cpm of test incubations, 6950±5468, 5779±4279; see figure 6.2) or the positive control tetanus toxoid (cpm, 7058±4319), but not to any of the other compounds tested (SMX, SMX-NO). In addition, lymphocyte cultures with 1mM GSH and TMP demonstrated comparable proliferation to lymphocyte cultures with TMP alone. No noteworthy difference, besides a small increase in magnitude, was seen between experiments performed on lymphocytes isolated at the time of the reaction and those undertaken on lymphocytes isolated 3 months subsequently (see figure 6.2).

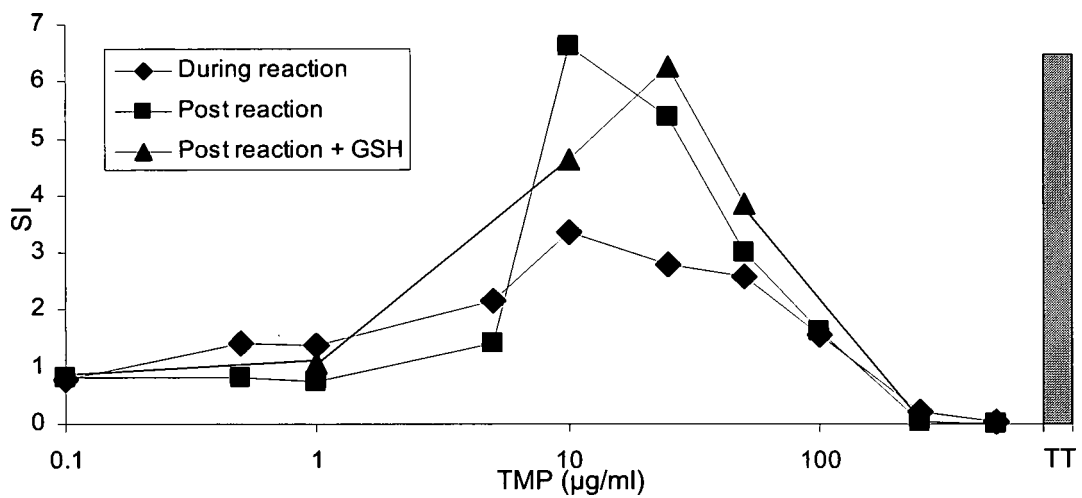Figure 6.2: Proliferation of lymphocytes from a TMP-hypersensitive patient stimulated with TMP either during or 3 months after the hypersensitivity reaction. Drug specific proliferation was determined by incubating lymphocytes with TMP for 5 days, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. TT: tetanus toxin. Results are expressed as the mean SI of triplicate cultures.

When lymphocytes were incubated with a TMP-iminoquinone methide generating system (TMP with 2mM NaOCl), proliferation was completely eliminated. However, this is likely due to a toxic mechanism related to the presence of the hypochlorite, so further experiments used this system simply as a source of antigen generation. In these experiments PBMCs were modified either with the metabolite generating system or TMP alone prior to being irradiated, washed, and added to fresh lymphocytes. A previous study has found that similarly metabolite-modified dead cells can represent a potent source of antigen (Naisbitt et al., 2002). However, although this did not inhibit proliferation to the positive control tetanus toxoid (indicating the absence of a functional inhibition), this was incapable of inducing T-cell proliferation (see figure 6.3).

6.3.2 Generation and characterisation of TMP-specific T-cell clones

Over 336 T-cell clones were investigated for TMP reactivity, of which 58 were found to respond to TMP (maximal response 50–100μg/ml; cpm of control incubations 1115$\pm$215, cpm of test incubations 8553$\pm$2930, 10125$\pm$2922; see figure 6.4). All of the clones thus identified were found to be CD4+, and all clones expressed a single, though varying, TCR Vβ chain, confirming the clonality of the lines. Only 3 of the clones expanded sufficiently to allow chemokine receptor analysis to be performed. Of these clones, 3/3 expressed both CXCR3 and CCR4, and 2/3 additionally expressed CCR8 (fold increase in median fluorescence $\geq$1.4; Wu et al., 2007). None of CXCR4, CCR5 or CCR6 were expressed by any of the clones examined. See figure 6.5 for flow cytometry traces from one representative clone. The 5 clones examined did not demonstrate any immune-mediated killing, even at the highest effector:target ratio of 30:1.

6.3.3 Role of metabolism and antigen processing in the activation of TMP-specific T-cell clones

The addition of TMP-iminoquinone methide modified B-LCLs to the standard proliferation assay was not able to induce a response in any of the T-cell clones tested, although they responded normally to the addition of free TMP (see figure 6.6). Simi-
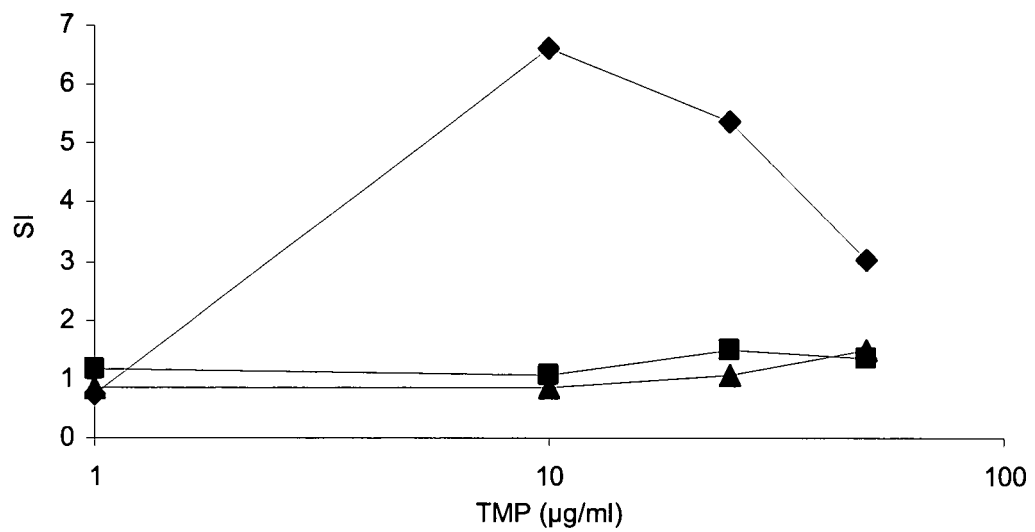
Figure 6.3: Proliferation of lymphocytes from a TMP-hypersensitive patient stimulated with either TMP (diamonds) or TMP-iminoquinone-methide-modified PBMCs $(0.5 \times 10^5$, squares; 1.5×10^5, triangles). Proliferation was determined by incubating lymphocytes with TMP for 5 days, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean SI of triplicate cultures.
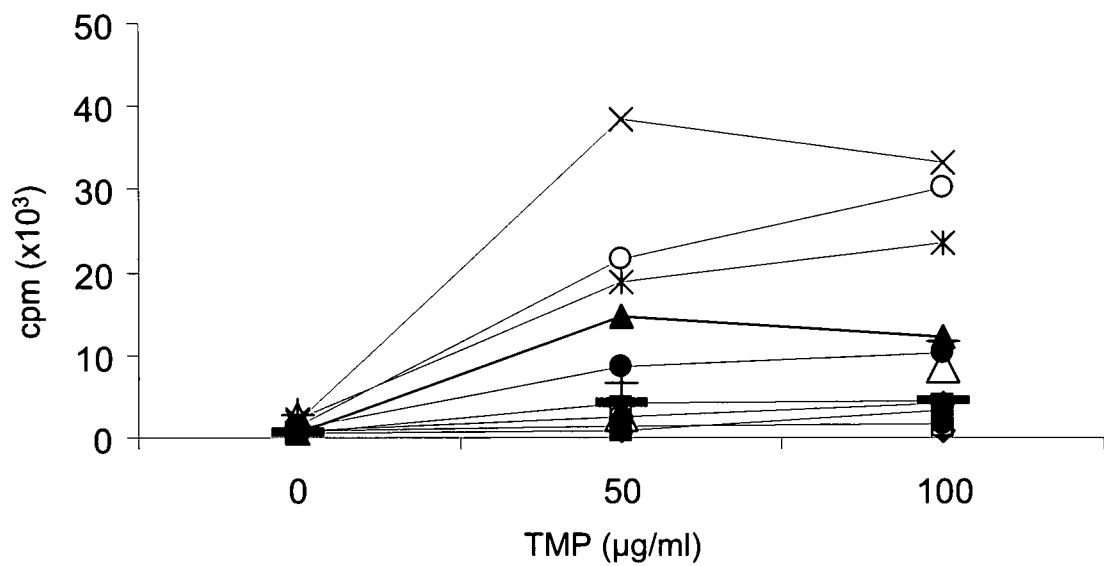
Figure 6.4: Proliferation of 12 T-cell clones isolated from a TMP hypersensitive patient and stimulated with TMP. Drug specific proliferation was determined by incubating T-cell clones with TMP and B-LCLs as APC for 48 h, followed by the addition of [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures.
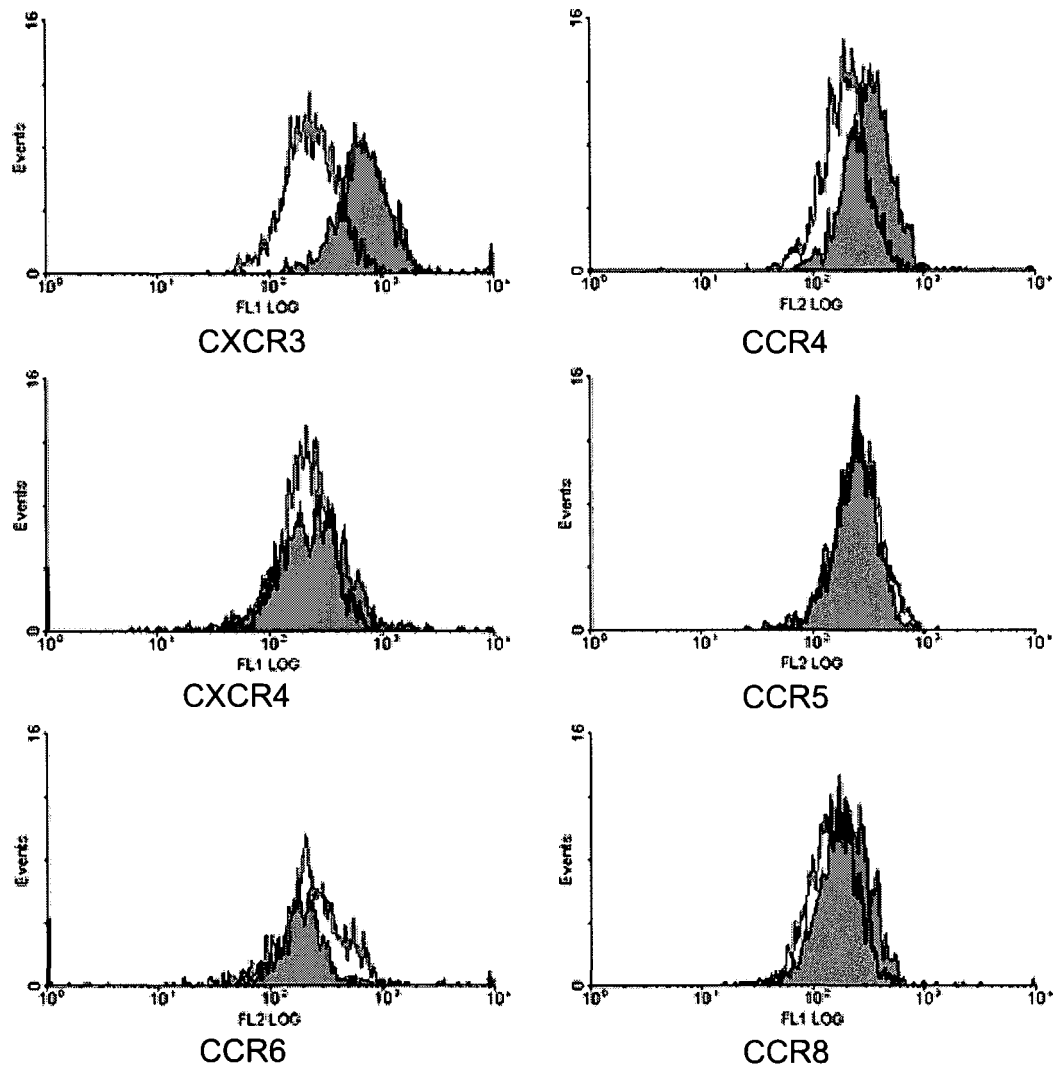
Figure 6.5: Flow cytometry traces from one representative T-cell clone demonstrating expression of CXCR3, CCR4 and CCR8. T-cell clones were incubated with labelled anti-chemokine receptor antibodies (filled histograms) or isotype-matched controls (unfilled histograms), and expression determined by comparison.

larly, B-LCLs pulsed with TMP and washed were incapable of activating T-cell clones which responded to free TMP (see figure 6.6). These data indicate that the T-cell clones required the simultaneous presence of T-cells, B-LCLs and free TMP to induce T-cell proliferation, in line with the p-i concept of T-cell activation by small molecules (Pichler, 2002). Furthermore, the addition of GSH, which would be expected to reduce the formation of protein adducts by the iminoquinone methide metabolite, had no effect on the proliferation of clones to TMP.

B-LCLs fixed with glutaraldehyde were found to be equally effective in the induction of T-cell proliferation as viable B-LCLs (see figure 6.6), suggesting that functional internal machinery for antigen processing is not required, and hence that the drug is presented on the surface of peptide/MHC molecules in the absence of antigen processing (Schnyder *et al.*, 1997).
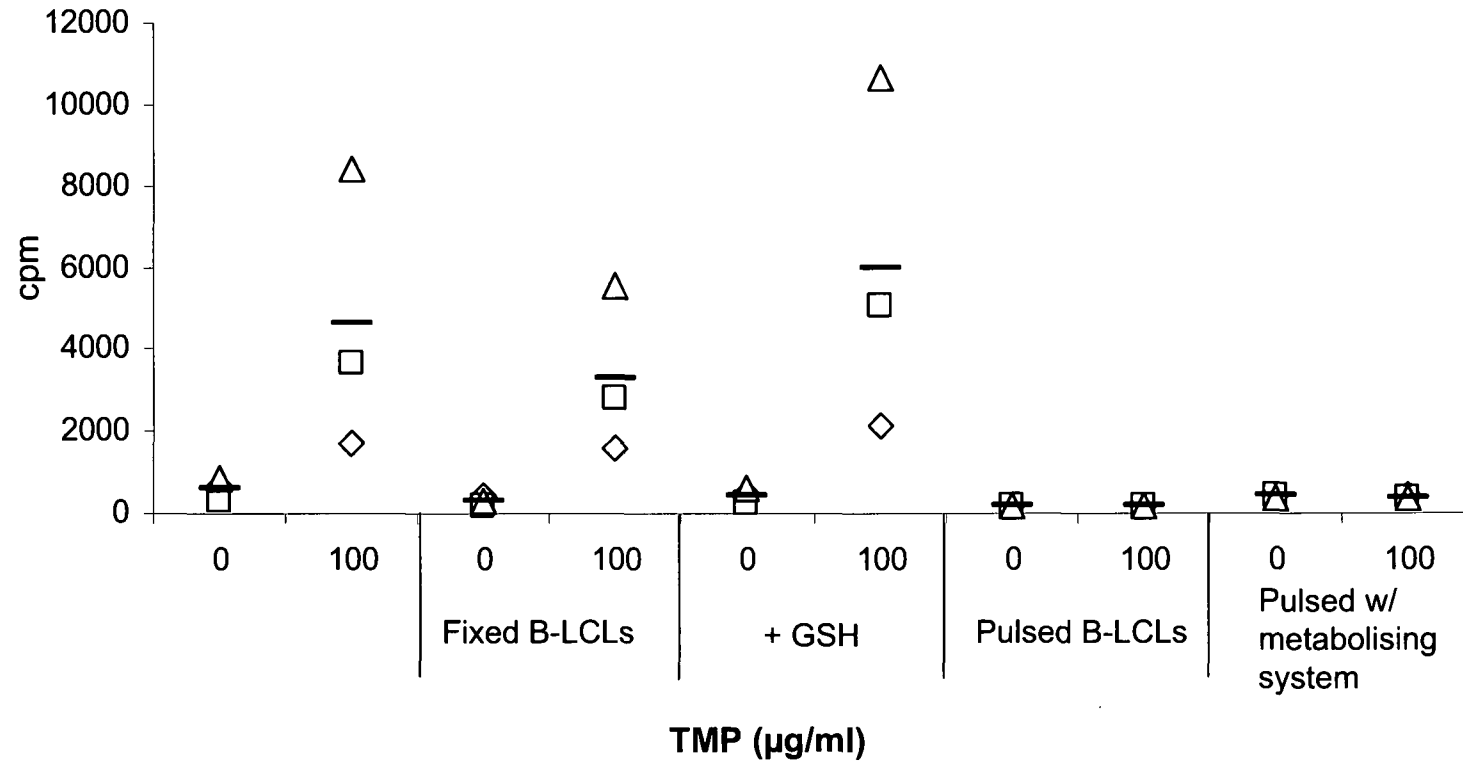
Figure 6.6: Proliferation of T-cell clones isolated from a TMP hypersensitive patient. Drug specific proliferation was determined by incubating T-cell clones either with TMP and B-LCLs as APC for 48 h, or with certain modifications as described in the text, followed by the addition of [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures. *: p<0.05 compared to control incubations

6.4 Discussion

While TMP is known to be associated with delayed-type hypersensitivity reactions, little work has been done on the mechanisms behind these reactions. As TMP is often used in conjunction with SMX, which induces very similar adverse reactions, in many cases it is hard to be sure which drug is the actual culprit. This case was particularly interesting as it not only offered a case where the culprit drug was unquestionably TMP, as the patient was solely exposed to this, but it also encompassed severe liver damage. While this is not unusual in drug hypersensitivity, it often requires transplantation and consequent immunosuppression, making future investigations of immune function difficult to perform and harder to interpret. Therefore, investigations into this case should shed some light on T-cell responses to TMP, as well as the mechanisms by which liver involvement is induced.

The clinical triad of fever, rash and systemic involvement is characteristic of drug hypersensitivity syndrome, and is often observed in conjunction with an elevated white cell count and eosinophilia. It is believed that these reactions have an immunological aetiology, although this has never been unquestionably demonstrated. T-cells isolated from the patient readily proliferated in response to *ex vivo* rechallenge with TMP, with a maximal response to TMP at $\sim$10–25μg/ml. This is in keeping with several previous studies with similar drugs (Naisbitt *et al.*, 2003a,b; Schnyder *et al.*, 1997), and with the studies with CBZ described earlier (see chapter 5). Furthermore, T-cell clones derived from the patient's PBMCs were also capable of reacting to TMP when cultured with autologous B-LCLs as antigen-presenting cells. These data, in conjunction with the clinical case history discussed earlier, strengthen the case that the adverse reactions experienced by the patient were immunological in nature.

One interesting facet of the LTT data was that a proliferative response to TMP was observed both during and, more strongly, following the reaction. This is unusual, as LTTs performed on PBMCs isolated at the time of the reaction are often unsuccessful (Naisbitt, D.J., unpublished observations). It is not known why this should be the case, despite speculation that it might be related either to extravascular compartmentalisation of drug-reactive T-cells or to excessive background activation of T-cells

leading to overwhelming of the drug-specific response, nor why this case in particular was unusual. However, attempts to clone T-cells from the PBMCs taken during the reaction were unsuccessful, and so all of the data generated from clones was performed with clones isolated post-reaction.

While the stable routes of oxidative TMP metabolism are relatively well understood (see figure 6.1), less is known about how TMP reactive metabolites are formed. One study identified a iminoquinone methide through oxidation of the pyrimidine ring, which reacted with biological nucleophiles to form cysteinyl adducts. Therefore, a metabolite-generating system was derived from this paper, consisting of TMP and NaOCl. This was intrinsically toxic, and was unsuitable for addition directly to lymphocyte assays, so an additional pulse methodology was attempted, based on previous findings that SMX-NO modified cells, both alive and dead, can act as a source of antigen for the activation of T-cells from SMX-NO treated animals (Naisbitt *et al.*, 2002). In this study, this involved modification of PBMCs or B-LCLs with TMP and NaOCl so as to use as a source of antigen in an incubation with either fresh PBMCs or T-cell clones and B-LCLs. However, this approach was unsuccessful at inducing a TMP-metabolite specific T-cell response. More work is required to determine if this is simply due to a failure of the experimental system to generate the appropriate, *in vivo*-equivalent, antigen (quite apart from any other consideration, the antigens in this case would be in large part localised on the surface rather than intracellularly), or was a more general failure of the experimental methodology, or actually reflected the genuine lack of antigenicity of TMP-modified proteins.

The addition of GSH had no effect on the proliferation of lymphocytes in response to TMP, giving further weight to the suggestion that the T-cells recognise the drug in the absence of metabolism. However, the reactions of GSH with TMP reactive metabolites have not been explored in sufficient detail to know whether this addition could be sure to completely eliminate all TMP-metabolite adduct formation.

Studies with T-cell clones isolated from the patient allow greater manipulation of the processes of T-cell activation than those performed with PBMCs, albeit at the expense of some proximity to the *in vivo* situation. The universal CD4+ status and lack

of cytotoxicity of the clones extracted was not unexpected, as CD4+ clones typically make up the majority of all clones isolated in this way. This is likely to be an artefact of the methodology; in particular, the requirement for *ex vivo* enrichment, which will give selective advantages to those clones which proliferate more rapidly in response to phytohaemagglutinin, and the use of TMP-specific proliferation as a marker of TMP-selectivity. A recent study by Wu *et al.* (2007) on CBZ hypersensitivity has attempted to address these concerns by various alterations to the cloning methodology, and was successful in generating significant numbers of drug-specific CD8+ clones. It might therefore be interesting to apply these methodologies to this case, in order to explore the antigen-specificity of TMP-specific CD8+ T-cells, as these are likely to be the final effector cells of the reaction.

Chemokines form a large family of >50 small proteins (8-10kDa), which play an important role in the migration, adhesion, and tissue infiltration of immune cells. In particular, chemokines presented on the luminal surface of the endothelium induce arrest of rolling lymphocytes and promote transendothelial migration. Once within the tissue, the cells undergo positive chemotaxis along a chemokine gradient towards the site of inflammation (Lalor & Adams, 2002). In the resting state, tissue infiltration is controlled both by reductions in the quantity of chemokines released from the tissue, but also through limited expression of chemokine receptors on the surface of resting T-cells. T-cell clones, as used in this study, are permanently activated, as they are maintained in medium containing IL-2, and so their chemokine receptor expression can be used as a marker to identify their particular tissue-homing characteristics.

All of the clones which were examined expressed the liver-homing chemokine receptor CXCR3, although not CCR5 or CXCR6, both of which are known to act as liver homing receptors (Lalor *et al.*, 2002). Furthermore, the skin homing receptor CCR4 was uniformly expressed, and 2 out of 3 clones examined expressed the skin homing receptor CCR8. This is in keeping with the known clinical characteristics of the reaction, with both skin and liver involvement. However, too few clones expanded sufficiently *in vitro* to allow solid conclusions to be drawn from this data, and further work is required, both to confirm the initial findings, and to contrast this pattern with those known from

both hypersensitivity reactions with different patterns of organ involvement, and from non-hypersensitive control individuals.

The nature of the antigen presented to TMP-specific T-cell clones was explored in a series of modifications to the standard proliferation assay. In these, TMP was successfully presented to T-cells on the surface of glutaraldehyde-fixed B-LCLs, and B-LCLs pulsed either with TMP alone or a TMP-iminoquinone methide generating system were found to be incapable of activating TMP-specific T-cell clones. These experiments are reminiscent to those performed earlier with CBZ-specific T-cells (see chapter 5), and many of the specific points raised in the discussion to that chapter relate equally to these experiments. Further work to determine the time-course of clonal activation would be interesting with regards to confirming the lack of dependence on processing, but this caveat notwithstanding, the data generated suggest that the clones respond to the parent drug via reversible interactions, as per the p-i concept, and as has been demonstrated both above, for CBZ, and previously, for a wide range of structurally unrelated drugs (Naisbitt et al., 2003b, 2005a; Schnyder et al., 1997; Zanni et al., 1998).

There are several possibilities by which reversible interactions could be formed between TMP and cellular macromolecules such as MHC-peptide or TCR complexes (see the discussion in 5.4 and table 5.3 for a fuller discussion of these interactions). These include the strong interactions afforded by ionic bonding, as TMP is a weak base with a pKa of 7.3 (Nowak et al., 1985) and hence the primary amine groups will be approximately 50% ionised at physiological pH; hydrogen bonds, both accepted by the nitrogen of the primary amines and the oxygens of the methoxy groups, and donated by the primary amine hydrogens; and Van der Waals forces, which require a close structural fit between the molecule and the 3-dimensional configuration of the macromolecule in question. Unlike for CBZ, it is unlikely that hydrophobic interactions will play a large role in this binding, as the two aromatic rings within the molecule are somewhat shielded by polar groups. A previous study has explored the fine specificity of TMP-specific antibodies using hapten inhibition studies (Pham et al., 1996), and has found that the sera from different patients fell into 3 groups: 2/8 responded solely to

the whole molecule, 1/8 recognised only TMP and diaveridine, a desmethoxy derivative of TMP, and the remaining 5/8 specifically recognised just the 3,4-dimethoxybenzene determinant. However, there are significant differences between the stringency of antibody binding and that of T-cell binding, and these data were produced from patients following immediate, type I immune reactions. Therefore, it would be interesting to use a similar panel of structural analogues of TMP in order to explore the precise specificities of 'TMP-specific' T-cell clones.

It is again worth mentioning that these findings relate solely to the antigen specificity of T-cell clones *subsequent* to the initiation of the reaction, and therefore can only inform us as to the *antigenicity* of TMP, not its *immunogenicity*. In other words, we are still left uncertain about the initial antigenic trigger which induced the reaction, and further work, with novel and innovative methodologies will be required in order to delve deeper into the fundamental mechanisms involved in the initiation and propagation of hypersensitivity reactions.

Chapter 7

Final Discussion

Adverse drug reactions are a major healthcare problem, both directly—that is to say, in the clinic—and indirectly, as regards drug development. Large-scale epidemiological studies place the total financial burden of adverse reactions on healthcare in this country at approaching £400m per annum, and claim that as many as 5% of all hospital inpatient episodes are directly due to them (Pirmohamed *et al.*, 2004). While the majority of these reactions are dose-dependent (i.e. Type A according to the Rawlins & Thompson (1991) scheme; see 1.1.1), a significant proportion (~20%) are idiosyncratic (Type B). While these are less common than type A reactions, they are often more serious, and have been the cause of several recent drug withdrawals (such as troglitazone or trovafloxacin). It is important to gain an immunotoxicological understanding of these reactions in order to better predict and prevent them. In order to advance this understanding, the aims of this study were to explore the possibility that drug metabolism and the generation of reactive drug metabolites play an important part either in the induction or propagation of drug hypersensitivity reactions.

In order to do this, three paradigm drugs with known associations with hypersensitivity reactions, SMX, CBZ and TMP, were investigated as sources of signal 1 or signal 2. This discussion will briefly recap the findings and conclusions drawn by the individual studies, and attempt to synthesise these so as to draw more wide-ranging conclusions about the impact these findings may have on our understanding of drug hypersensitivity reactions in general.

Firstly, SMX was studied in order to explore the possibility that either it or its known reactive metabolite SMX-NO can supply the requisite signal 2 for the induction of an immune response. In Chapter 2, this hypothesis was investigated through incubating monocyte-derived dendritic cells, used as a model of an antigen-presenting cell, with either SMX or SMX-NO, and looking for changes in the expression of surface co-stimulatory markers which are associated with maturation and activation of dendritic cells. These changes are an essential part of a full immune response to antigen, and in their absence, antigen presentation is tolerogenic, not immunogenic. It was found that incubation of dendritic cells with either SMX or SMX-NO, at concentrations within the therapeutic range, induced up-regulation of a key co-stimulatory molecule, CD40. This surface marker acts as the natural receptor for the CD154 ligand, which is expressed on the surface of activated T-cells, and induces full maturation of the dendritic cell affected (see 1.3.3 for a fuller treatment of these responses). Up-regulation of CD40, therefore, is believed to be an important step in the induction of an immune response. This proposition, with regards to SMX hypersensitivity, was tested by means of two models of SMX-NO immunogenicity: the murine local lymph node assay; and an *in vitro* primary stimulation assay. In both cases inhibition of CD40 signalling prevented the generation of a primary T-cell response to SMX-NO, and enhancement of CD40 signalling led to a significantly enhanced primary stimulation *in vitro*. These data provide compelling reasons to believe that CD40 signalling plays an important role in the transduction of co-stimulatory signals between T-cells and APCs, and therefore that the observed modulation of the expression of these molecules by SMX and SMX-NO may have immunotoxicological consequences.

The mechanism by which this phenomenon occurs was explored in a later study (see Chapter 3). Here, three possible mechanisms were systematically considered and tested: cell death, either necrotic or apoptotic; glutathione depletion; or covalent modification of protein. Of the mechanisms tested, two, glutathione depletion and cell death, required greatly increased concentrations of SMX-NO and were not observed at any concentration of SMX examined. Protein adduct formation, by contrast, was observed both with SMX and SMX-NO, and both at concentrations comparable to

those which had earlier been found to induce CD40 up-regulation on dendritic cells. As SMX is incapable of directly binding to protein, this is indirect evidence of oxidative metabolism within the incubation. In addition, the preincubation of dendritic cells with ABT, a metabolic enzyme inhibitor with a broad specificity was found to inhibit the CD40 up-regulation induced by SMX, but not that produced by SMX-NO or LPS, offering further evidence that the response to SMX was secondary to oxidative metabolism within the cell. It is also possible that production of reactive oxygen species as a consequence of SMX metabolism may play an additional role, and further work is required to investigate this possibility.

In order to explore the possibility of dendritic cell metabolism, the dendritic cell expression of a panel of metabolic enzymes, including several CYPs and peroxidases, was determined, along with the activities of the enzyme to catalyse SMX N-hydroxylation (although this was not as clear-cut as previous studies might have suggested—see Chapter 4 for details). It was found that the only enzyme on the panel which was expressed in dendritic cells, active with respect to SMX N-hydroxylation, and readily inhibited by ABT, was MPO. This does not preclude the possibility that an unexamined enzyme, for instance, FMO-3 or lactoperoxidase, may also be expressed in dendritic cells and be active in this regard, but it does demonstrate that MPO is likely to be the enzyme responsible, at least in part.

The main conclusion from these studies is that localised bioactivation and metabolism can induce dendritic cell maturation, and hence has the potential to supply the requisite signal 2 for a immune reaction. We have demonstrated this for SMX, however, further work is now needed to see if this concept extends to other drugs: importantly, many other drugs which are associated with immune-mediated adverse reactions are also known to be metabolised to reactive intermediates by MPO, such as, *inter alia*, CBZ (Furst & Uetrecht, 1993), TMP (Lai *et al.*, 1999), phenytoin (Uetrecht & Zahid, 1988), and lamotrigine (Lu & Uetrecht, 2007). This work will explore the possibility that these compounds have similar effects to SMX when incubated with dendritic cells. Were it to be the case, then it is not hard to see how this would be useful for the purposes of drug discovery, as tests for the potential to cause hypersensitivity are poor at

present. For instance, *in vitro* testing for the ability of novel drugs or drug metabolites to activate dendritic cells may offer insights into their potential to induce hypersensitivity reactions. A parallel for this would be the recent studies into the possibility of using dendritic cell activation as a marker for the potential of a chemical to cause contact sensitisation, and hence to replace *in vivo* testing for cosmetics and related household goods (Python *et al.*, 2007; Sakaguchi *et al.*, 2006). However, it is likely that the complexity of hypersensitivity reactions will continue to confound attempts to accurately predict them for the near future.

While these studies have concentrated on the possibility that localised SMX metabolism and covalent binding by dendritic cells can provide a source of signal 2 in the induction of an immune response, they have not addressed the equally compelling question of whether or not this localised metabolism and covalent binding might be able to generate sufficient covalent adducts to act as a source of antigen, or signal 1. SMX-NO has been found to activate T-cells isolated from SMX-hypersensitive patients, and so, if the quantity of SMX-NO generated in dendritic cells is sufficient, it appears quite plausible that dendritic cells incubated with SMX might be able to activate T-cells via a covalent-binding-dependent hapten-like mechanism. However, the only time this proposition has been tested, dendritic cells were found to present a drug (CBZ) to T-cells in a metabolism- and processing-independent fashion (Wu, 2006), indistinguishable from that observed with EBV-transformed B-LCLs. In this study, the clones used were derived by *ex vivo* enrichment with parent CBZ, and so it could be argued that they do not satisfactorily reflect the *in vivo* scenario. Further work is clearly required in order to thoroughly test this proposition. In particular, this work will require the assessment of parallel *in vitro* and *in vivo* metabolism and immunogenicity, including analysis of MHC-presented peptide antigens, and their use in order to interrogate the T-cell response *in vivo*. Analysis of such low-abundance peptides presents significant technical difficulties, and advances in proteomic technologies may be required in order to isolate and identify them, but this will potentially answer many of the important questions about drug hypersensitivity. A recent study (Yang *et al.*, 2007) has attempted to explore these issues with regard to CBZ hypersensitivity by looking for differences

between the antigenic peptide library presented by an HLA-B*1502 transgenic B-LCL both with and without CBZ treatment, including attempts to identify CBZ-modified peptides. Although this study was unsuccessful, it stands as an example of the kind of study which will be required in order to truly explore the fundamental mechanisms of drug hypersensitivity.

One of the key unanswered questions in the field of drug hypersensitivity reactions concerns the nature of the antigen(s) involved, and whether the original antigen—that to which the immune reaction is first stimulated—and the ultimate antigen—that which stimulates propagation of the response, and is involved in rechallenge—are necessarily identical. Chapters 5 and 6 describe studies which were performed to explore the characteristics of the ultimate antigen involved in cases of CBZ and TMP hypersensitivity.

CBZ was found to activate T-cells in a metabolism- or covalent binding-independent fashion, as pulsed APCs were unable to present antigen and neither ABT or GSH inhibited the response. The response also appeared to be processing-independent, as it happened more rapidly than the time required for the internal processing machinery and was not inhibited by fixation of the APCs. This has been postulated for several drugs previously, but rarely with such a detailed study of the mechanisms as was performed in chapter 5. TMP was also found to activate T-cells in a processing- and metabolism-independent fashion, and TMP-iminoquinone methide modified cells, used as a source of antigen, were ineffective at T-cell activation. This demonstrates that, like CBZ, and several previously studied drugs, TMP can activate T-cells in the absence of covalent binding and hapten formation. However, these findings only offer guides as to what the initial antigen might be, as in all similar cases they require T-cells isolated from patients subsequent to the initiation of the reaction.

These studies allow a greater knowledge of the range of potential antigens the immune system can 'see'. This is important, since early arguments for the hapten hypothesis relied upon the perceived impossibility of TCR recognition of small molecules. Clearly then, if the conclusions from these studies—that small molecules can be recognised by specific TCRs—are valid, then these arguments for the importance of the hap-

ten hypothesis are not. Of course, there are many other arguments for the importance of covalent binding in drug hypersensitivity, such as the considerable immunogenicity of many reactive metabolites *in vivo*, the existence of SMX-NO reactive T-cells in SMX-hypersensitive patients, and the associations between metabolite exposure and hypersensitivity, and so these data can only be seen as a step towards a greater understanding, but this story still illustrates how these findings can have implications, even if questions remain about their relevance to the *in vivo* situation at the initiation of a reaction.

The chemical requirements for binding of CBZ to the TCR or MHC-peptide complex were also investigated in chapter 5 through structure-activity relationship analysis. The findings here suggested that certain regions of the molecule, such as the aromatic rings, appeared to predominate with respect to this binding, in that modifications there were less well tolerated than other sites of modification such as the unsaturated bond within the iminostilbene moiety. These allow a small insight into the mechanisms at play within the immunological synapse, and further work, with novel compounds— for instance, halogenated CBZ derivatives—will lead to a better understanding of the requirements for TCR-MHC binding, and maybe even help to design drugs without the potentiality for these kinds of reactions.

The possibility that T-cells can interact with relatively small parts of a molecule is reminiscent of antibody binding, in which a similar phenomenon is well recognised. Although the binding between TCR and antigen is significantly more stringent than that between antibody and antigen, there are a number of interesting parallels which will be explored here. The specificity of antibodies to small molecules has been investigated in some detail, including that to ethinyloestradiol (Monnet *et al.*, 2002; Park & Whittaker, 1978), TMP (Pham *et al.*, 1996), penicillins (Moreno *et al.*, 1995) and SMX (Gill *et al.*, 1997). The main finding of interest from these studies, at least for the purposes of this discussion, is that there is a considerable degree of degeneracy and promiscuity within the system, i.e. individual monoclonal antibodies are cross-reactive to different structures, and different structures are recognised by multiple clones of antibodies (Parnes, 2004). Immunisation with a given antigen thus leads to a polyclonal response,

involving multiple antibodies each with subtly differing fine specificities. Similarly, this has been observed amongst antigen-responding populations of T-cells (Wucherpfennig, 2004), and is believed to be an intrinsic element in the immune system. However, several studies have looked for the degree of clonality amongst drug-responsive populations of T-cells, and have found considerable heterogeneity (Pichler *et al.*, 1997); some studies have found the expected polyclonality (Naisbitt *et al.*, 2003a), however, many other studies have found suggestions of oligoclonality, with few Vβ TCR chains involved (Mauri-Hellweg *et al.*, 1995; Nassif *et al.*, 2002). In these cases, however, the responses have been directed at the free drug in solution, and so may have missed T-cells directed at haptenic derivatives. In order to fully explore this possibility, and to define the limits of cross-reactivity between free drug and covalently-bound material, it may be necessary to design and synthesise modified peptides, which can be used directly in order to bypass the cellular mechanisms of antigen processing. This approach has been previously successful when applied to the study of certain haptens, such as dinitrohalobenzenes (Kohler *et al.*, 1995; Martin *et al.*, 1993) and penicillins (Padovan *et al.*, 1996, 1997), but has never been applied to the study of pro-haptens such as the drugs used in this study.

In the general introduction to this thesis, the events which take place during the course of an immune reaction to a drug were sketched out (see 1.3.1). To conclude, I will describe how the findings reported in this thesis enhance our understanding, and try to answer some of the questions which were left unanswered originally.

The source of the danger signals involved in the activation of dendritic cells during the initiation of an immune response to a drug was considered, and three possibilities suggested—viral or bacterial infection, or the cellular damage produced by reactive metabolites. Of these, it was noted that dendritic cell activation by the reactive metabolites of drugs was plausible, but that there was no evidence to suggest it occurred. The work presented in chapters 2 and 3 indicate that, at least for SMX, the process of localised metabolism can generate a toxic signal which leads to dendritic cell activation. This is likely to not be the only mechanism, as bacterial and viral infection may both occur concurrently.
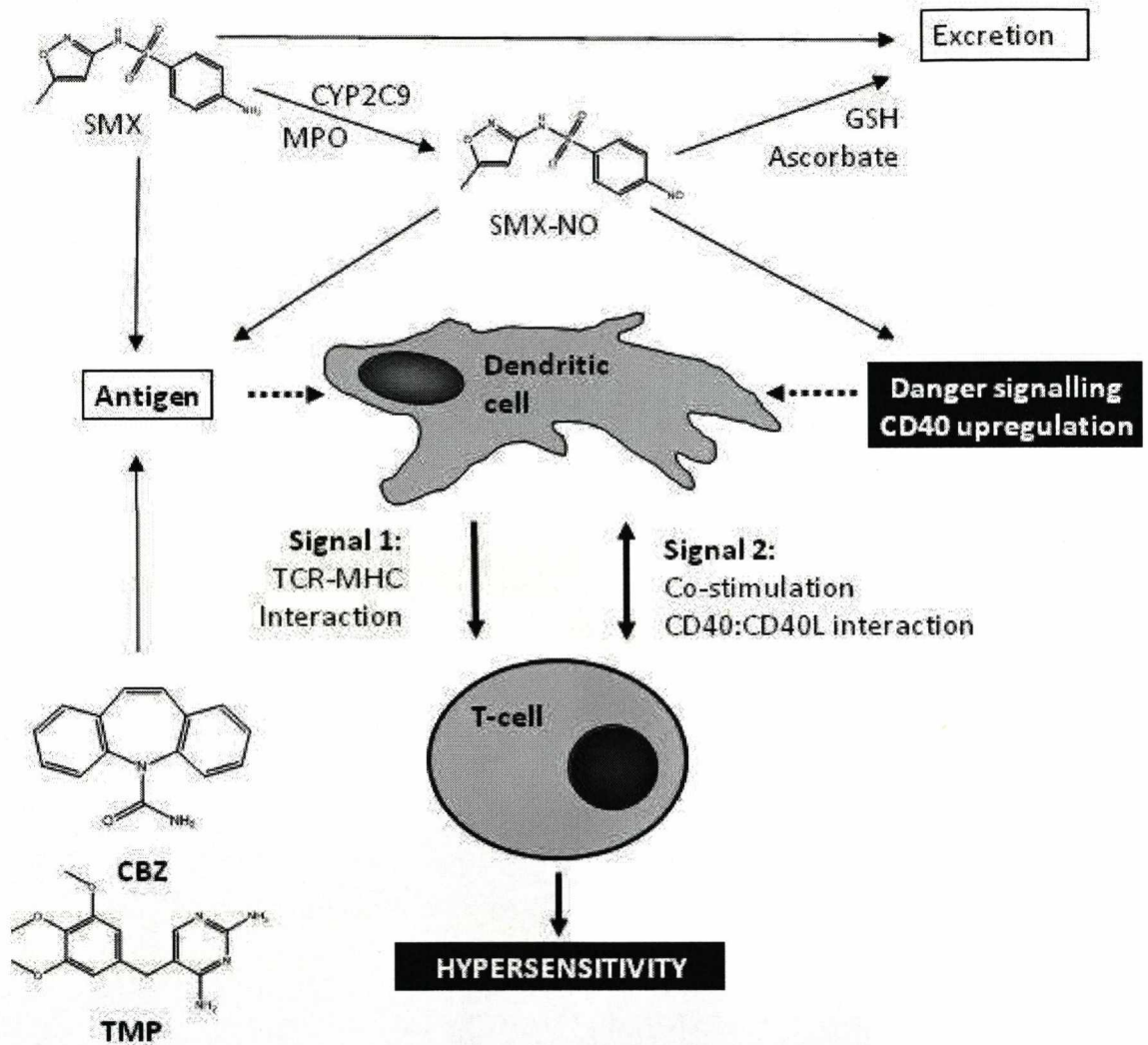
Figure 7.1: Routes by which the drugs studied herein have been found to activate the immune system in the induction of a hypersensitivity reaction

The next important question arising from our discussion of the processes involved in hypersensitivity was regarding the nature of the antigen recognised by T-cells. Whether effector T-cells in drug hypersensitivity respond to free drug or haptens, or both, is one of the most important unanswered questions in the field of hypersensitivity. The data presented in chapters 5 and 6 provide evidence that, at least for T-cells isolated following the initial sensitisation event, they react *in vitro* to the parent drug in a processing- and metabolism-independent fashion.

In conclusion then, these studies offer some advances towards a greater understanding of the role of metabolism in drug hypersensitivity, in that they show that metabolism may not be necessary for the source of signal 1, the T-cell specific antigenic signal, yet appears able to induce signal 2, or dendritic cell activation. Further work will better delineate these two important mechanisms, and allow insight into possible crosstalk between them.

Bibliography

ABBAS, A. & LICHTMAN, A. (2003). *Cellular and Molecular Immunology*. 5th edn. Philadelphia: Saunders.

ABE, R., SHIMIZU, T., SHIBAKI, A., NAKAMURA, H., WATANABE, H., & SHIMIZU, H. (2003). Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. *Am J Pathol*, **162**, 1515–1520.

ACHARYA, N., SMITH, A., HAWKINS, Z., STRICKLAND, I., & FRIEDMANN, P. S. (2001). Carbamazepine-specific T cells are detected in drug-allergic patients after metabolic bioactivation by peripheral blood mononuclear cells. *J Invest Dermatol*, **117**, 800.

AHLSTEDT, S. & KRISTOFFERSON, A. (1982). Immune mechanisms for induction of penicillin allergy. *Prog Allergy*, **30**, 67–134.

AIBA, S. & KATZ, S. I. (1990). Phenotypic and functional characteristics of in vivo-activated langerhans cells. *J Immunol*, **145**, 2791–6.

AIBA, S., MANOME, H., NAKAGAWA, S., MOLLAH, Z. U., MIZUASHI, M., OHTANI, T., YOSHINO, Y., & TAGAMI, H. (2003). p38 mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl2 and 2,4-dinitrochlorobenzene. *J Invest Dermatol*, **120**, 390–9.

AIBA, S., MANOME, H., YOSHINO, Y., & TAGAMI, H. (2000). In vitro treatment of human transforming growth factor-beta1-treated monocyte-derived dendritic cells with haptens can induce the phenotypic and functional changes similar to epider-

mal langerhans cells in the initiation phase of allergic contact sensitivity reaction. *Immunology*, **101**, 68–75.

AIBA, S., TERUNUMA, A., MANOME, H., & TAGAMI, H. (1997). Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur J Immunol*, **27**, 3031–8.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R., & FLAVELL, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, **413**, 732–738.

ALFIREVIC, A., JORGENSEN, A. L., WILLIAMSON, P. R., CHADWICK, D. W., PARK, B. K., & PIRMOHAMED, M. (2006). HLA-B locus in Caucasian patients with carbamazepine hypersensitivity. *Pharmacogenomics*, **7**, 813–818.

ALFIREVIC, A., STALFORD, A. C., VILAR, F. J., WILKINS, E. G. L., PARK, B. K., & PIRMOHAMED, M. (2003). Slow acetylator phenotype and genotype in HIV-positive patients with sulphamethoxazole hypersensitivity. *Br J Clin Pharmacol*, **55**, 158–165.

AMRANI, A., VERDAGUER, J., SERRA, P., TAFURO, S., TAN, R., & SANTAMARIA, P. (2000). Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature*, **406**, 739–742.

APPLEMAN, L. J. & BOUSSIOTIS, V. A. (2003). T cell anergy and costimulation. *Immunol Rev*, **192**, 161–80.

ARDESHNA, K. M., PIZZEY, A. R., DEVEREUX, S., & KHWAJA, A. (2000). ThePI3 kinase, p38 MAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood*, **96**, 1039–46.

ARRIGHI, J. F., REBSAMEN, M., ROUSSET, F., KINDLER, V., & HAUSER, C. (2001). A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J Immunol*, **166**, 3837–45.

BIBLIOGRAPHY

ASEA, A., KRAEFT, S. K., KURT-JONES, E. A., STEVENSON, M. A., CHEN, L. B., FINBERG, R. W., KOO, G. C., & CALDERWOOD, S. K. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med*, **6**, 435–442.

BALANI, S. K., ZHU, T., YANG, T. J., LIU, Z., HE, B., & LEE, F. W. (2002). Effective dosing regimen of 1-aminobenzotriazole for inhibition of antipyrine clearance in rats, dogs, and monkeys. *Drug Metab Dispos*, **30**, 1059–1062.

BARKER, R. N., ERWIG, L.-P., HILL, K. S. K., DEVINE, A., PEARCE, W. P., & REES, A. J. (2002). Antigen presentation by macrophages is enhanced by the uptake of necrotic, but not apoptotic, cells. *Clin Exp Immunol*, **127**, 220–225.

BARON, J. M., HOLLER, D., SCHIFFER, R., FRANKENBERG, S., NEIS, M., MERK, H. F., & JUGERT, F. K. (2001). Expression of multiple cytochrome p450 enzymes and multidrug resistance-associated transport proteins in human skin keratinocytes. *J Invest Dermatol*, **116**, 541–8.

BARON, J. M., ZWADLO-KLARWASSER, G., JUGERT, F., HAMANN, W., RUBBEN, A., MUKHTAR, H., & MERK, H. F. (1998). Cytochrome P450 1B1: a major P450 isoenzyme in human blood monocytes and macrophage subsets. *Biochem Pharmacol*, **56**, 1105–10.

BAUSINGER, H., LIPSKER, D., ZIYLAN, U., MANIE, S., BRIAND, J.-P., CAZENAVE, J.-P., MULLER, S., HAEUW, J.-F., RAVANAT, C., DE LA SALLE, H., & HANAU, D. (2002). Endotoxin-free heat-shock protein 70 fails to induce APC activation. *Eur J Immunol*, **32**, 3708–3713.

BECKER, D., VALK, E., ZAHN, S., BRAND, P., & KNOP, J. (2003). Coupling of contact sensitizers to thiol groups is a key event for the activation of monocytes and monocyte-derived dendritic cells. *J Invest Dermatol*, **120**, 233–8.

BELLO, J., IIJIMA, H., & KARTHA, G. (1979). A new arylating agent, 2-carboxy-4,6-dinitrochlorobenzene. Reaction with model compounds and bovine pancreatic ribonuclease. *Int J Pept Protein Res*, **14**, 199–212.

BIBLIOGRAPHY

BERAN, R. G. (1993). Cross-reactive skin eruption with both carbamazepine and oxcarbazepine. *Epilepsia*, **34**, 163–165.

BERNHARD, H., DISIS, M. L., HEIMFELD, S., HAND, S., GRALOW, J. R., & CHEEVER, M. A. (1995). Generation of immunostimulatory dendritic cells from human CD34+ hematopoietic progenitor cells of the bone marrow and peripheral blood. *Cancer Res*, **55**, 1099–1104.

BONFANTI, P., PUSTERLA, L., PARAZZINI, F., LIBANORE, M., CAGNI, A. E., FRANZETTI, M., FAGGION, I., LANDONIO, S., & QUIRINO, T. (2000). The effectiveness of desensitization versus rechallenge treatment in HIV-positive patients with previous hypersensitivity to TMP-SMX: a randomized multicentric study. C.I.S.A.I. Group. *Biomed Pharmacother*, **54**, 45–49.

BOROVSKY, Z., MISHAN-EISENBERG, G., YANIV, E., & RACHMILEWITZ, J. (2002). Serial triggering of T cell receptors results in incremental accumulation of signaling intermediates. *J Biol Chem*, **277**, 21529–21536.

BOURS, M. J. L., SWENNEN, E. L. R., VIRGILIO, F. D., CRONSTEIN, B. N., & DAGNELIE, P. C. (2006). Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther*, **112**, 358–404.

BRACHO, F., VAN DE VEN, C., AREMAN, E., HUGHES, R. M., DAVENPORT, V., BRADLEY, M. B., CAI, J.-W., & CAIRO, M. S. (2003). A comparison of ex vivo expanded DCs derived from cord blood and mobilized adult peripheral blood plastic-adherent mononuclear cells: decreased alloreactivity of cord blood DCs. *Cytotherapy*, **5**, 349–361.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248–54.

BRANDER, C., MAURI-HELLWEG, D., BETTENS, F., ROLLI, H., GOLDMAN, M., & PICHLER, W. J. (1995). Heterogeneous T cell responses to beta-lactam-modified

self-structures are observed in penicillin-allergic individuals. *J Immunol*, **155**, 2670–2678.

BRETON, H., COCIGLIO, M., BRESSOLLE, F., PEYRIERE, H., BLAYAC, J. P., & HILLAIRE-BUYS, D. (2005). Liquid chromatography-electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*, **828**, 80–90.

BRUCHHAUSEN, S., ZAHN, S., VALK, E., KNOP, J., & BECKER, D. (2003). Thiol antioxidants block the activation of antigen-presenting cells by contact sensitizers. *J Invest Dermatol*, **121**, 1039–44.

BU, H.-Z., KANG, P., DEESE, A. J., ZHAO, P., & POOL, W. F. (2005). Human in vitro glutathionyl and protein adducts of carbamazepine-10,11-epoxide, a stable and pharmacologically active metabolite of carbamazepine. *Drug Metab Dispos*, **33**, 1920–1924.

BURKHART, C., BRITSCHGI, M., STRASSER, I., DEPTA, J. P. H., VON GREYERZ, S., BARNABA, V., & PICHLER, W. J. (2002). Non-covalent presentation of sulfamethoxazole to human CD4+ T cells is independent of distinct human leucocyte antigen-bound peptides. *Clin Exp Allergy*, **32**, 1635–1643.

BURKHART, C., VON GREYERZ, S., DEPTA, J. P., NAISBITT, D. J., BRITSCHGI, M., PARK, K. B., & PICHLER, W. J. (2001). Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. *Br J Pharmacol*, **132**, 623–630.

BUSCH, D. H. & PAMER, E. G. (1999). T cell affinity maturation by selective expansion during infection. *J Exp Med*, **189**, 701–710.

CALDWELL, G. W., RITCHIE, D. M., MASUCCI, J. A., HAGEMAN, W., & YAN, Z. (2001). The new pre-preclinical paradigm: compound optimization in early and late phase drug discovery. *Curr Top Med Chem*, **1**, 353–366.

CALDWELL, G. W. & YAN, Z. (2006). Screening for reactive intermediates and toxicity assessment in drug discovery. *Curr Opin Drug Discov Devel*, **9**, 47–60.

CARR, A., SWANSON, C., PENNY, R., & COOPER, D. A. (1993a). Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with Pneumocystis carinii pneumonia and AIDS. *J Infect Dis*, **167**, 180–185.

CARR, A., TINDALL, B., PENNY, R., & COOPER, D. A. (1993b). In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin Exp Immunol*, **94**, 21–25.

CASHMAN, J. R. (2000). Human flavin-containing monooxygenase: substrate specificity and role in drug metabolism. *Curr Drug Metab*, 1, 181–191.

CASHMAN, J. R. & ZHANG, J. (2006). Human flavin-containing monooxygenases. *Annu Rev Pharmacol Toxicol*, **46**, 65–100.

CAUX, C., MASSACRIER, C., VANBERVLIET, B., DUBOIS, B., VAN KOOTEN, C., DURAND, I., & BANCHEREAU, J. (1994). Activation of human dendritic cells through CD40 cross-linking. *J Exp Med*, **180**, 1263–1272.

CAVANI, A., HACKETT, C. J., WILSON, K. J., ROTHBARD, J. B., & KATZ, S. I. (1995). Characterization of epitopes recognized by hapten-specific CD4+ T cells. *J Immunol*, **154**, 1232–1238.

CAVANI, A., NASORRI, F., OTTAVIANI, C., SEBASTIANI, S., PIT, O. D., & GIROLOMONI, G. (2003). Human CD25+ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. *J Immunol*, **171**, 5760–5768.

CELLA, M., SCHEIDEGGER, D., PALMER-LEHMANN, K., LANE, P., LANZAVECCHIA, A., & ALBER, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med*, **184**, 747–752.

CHRISTIANSEN, C., PICHLER, W. J., & SKOTLAND, T. (2000). Delayed allergy-like reactions to X-ray contrast media: mechanistic considerations. *Eur Radiol*, **10**, 1965–1975.

CHUNG, W.-H., HUNG, S.-I., HONG, H.-S., HSIH, M.-S., YANG, L.-C., HO, H.-C., WU, J.-Y., & CHEN, Y.-T. (2004). Medical genetics: a marker for Stevens-Johnson syndrome. *Nature*, **428**, 486.

COHEN, E. N., TRUDELL, J. R., EDMUNDS, H. N., & WATSON, E. (1975). Urinary metabolites of halothane in man. *Anesthesiology*, **43**, 392–401.

COULTER, E. M., FARRELL, J., MATHEWS, K. L., MAGGS, J. L., PEASE, C. K., LOCKLEY, D. J., BASKETTER, D. A., PARK, B. K., & NAISBITT, D. J. (2006). Activation of human dendritic cells by p-phenylenediamine. *J Pharmacol Exp Ther*, **320**, 885–892.

COUTANT, K. D., DE FRAISSINETTE, A. B., CORDIER, A., & ULRICH, P. (1999). Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol Sci*, **52**, 189–98.

CRIBB, A. E., MILLER, M., LEEDER, J. S., HILL, J., & SPIELBERG, S. P. (1991). Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. Implications for idiosyncratic toxicity. *Drug Metab Dispos*, **19**, 900–906.

CRIBB, A. E., MILLER, M., TESORO, A., & SPIELBERG, S. P. (1990). Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. *Mol Pharmacol*, **38**, 744–51.

CRIBB, A. E., POHL, L. R., SPIELBERG, S. P., & LEEDER, J. S. (1997). Patients with delayed-onset sulfonamide hypersensitivity reactions have antibodies recognizing endoplasmic reticulum luminal proteins. *J Pharmacol Exp Ther*, **282**, 1064–1071.

CRIBB, A. E. & SPIELBERG, S. P. (1990). Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine. *Drug Metab Dispos*, **18**, 784–787.

CRIBB, A. E. & SPIELBERG, S. P. (1992). Sulfamethoxazole is metabolized to the hydroxylamine in humans. *Clin Pharmacol Ther*, **51**, 522–6.

CRIBB, A. E., SPIELBERG, S. P., & GRIFFIN, G. P. (1995). N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab Dispos*, **23**, 406–14.

CSETENYI, J., BAKER, K. M., FRIGERIO, A., & MORSELLI, P. L. (1973). Iminostilbene–a metabolite of carbamazepine isolated from rat urine. *J Pharm Pharmacol*, **25**, 340–341.

CUTTLE, L., MUNNS, A. J., HOGG, N. A., SCOTT, J. R., HOOPER, W. D., DICKINSON, R. G., & GILLAM, E. M. (2000). Phenytoin metabolism by human cytochrome P450: involvement of P450 3A and 2C forms in secondary metabolism and drug-protein adduct formation. *Drug Metab Dispos*, **28**, 945–50.

DAVIDSON, A., WANG, X., MIHARA, M., RAMANUJAM, M., HUANG, W., SCHIFFER, L., & SINHA, J. (2003). Co-stimulatory blockade in the treatment of murine systemic lupus erythematosus (SLE). *Ann N Y Acad Sci*, **987**, 188–98.

DAVIES, E. C., GREEN, C. F., MOTTRAM, D. R., & PIRMOHAMED, M. (2006). Adverse drug reactions in hospital in-patients: a pilot study. *J Clin Pharm Ther*, **31**, 335–341.

DEARMAN, R. J., HEGARTY, J. M., & KIMBER, I. (1991). Inhalation exposure of mice to trimellitic anhydride induces both IgG and IgE anti-hapten antibody. *Int Arch Allergy Appl Immunol*, **95**, 70–6.

DEARMAN, R. J., WARBRICK, E. V., HUMPHREYS, I. R., & KIMBER, I. (2000). Characterization in mice of the immunological properties of five allergenic acid anhydrides. *J Appl Toxicol*, **20**, 221–30.

DEPTA, J. P., ALTZNAUER, F., GAMERDINGER, K., BURKHART, C., WELTZIEN, H. U., & PICHLER, W. J. (2004). Drug interaction with T-cell receptors: T-cell receptor density determines degree of cross-reactivity. *J Allergy Clin Immunol*, **113**, 519–27.

DEY, A., PARMAR, D., DAYAL, M., DHAWAN, A., & SETH, P. K. (2001). Cytochrome P450 1A1 (CYP1A1) in blood lymphocytes evidence for catalytic activity and mRNA expression. *Life Sci*, **69**, 383–93.

DIEBOLD, S. S., KAISHO, T., HEMMI, H., AKIRA, S., & REIS E SOUSA, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science*, **303**, 1529–1531.

DIECKHAUS, C. M., FERNNDEZ-METZLER, C. L., KING, R., KROLIKOWSKI, P. H., & BAILLIE, T. A. (2005). Negative ion tandem mass spectrometry for the detection of glutathione conjugates. *Chem Res Toxicol*, **18**, 630–638.

DIMASI, J. A. (1995). Success rates for new drugs entering clinical testing in the United States. *Clin Pharmacol Ther*, **58**, 1–14.

DIMASI, J. A., HANSEN, R. W., & GRABOWSKI, H. G. (2003). The price of innovation: new estimates of drug development costs. *J Health Econ*, **22**, 151–185.

DIMASI, J. A., HANSEN, R. W., GRABOWSKI, H. G., & LASAGNA, L. (1991). Cost of innovation in the pharmaceutical industry. *J Health Econ*, **10**, 107–142.

DOERGE, D. R., DIVI, R. L., DECK, J., & TAUROG, A. (1997). Mechanism for the anti-thyroid action of minocycline. *Chem Res Toxicol*, **10**, 49–58.

DOYLE, H. A. & MAMULA, M. J. (2001). Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol*, **22**, 443–9.

DU, L., HOFFMAN, S. M., & KEENEY, D. S. (2004). Epidermal CYP2 family cytochromes P450. *Toxicol Appl Pharmacol*, **195**, 278–87.

EDWARDS, I. R. & ARONSON, J. K. (2000). Adverse drug reactions: definitions, diagnosis, and management. *Lancet*, **356**, 1255–1259.

EGGENA, M. P., BARUGAHARE, B., JONES, N., OKELLO, M., MUTALYA, S., KITYO, C., MUGYENYI, P., & CAO, H. (2005). Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol*, **174**, 4407–14.

EICHELBAUM, M., TOMSON, T., TYBRING, G., & BERTILSSON, L. (1985). Carbamazepine metabolism in man. Induction and pharmacogenetic aspects. *Clin Pharmacokinet*, **10**, 80–90.

ELIASSON, E., GARDNER, I., HUME-SMITH, H., DE WAZIERS, I., BEAUNE, P., & KENNA, J. G. (1998). Interindividual variability in P450-dependent generation of neoantigens in halothane hepatitis. *Chem Biol Interact*, **116**, 123–41.

ELIASZEWICZ, M., FLAHAULT, A., ROUJEAU, J. C., FILLET, A. M., CHALLINE, D., MANSOURI, S., WOLKENSTEIN, P., ARACTINGI, S., PENSO-ASSATHIANY, D., MASLO, C., BOURGAULT-VILLADA, I., CHOSIDOW, O., & CAUMES, E. (2002). Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. *J Am Acad Dermatol*, **47**, 40–6.

EMOTO, C., MURASE, S., SAWADA, Y., & IWASAKI, K. (2005). In vitro inhibitory effect of 1-aminobenzotriazole on drug oxidations in human liver microsomes: a comparison with SKF-525A. *Drug Metab Pharmacokinet*, **20**, 351–357.

EMOTO, C., MURASE, S., SAWADA, Y., JONES, B. C., & IWASAKI, K. (2003). In vitro inhibitory effect of 1-aminobenzotriazole on drug oxidations catalyzed by human cytochrome P450 enzymes: a comparison with SKF-525A and ketoconazole. *Drug Metab Pharmacokinet*, **18**, 287–295.

ENGLER, O. B., STRASSER, I., NAISBITT, D. J., CERNY, A., & PICHLER, W. J. (2004). A chemically inert drug can stimulate T cells in vitro by their T cell receptor in non-sensitised individuals. *Toxicology*, **197**, 47–56.

ESWARAMOORTHY, S., BONANNO, J. B., BURLEY, S. K., & SWAMINATHAN, S. (2006). Mechanism of action of a flavin-containing monooxygenase. *Proc Natl Acad Sci U S A*, **103**, 9832–9837.

EVANS, D. C., WATT, A. P., NICOLL-GRIFFITH, D. A., & BAILLIE, T. A. (2004). Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem Res Toxicol*, **17**, 3–16.

FARRELL, J., NAISBITT, D. J., DRUMMOND, N. S., DEPTA, J. P., VILAR, F. J., PIRMOHAMED, M., & PARK, B. K. (2003). Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *J Pharmacol Exp Ther*, **306**, 229–37.

FDA (2004). Innovation or stagnation: challenge and innovation on the critical path to new medical products. Tech. rep., US Food and Drug Administration.

FINNSTROM, N., ASK, B., DAHL, M. L., GADD, M., & RANE, A. (2002). Intra-individual variation and sex differences in gene expression of cytochromes P450 in circulating leukocytes. *Pharmacogenomics J*, **2**, 111–6.

FRENCH, L. E. & TSCHOPP, J. (1996). Constitutive Fas ligand expression in several non-lymphoid mouse tissues: implications for immune-protection and cell turnover. *Behring Inst Mitt*, pp. 156–60.

FRIEDMANN, P. S., STRICKLAND, I., PIRMOHAMED, M., & PARK, B. K. (1994). Investigation of mechanisms in toxic epidermal necrolysis induced by carbamazepine. *Arch Dermatol*, **130**, 598–604.

FURST, S. M. & GANDOLFI, A. J. (1997). Interaction of lymphocytes with Kupffer cells from halothane-exposed guinea pigs. *Int Arch Allergy Immunol*, **114**, 46–53.

FURST, S. M., LUEDKE, D., GAW, H. H., REICH, R., & GANDOLFI, A. J. (1997). Demonstration of a cellular immune response in halothane-exposed guinea pigs. *Toxicol Appl Pharmacol*, **143**, 245–55.

FURST, S. M., SUKHAI, P., McCLELLAND, R. A., & UETRECHT, J. P. (1995). Covalent binding of carbamazepine oxidative metabolites to neutrophils. *Drug Metab Dispos*, **23**, 590–594.

FURST, S. M. & UETRECHT, J. P. (1993). Carbamazepine metabolism to a reactive intermediate by the myeloperoxidase system of activated neutrophils. *Biochem Pharmacol*, **45**, 1267–75.

GAEDIGK, A., SPIELBERG, S. P., & GRANT, D. M. (1994). Characterization of the microsomal epoxide hydrolase gene in patients with anticonvulsant adverse drug reactions. *Pharmacogenetics*, **4**, 142–153.

GALLUCCI, S., LOLKEMA, M., & MATZINGER, P. (1999). Natural adjuvants: endogenous activators of dendritic cells. *Nat Med*, **5**, 1249–55.

GAMERDINGER, K., MOULON, C., KARP, D. R., VAN BERGEN, J., KONING, F., WILD, D., PFLUGFELDER, U., & WELTZIEN, H. U. (2003). A new type of metal recognition by human T cells: contact residues for peptide-independent bridging of T cell receptor and major histocompatibility complex by nickel. *J Exp Med*, **197**, 1345–1353.

GAO, B. & TSAN, M.-F. (2003). Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J Biol Chem*, **278**, 174–179.

GARZA, K. M., CHAN, S. M., SURI, R., NGUYEN, L. T., ODERMATT, B., SCHOEN-BERGER, S. P., & OHASHI, P. S. (2000). Role of antigen-presenting cells in mediating tolerance and autoimmunity. *J Exp Med*, **191**, 2021–2027.

GELL, P. & COOMBS, R. (1963). *Clinical aspects of immunology*. Blackwell, Oxford.

GERBER, B. O. & PICHLER, W. J. (2006). Noncovalent interactions of drugs with immune receptors may mediate drug-induced hypersensitivity reactions. *AAPS J*, **8**, 160–165.

GERBERICK, G. F., RYAN, C. A., DEARMAN, R. J., & KIMBER, I. (2007). Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods*, **41**, 54–60.

GERBERICK, G. F., VASSALLO, J. D., BAILEY, R. E., CHANEY, J. G., MORRALL, S. W., & LEPOITTEVIN, J.-P. (2004). Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci*, **81**, 332–43.

GILL, H. J., HOUGH, S. J., NAISBITT, D. J., MAGGS, J. L., KITTERINGHAM, N. R., PIRMOHAMED, M., & PARK, B. K. (1997). The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. *J Pharmacol Exp Ther*, **282**, 795–801.

GILL, H. J., MAGGS, J. L., MADDEN, S., PIRMOHAMED, M., & PARK, B. K. (1996). The effect of fluconazole and ketoconazole on the metabolism of sulphamethoxazole. *Br J Clin Pharmacol*, **42**, 347–53.

GOLDRING, C., KITTERINGHAM, N., JENKINS, R., COPPLE, I., JEANNIN, J.-F., & PARK, B. K. (2006). Plasticity in cell defence: access to and reactivity of critical protein residues and DNA response elements. *J Exp Biol*, **209**, 2337–2343.

GOODWIN, D. C., GROVER, T. A., & AUST, S. D. (1996). Redox mediation in the peroxidase-catalyzed oxidation of aminopyrine: possible implications for drug-drug interactions. *Chem Res Toxicol*, **9**, 476–483.

GREEN, V. J., PIRMOHAMED, M., KITTERINGHAM, N. R., GAEDIGK, A., GRANT, D. M., BOXER, M., BURCHELL, B., & PARK, B. K. (1995). Genetic analysis of microsomal epoxide hydrolase in patients with carbamazepine hypersensitivity. *Biochem Pharmacol*, **50**, 1353–1359.

GROHMANN, U., ORABONA, C., FALLARINO, F., VACCA, C., CALCINARO, F., FALORNI, A., CANDELORO, P., BELLADONNA, M. L., BIANCHI, R., FIORETTI, M. C., & PUCCETTI, P. (2002). CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol*, **3**, 1097–1101.

GRUCHALLA, R. S., PESENKO, R. D., DO, T. T., & SKIEST, D. J. (1998). Sulfonamide-induced reactions in desensitized patients with AIDS–the role of covalent protein haptenation by sulfamethoxazole. *J Allergy Clin Immunol*, **101**, 371–378.

GU, Y., HERSHFIELD, M. S., & COHEN, A. (2004). The danger within. *N Engl J Med*, **350**, 2721–2; author reply 2721–2.

GUT, J., CHRISTEN, U., & HUWYLER, J. (1993). Mechanisms of halothane toxicity: novel insights. *Pharmacol Ther*, **58**, 133–55.

HABIG, W. H., PABST, M. J., & JAKOBY, W. B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem*, **249**, 7130–9.

HADLEY, G., DERRY, S., & MOORE, R. A. (2006). Imiquimod for actinic keratosis: systematic review and meta-analysis. *J Invest Dermatol*, **126**, 1251–1255.

HALEBIAN, P. H., CORDER, V. J., MADDEN, M. R., FINKLESTEIN, J. L., & SHIRES, G. T. (1986). Improved burn center survival of patients with toxic epidermal necrolysis managed without corticosteroids. *Ann Surg*, **204**, 503–512.

HAMILTON, G. A. (1962). *Oxygenases*, chap. Chemical models and mechanisms for oxygenases, pp. 419–431. Academic Press, New York.

HAN, B., SERRA, P., YAMANOUCHI, J., AMRANI, A., ELLIOTT, J. F., DICKIE, P., DILORENZO, T. P., & SANTAMARIA, P. (2005). Developmental control of CD8 T cell-avidity maturation in autoimmune diabetes. *J Clin Invest*, **115**, 1879–1887.

HANZLIK, R. P., KOEN, Y. M., THEERTHAM, B., DONG, Y., & FANG, J. (2007). The reactive metabolite target protein database (TPDB)–a web-accessible resource. *BMC Bioinformatics*, **8**, 95.

HARDING, F. A., MCARTHUR, J. G., GROSS, J. A., RAULET, D. H., & ALLISON, J. P. (1992). CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*, **356**, 607–609.

HASAN, U., CHAFFOIS, C., GAILLARD, C., SAULNIER, V., MERCK, E., TANCREDI, S., GUIET, C., BRIERE, F., VLACH, J., LEBECQUE, S., TRINCHIERI, G., & BATES, E. E. M. (2005). Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol*, **174**, 2942–2950.

HASHIMOTO, K., YASUKAWA, M., & TOHYAMA, M. (2003). Human herpesvirus 6 and drug allergy. *Curr Opin Allergy Clin Immunol*, **3**, 255–260.

HAYASHI, F., SMITH, K. D., OZINSKY, A., HAWN, T. R., YI, E. C., GOODLETT, D. R., ENG, J. K., AKIRA, S., UNDERHILL, D. M., & ADEREM, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, **410**, 1099–1103.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H., & BAUER, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*, **303**, 1526–1529.

HEMMI, H., KAISHO, T., TAKEUCHI, O., SATO, S., SANJO, H., HOSHINO, K., HORIUCHI, T., TOMIZAWA, H., TAKEDA, K., & AKIRA, S. (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol*, **3**, 196–200.

HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H., TAKEDA, K., & AKIRA, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature*, **408**, 740–745.

HEYDTMANN, M., SHIELDS, P., McCAUGHAN, G., & ADAMS, D. (2001). Cytokines and chemokines in the immune response to hepatitis C infection. *Curr Opin Infect Dis*, **14**, 279–287.

HOLZER, A. M., KAPLAN, L. L., & LEVIS, W. R. (2006). Haptens as drugs: contact allergens are powerful topical immunomodulators. *J Drugs Dermatol*, **5**, 410–416. Comparative Study.

HULETTE, B. C., RYAN, C. A., GILDEA, L. A., & GERBERICK, G. F. (2005). Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. *Toxicol Appl Pharmacol*, pp. 159–66.

HUNG, S.-I., CHUNG, W.-H., JEE, S.-H., CHEN, W.-C., CHANG, Y.-T., LEE, W.-R., HU, S.-L., WU, M.-T., CHEN, G.-S., WONG, T.-W., HSIAO, P.-F., CHEN, W.-H., SHIH, H.-Y., FANG, W.-H., WEI, C.-Y., LOU, Y.-H., HUANG, Y.-L., LIN, J.-J., & CHEN, Y.-T. (2006). Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. *Pharmacogenet Genomics*, **16**, 297–306.

ICHIKAWA, H. T., WILLIAMS, L. P., & SEGAL, B. M. (2002). Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J Immunol*, **169**, 2781-7.

IIJIMA, N., YANAGAWA, Y., & ONOE, K. (2003). Role of early- or late-phase activation of p38 mitogen-activated protein kinase induced by tumour necrosis factor-alpha or 2,4-dinitrochlorobenzene during maturation of murine dendritic cells. *Immunology*, **110**, 322-8.

IRVINE, D. J., PURBHOO, M. A., KROGSGAARD, M., & DAVIS, M. M. (2002). Direct observation of ligand recognition by t cells. *Nature*, **419**, 845-9.

ITO, S., UENO, K., MITARAI, A., & SASAKI, K. (1993). Evidence for hydroxyl radicals as an active species generated from Udenfriend's reagent. *J Chem Soc Perk T 2*, **2**, 255-259.

JACKSON, G. E. & YOUNG, N. M. (1986). Determination of chemical properties of individual histidine and tyrosine residues of concanavalin A by competitive labeling with 1-fluoro-2,4-dinitrobenzene. *Biochemistry*, **25**, 1657-62.

JANEWAY, C. A. J. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today*, **13**, 11-16.

JANMOHAMED, A., DOLPHIN, C. T., PHILLIPS, I. R., & SHEPHARD, E. A. (2001). Quantification and cellular localization of expression in human skin of genes encoding flavin-containing monooxygenases and cytochromes P450. *Biochem Pharmacol*, **62**, 777-786.

JANSSEN, E., TABETA, K., BARNES, M. J., RUTSCHMANN, S., MCBRIDE, S., BAHJAT, K. S., SCHOENBERGER, S. P., THEOFILOPOULOS, A. N., BEUTLER, B., & HOEBE, K. (2006). Efficient T cell activation via a Toll-Interleukin 1 Receptor-independent pathway. *Immunity*, **24**, 787-799.

JENKINS, M. K., TAYLOR, P. S., NORTON, S. D., & URDAHL, K. B. (1991). CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol*, **147**, 2461-2466.

JIANG, X., KHURSIGARA, G., & RUBIN, R. L. (1994). Transformation of lupus-inducing drugs to cytotoxic products by activated neutrophils. *Science*, **266**, 810–3.

JOHNSON-LGER, C., CHRISTENSEN, J., & KLAUS, G. G. (1998). CD28 co-stimulation stabilizes the expression of the CD40 ligand on T cells. *Int Immunol*, **10**, 1083–1091.

JONES, W. G., HALEBIAN, P., MADDEN, M., FINKELSTEIN, J., & GOODWIN, C. W. (1989). Drug-induced toxic epidermal necrolysis in children. *J Pediatr Surg*, **24**, 167–170.

JU, C. & POHL, L. R. (2005). Tolerogenic role of Kupffer cells in immune-mediated adverse drug reactions. *Toxicology*, **209**, 109–12.

JU, C. & UETRECHT, J. P. (1998). Oxidation of a metabolite of indomethacin (Desmethyldeschlorobenzoylindomethacin) to reactive intermediates by activated neutrophils, hypochlorous acid, and the myeloperoxidase system. *Drug Metab Dispos*, **26**, 676–680.

JU, C. & UETRECHT, J. P. (1999). Detection of 2-hydroxyiminostilbene in the urine of patients taking carbamazepine and its oxidation to a reactive iminoquinone intermediate. *J Pharmacol Exp Ther*, **288**, 51–6.

JUGDE, F., BOISSIER, C., ROUGIER-LARZAT, N., CORLU, A., CHESNE, C., SEMANA, G., & HERESBACH, D. (2005). Regulation by allergens of chemokine receptor expression on in vitro-generated dendritic cells. *Toxicology*.

KABE, Y., ANDO, K., HIRAO, S., YOSHIDA, M., & HANDA, H. (2005). Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal*, **7**, 395–403.

KALERGIS, A. M., BOUCHERON, N., DOUCEY, M. A., PALMIERI, E., GOYARTS, E. C., VEGH, Z., LUESCHER, I. F., & NATHENSON, S. G. (2001). Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nat Immunol*, **2**, 229–234.

KANTENGWA, S., JORNOT, L., DEVENOGES, C., & NICOD, L. P. (2003). Superoxide anions induce the maturation of human dendritic cells. *Am J Respir Crit Care Med*, **167**, 431–437.

KASSEL, D. B. (2004). Applications of high-throughput ADME in drug discovery. *Curr Opin Chem Biol*, **8**, 339–345.

KENNA, J. G., NEUBERGER, J., & WILLIAMS, R. (1988a). Evidence for expression in human liver of halothane-induced neoantigens recognized by antibodies in sera from patients with halothane hepatitis. *Hepatology*, **8**, 1635–41.

KENNA, J. G., SATOH, H., CHRIST, D. D., & POHL, L. R. (1988b). Metabolic basis for a drug hypersensitivity: antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane. *J Pharmacol Exp Ther*, **245**, 1103–9.

KENNEDY, T. (1997). Managing the drug discovery/development interface. *Drug Discov Today*, **2**, 436–44.

KENNY, J. R., MAGGS, J. L., MENG, X., SINNOTT, D., CLARKE, S. E., PARK, B. K., & STACHULSKI, A. V. (2004). Syntheses and characterization of the acyl glucuronide and hydroxy metabolites of diclofenac. *J Med Chem*, **47**, 2816–2825.

KERR, B. M., THUMMEL, K. E., WURDEN, C. J., KLEIN, S. M., KROETZ, D. L., GONZALEZ, F. J., & LEVY, R. H. (1994). Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochem Pharmacol*, **47**, 1969–1979.

KIMBER, I. & DEARMAN, R. J. (2002). Immunologic basis for autoimmunity and the potential influences of xenobiotics. *Toxicol Lett*, **127**, 77–81.

KITTERINGHAM, N. R., MAGGS, J. L., NEWBY, S., & PARK, B. K. (1985). Drug-protein conjugates–VIII. The metabolic fate of the dinitrophenyl hapten conjugated to albumin. *Biochem Pharmacol*, **34**, 1763–71.

KLEINDIENST, P., WIETHE, C., LUTZ, M. B., & BROCKER, T. (2005). Simultaneous induction of CD4 T cell tolerance and CD8 T cell immunity by semimature dendritic cells. *J Immunol*, **174**, 3941–3947.

KNOWLES, S. R., SHAPIRO, L. E., & SHEAR, N. H. (1999). Anticonvulsant hypersensitivity syndrome: incidence, prevention and management. *Drug Saf*, **21**, 489–501.

KOHLER, J., MARTIN, S., PFLUGFELDER, U., RUH, H., VOLLMER, J., & WELTZIEN, H. U. (1995). Cross-reactive trinitrophenylated peptides as antigens for class II major histocompatibility complex-restricted T cells and inducers of contact sensitivity in mice. Limited T cell receptor repertoire. *Eur J Immunol*, **25**, 92–101.

KROVAT, B. C., TRACY, J. H., & OMIECINSKI, C. J. (2000). Fingerprinting of cytochrome P450 and microsomal epoxide hydrolase gene expression in human blood cells. *Toxicol Sci*, **55**, 352–60.

KURIAN, J. R., BAJAD, S. U., MILLER, J. L., CHIN, N. A., & TREPANIER, L. A. (2004). NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther*, **311**, 1171–1178.

KVASZ, M., ALLEN, I. E., GORDON, M. J., RO, E. Y., ESTOK, R., OLKIN, I., & ROSS, S. D. (2000). Adverse drug reactions in hospitalized patients: A critique of a meta-analysis. *MedGenMed*, **2**, E3.

LAI, W. G., ZAHID, N., & UETRECHT, J. P. (1999). Metabolism of trimethoprim to a reactive iminoquinone methide by activated human neutrophils and hepatic microsomes. *J Pharmacol Exp Ther*, **291**, 292–9.

LALOR, P. F., SHIELDS, P., GRANT, A., & ADAMS, D. H. (2002). Recruitment of lymphocytes to the human liver. *Immunol Cell Biol*, **80**, 52–64.

LALOR, T. & ADAMS, D. (2002). The liver: a model of organ-specific lymphocyte recruitment. *Expert Rev Mol Med*, **2002**, 1–15.

LANDSTEINER, K. & JACOBS, J. (1935). Studies on the sensitisation of animals with simple chemical compounds. *J Exp Med*, **61**, 643–56.

BIBLIOGRAPHY

LANDSTEINER, K. & JACOBS, J. (1936). Studies on the sensitisation of animals with simple chemical compounds: II. *J Exp Med*, **64**, 625–39.

LAVERGNE, S. N., KURIAN, J. R., BAJAD, S. U., MAKI, J. E., YODER, A. R., GUZINSKI, M. V., GRAZIANO, F. M., & TREPANIER, L. A. (2006). Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. *Toxicology*, **222**, 25–36.

LAZAROU, J., POMERANZ, B. H., & COREY, P. N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA*, **279**, 1200–5.

LEBLANC, R., HIDESHIMA, T., CATLEY, L. P., SHRINGARPURE, R., BURGER, R., MITSIADES, N., MITSIADES, C., CHEEMA, P., CHAUHAN, D., RICHARDSON, P. G., ANDERSON, K. C., & MUNSHI, N. C. (2004). Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. *Blood*, **103**, 1787–90.

LERTRATANANGKOON, K. & HORNING, M. G. (1982). Metabolism of carbamazepine. *Drug Metab Dispos*, **10**, 1–10.

LEYVA, L., TORRES, M. J., POSADAS, S., BLANCA, M., BESSO, G., O'VALLE, F., DEL MORAL, R. G., SANTAMARIA, L. F., & JUAREZ, C. (2000). Anticonvulsant-induced toxic epidermal necrolysis: monitoring the immunologic response. *J Allergy Clin Immunol*, **105**, 157–165.

LIN, D., TUCKER, M. J., & RIEDER, M. J. (2006). Increased adverse drug reactions to antimicrobials and anticonvulsants in patients with HIV infection. *Ann Pharmacother*, **40**, 1594–1601.

LING, E. M., SMITH, T., NGUYEN, X. D., PRIDGEON, C., DALLMAN, M., ARBERY, J., CARR, V. A., & ROBINSON, D. S. (2004). Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet*, **363**, 608–615.

BIBLIOGRAPHY

LIU, J., LIU, Y., HARTLEY, D., KLAASSEN, C. D., SHEHIN-JOHNSON, S. E., LUCAS, A., & COHEN, S. D. (1999). Metallothionein-I/II knockout mice are sensitive to acetaminophen-induced hepatotoxicity. *J Pharmacol Exp Ther*, **289**, 580–586.

LIU, L. F., HONG, J. L., TSAI, S. P., HSIEH, J. C., & TAM, M. F. (1993). Reversible modification of rat liver glutathione S-transferase 3-3 with 1-chloro-2,4-dinitrobenzene: specific labelling of Tyr-115. *Biochem J*, **296** (**Pt 1**), 189–97.

LIU, Z. C. & UETRECHT, J. P. (1995). Clozapine is oxidized by activated human neutrophils to a reactive nitrenium ion that irreversibly binds to the cells. *J Pharmacol Exp Ther*, **275**, 1476–1483.

LIU, Z. C. & UETRECHT, J. P. (2000). Metabolism of ticlopidine by activated neutrophils: implications for ticlopidine-induced agranulocytosis. *Drug Metab Dispos*, **28**, 726–730.

LIU, Z.-X. & KAPLOWITZ, N. (2006). Role of innate immunity in acetaminophen-induced hepatotoxicity. *Expert Opin Drug Metab Toxicol*, **2**, 493–503.

LONJOU, C., THOMAS, L., BOROT, N., LEDGER, N., DE TOMA, C., LELOUET, H., GRAF, E., SCHUMACHER, M., HOVNANIAN, A., MOCKENHAUPT, M., ROUJEAU, J.-C., & GROUP, R. C. A. R. (2006). A marker for Stevens-Johnson syndrome ...: ethnicity matters. *Pharmacogenomics J*, **6**, 265–268.

LU, L., VOLLMER, J., MOULON, C., WELTZIEN, H. U., MARRACK, P., & KAPPLER, J. (2003). Components of the ligand for a Ni++ reactive human T cell clone. *J Exp Med*, **197**, 567–574.

LU, W. & UETRECHT, J. P. (2007). Possible bioactivation pathways of lamotrigine. *Drug Metab Dispos*, **35**, 1050–1056.

LUDEWIG, B., GRAF, D., GELDERBLOM, H. R., BECKER, Y., KROCZEK, R. A., & PAULI, G. (1995). Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10. *Eur J Immunol*, **25**, 1943–1950. In Vitro.

LUTZ, M. B. & SCHULER, G. (2002). Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol*, **23**, 445-9.

MADDEN, S., MAGGS, J. L., & PARK, B. K. (1996). Bioactivation of carbamazepine in the rat in vivo. Evidence for the formation of reactive arene oxide(s). *Drug Metab Dispos*, **24**, 469-479.

MAEMURA, K., ZHENG, Q., WADA, T., OZAKI, M., TAKAO, S., AIKOU, T., BULKLEY, G. B., KLEIN, A. S., & SUN, Z. (2005). Reactive oxygen species are essential mediators in antigen presentation by Kupffer cells. *Immunol Cell Biol*, **83**, 336-343.

MAGGS, J. L., KITTERINGHAM, N. R., GRABOWSKI, P. S., & PARK, B. K. (1986). Drug-protein conjugates-X. The role of protein conjugation in the disposition of dinitrofluorobenzene. *Biochem Pharmacol*, **35**, 505-13.

MAJUMDER, B., JANKET, M. L., SCHAFER, E. A., SCHAUBERT, K., HUANG, X.-L., KAN-MITCHELL, J., RINALDO, C. R. J., & AYYAVOO, V. (2005). Human immunodeficiency virus type 1 Vpr impairs dendritic cell maturation and T-cell activation: implications for viral immune escape. *J Virol*, **79**, 7990-8003.

MAN, C. B. L., KWAN, P., BAUM, L., YU, E., LAU, K. M., CHENG, A. S. H., & NG, M. H. L. (2007). Association between HLA-B*1502 allele and antiepileptic drug-induced cutaneous reactions in Han Chinese. *Epilepsia*, **48**, 1015-1018.

MANCHANDA, T., HESS, D., DALE, L., FERGUSON, S. G., & RIEDER, M. J. (2002). Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol Pharmacol*, **62**, 1011-1026.

MANOME, H., AIBA, S., & TAGAMI, H. (1999). Simple chemicals can induce maturation and apoptosis of dendritic cells. *Immunology*, **98**, 481-90.

MARTIN, E., O'SULLIVAN, B., LOW, P., & THOMAS, R. (2003). Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity*, **18**, 155-167.

MARTIN, S., VON BONIN, A., FESSLER, C., PFLUGFELDER, U., & WELTZIEN, H. U. (1993). Structural complexity of antigenic determinants for class I MHC-restricted, hapten-specific T cells. Two qualitatively differing types of H-2Kb-restricted TNP epitopes. *J Immunol*, **151**, 678–87.

MATSUE, H., EDELBAUM, D., SHALHEVET, D., MIZUMOTO, N., YANG, C., MUMMERT, M. E., OEDA, J., MASAYASU, H., & TAKASHIMA, A. (2003). Generation and function of reactive oxygen species in dendritic cells during antigen presentation. *J Immunol*, **171**, 3010–3018.

MATZINGER, P. (1994). Tolerance, danger, and the extended family. *Annu Rev Immunol*, **12**, 991–1045.

MAURI-HELLWEG, D., BETTENS, F., MAURI, D., BRANDER, C., HUNZIKER, T., & PICHLER, W. J. (1995). Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol*, **155**, 462–472.

McCONNACHIE, L. A., PHILLIPS, B., BAJPAI, M., SHEN, D. D., & HO, R. J. (2003). Only truncated, not complete cytochrome p450 2D6 RNA transcript and no detectable enzyme activity are expressed in human lymphocytes. *Drug Metab Dispos*, **31**, 1103–7.

McMAHAN, C. J. & FINK, P. J. (2000). Receptor revision in peripheral T cells creates a diverse V beta repertoire. *J Immunol*, **165**, 6902–6907.

MEDZHITOV, R., PRESTON-HURLBURT, P., & JANEWAY, C. A. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, **388**, 394–397.

MENNE, T. (1996). Prevention of nickel allergy by regulation of specific exposures. *Ann Clin Lab Sci*, **26**, 133–138.

MERK, H. F. (2005). Diagnosis of drug hypersensitivity: lymphocyte transformation test and cytokines. *Toxicology*, **209**, 217–220.

MEYER, D. & THOMSON, G. (2001). How selection shapes variation of the human major histocompatibility complex: a review. *Ann Hum Genet*, **65**, 1–26.

MIELI-VERGANI, G., VERGANI, D., TREDGER, J. M., EDDLESTON, A. L., DAVIS, M., & WILLIAMS, R. (1980). Lymphocyte cytotoxicity to halothane altered hepatocytes in patients with severe hepatic necrosis following halothane anaesthesia. *J Clin Lab Immunol*, **4**, 49–51.

MIGA, A. J., MASTERS, S. R., DURELL, B. G., GONZALEZ, M., JENKINS, M. K., MALISZEWSKI, C., KIKUTANI, H., WADE, W. F., & NOELLE, R. J. (2001). Dendritic cell longevity and T cell persistence is controlled by CD154-CD40 interactions. *Eur J Immunol*, **31**, 959–965.

MISRA, U. K., KALITA, J., & RATHORE, C. (2003). Phenytoin and carbamazepine cross reactivity: report of a case and review of literature. *Postgrad Med J*, **79**, 703–704.

MITRA, A. K., THUMMEL, K. E., KALHORN, T. F., KHARASCH, E. D., UNADKAT, J. D., & SLATTERY, J. T. (1996). Inhibition of sulfamethoxazole hydroxylamine formation by fluconazole in human liver microsomes and healthy volunteers. *Clin Pharmacol Ther*, **59**, 332–340. Clinical Trial.

MIZUASHI, M., OHTANI, T., NAKAGAWA, S., & AIBA, S. (2005). Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *J Invest Dermatol*, **124**, 579–86.

MONNET, C., BETTSWORTH, F., STURA, E. A., DU, M. H. L., MNEZ, R., DERRIEN, L., ZINN-JUSTIN, S., GILQUIN, B., SIBA, G., BATTAIL-POIROT, N., JOLIVET, M., MNEZ, A., ARNAUD, M., DUCANCEL, F., & CHARBONNIER, J. B. (2002). Highly specific anti-estradiol antibodies: structural characterisation and binding diversity. *J Mol Biol*, **315**, 699–712.

MORENO, F., BLANCA, M., MAYORGA, C., TERRADOS, S., MOYA, M., PREZ, E., SUAU, R., VEGA, J. M., GARCA, J., & MIRANDA, A. (1995). Studies of the

specificities of IgE antibodies found in sera from subjects with allergic reactions to penicillins. *Int Arch Allergy Immunol*, **108**, 74–81.

MORTIMER, N. J., BERMINGHAM, M. R., CHAPPLE, S. J., & SLADDEN, M. J. (2005). Fatal adverse drug reaction to trimethoprim. *Aust Fam Physician*, **34**, 345–346.

MOSTELLA, J., PIERONI, R., JONES, R., & FINCH, C. K. (2004). Anticonvulsant hypersensitivity syndrome: treatment with corticosteroids and intravenous immunoglobulin. *South Med J*, **97**, 319–321.

MOULON, C., VOLLMER, J., & WELTZIEN, H. U. (1995). Characterization of processing requirements and metal cross-reactivities in T cell clones from patients with allergic contact dermatitis to nickel. *Eur J Immunol*, **25**, 3308–3315.

MUNN, D. H., SHARMA, M. D., & MELLOR, A. L. (2004). Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. *J Immunol*, **172**, 4100–4110.

MUTHUMANI, K., HWANG, D. S., CHOO, A. Y., MAYILVAHANAN, S., DAYES, N. S., THIEU, K. P., & WEINER, D. B. (2005). HIV-1 Vpr inhibits the maturation and activation of macrophages and dendritic cells in vitro. *Int Immunol*, **17**, 103–116.

NAISBITT, D. J., BRITSCHGI, M., WONG, G., FARRELL, J., DEPTA, J. P., CHADWICK, D. W., PICHLER, W. J., PIRMOHAMED, M., & PARK, B. K. (2003a). Hypersensitivity reactions to carbamazepine: characterization of the specificity, phenotype, and cytokine profile of drug-specific T cell clones. *Mol Pharmacol*, **63**, 732–41.

NAISBITT, D. J., FARRELL, J., CHAMBERLAIN, P. J., HOPKINS, J. E., BERRY, N. G., PIRMOHAMED, M., & PARK, B. K. (2005a). Characterization of the T-cell response in a patient with phenindione hypersensitivity. *J Pharmacol Exp Ther*, **313**, 1058–65.

NAISBITT, D. J., FARRELL, J., GORDON, S. F., MAGGS, J. L., BURKHART, C., PICHLER, W. J., PIRMOHAMED, M., & PARK, B. K. (2002). Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol*, **62**, 628–637.

NAISBITT, D. J., FARRELL, J., WONG, G., DEPTA, J. P., DODD, C. C., HOPKINS, J. E., GIBNEY, C. A., CHADWICK, D. W., PICHLER, W. J., PIRMOHAMED, M., & PARK, B. K. (2003b). Characterization of drug-specific T cells in lamotrigine hypersensitivity. *J Allergy Clin Immunol*, **111**, 1393–403.

NAISBITT, D. J., GORDON, S. F., PIRMOHAMED, M., BURKHART, C., CRIBB, A. E., PICHLER, W. J., & PARK, B. K. (2001). Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br J Pharmacol*, **133**, 295–305.

NAISBITT, D. J., GORDON, S. F., PIRMOHAMED, M., & PARK, B. K. (2000a). Immunological principles of adverse drug reactions: the initiation and propagation of immune responses elicited by drug treatment. *Drug Saf*, **23**, 483–507.

NAISBITT, D. J., HOUGH, S. J., GILL, H. J., PIRMOHAMED, M., KITTERINGHAM, N. R., & PARK, B. K. (1999). Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. *Br J Pharmacol*, **126**, 1393–407.

NAISBITT, D. J., O'NEILL, P. M., PIRMOHAMED, M., & PARK, B. K. (1996). Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: Implications for immune mediated toxicity. *Bioorg Med Chem Lett*, **6**, 1511–1516.

NAISBITT, D. J., PIRMOHAMED, M., & PARK, B. K. (2005b). Drug presentation to T cells. *J Allergy Clin Immunol*, **115**, 876–7; author reply 877–8.

NAISBITT, D. J., RUSCOE, J. E., WILLIAMS, D., O'NEILL, P. M., PIRMOHAMED, M., & PARK, B. K. (1997). Disposition of amodiaquine and related antimalarial agents in human neutrophils: implications for drug design. *J Pharmacol Exp Ther*, **280**, 884–893.

NAISBITT, D. J., VILAR, F. J., STALFORD, A. C., WILKINS, E. G., PIRMOHAMED, M., & PARK, B. K. (2000b). Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. *AIDS Res Hum Retroviruses*, **16**, 1929–38.

NANJI, S. A., HANCOCK, W. W., LUO, B., SCHUR, C. D., PAWLICK, R. L., ZHU, L. F., ANDERSON, C. C., & SHAPIRO, A. M. (2006). Costimulation blockade of both inducible costimulator and CD40 ligand induces dominant tolerance to islet allografts and prevents spontaneous autoimmune diabetes in the NOD mouse. *Diabetes*, **55**, 27–33.

NASSIF, A., BENSUSSAN, A., BOUMSELL, L., DENIAUD, A., MOSLEHI, H., WOLKEN-STEIN, P., BAGOT, M., & ROUJEAU, J. C. (2004). Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *J Allergy Clin Immunol*, **114**, 1209–15.

NASSIF, A., BENSUSSAN, A., DOROTHE, G., MAMI-CHOUAIB, F., BACHOT, N., BAGOT, M., BOUMSELL, L., & ROUJEAU, J.-C. (2002). Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. *J Invest Dermatol*, **118**, 728–733.

NEBERT, D. W., ADESNIK, M., COON, M. J., ESTABROOK, R. W., GONZALEZ, F. J., GUENGERICH, F. P., GUNSALUS, I. C., JOHNSON, E. F., KEMPER, B., & LEVIN, W. (1987). The P450 gene superfamily: recommended nomenclature. *DNA*, **6**, 1–11. Comparative Study.

NEUBERGER, J. M. (1990). Halothane and hepatitis. Incidence, predisposing factors and exposure guidelines. *Drug Saf*, **5**, 28–38.

NIELSEN, N. H., LINNEBERG, A., MENNE, T., MADSEN, F., FROLUND, L., DIRKSEN, A., & JORGENSEN, T. (2002). Incidence of allergic contact sensitization in Danish adults between 1990 and 1998; the Copenhagen Allergy Study, Denmark. *Br J Dermatol*, **147**, 487–492.

NIIYA, H., LEI, J., GUO, Y., AZUMA, T., YAKUSHIJIN, Y., SAKAI, I., HATO, T., TOHYAMA, M., HASHIMOTO, K., & YASUKAWA, M. (2006). Human herpesvirus 6 impairs differentiation of monocytes to dendritic cells. *Exp Hematol*, **34**, 642–653.

NIKULINA, M., HABICH, C., FLOHE, S. B., SCOTT, F. W., & KOLB, H. (2004). Wheat gluten causes dendritic cell maturation and chemokine secretion. *J Immunol*, **173**, 1925–1933. Comparative Study.

NJOKU, D., LASTER, M. J., GONG, D. H., E. I., N. E., REED, G. F., & MARTIN, J. L. (1997). Biotransformation of halothane, enflurane, isoflurane, and desflurane to trifluoroacetylated liver proteins: association between protein acylation and hepatic injury. *Anesth Analg*, **84**, 173–8.

NORDBERG, J. & ARNER, E. S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med*, **31**, 1287–312.

NORGAUER, J., IBIG, Y., GMEINER, D., HEROUY, Y., & FIEBICH, B. L. (2003). Prostaglandin E2 synthesis in human monocyte-derived dendritic cells. *Int J Mol Med*, **12**, 83–6.

NOWAK, A., KLIMOWICZ, A., & KADYKW, M. (1985). Distribution of trimethoprim and sulphamethoxazole in blood during treatment with co-trimoxazole. *Eur J Clin Pharmacol*, **29**, 231–234.

NURNBERGER, T., BRUNNER, F., KEMMERLING, B., & PIATER, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev*, **198**, 249–266.

NYFELER, B. & PICHLER, W. J. (1997). The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Allergy*, **27**, 175–181.

OECD (2002). Guideline No. 429. Skin sensitization: Local lymph node assay. Tech. rep., Guidelines for Testing of Chemicals.

OHASHI, K., BURKART, V., FLOHE, S., & KOLB, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol*, **164**, 558–561.

OHISHI, K., KATAYAMA, N., MITANI, H., ARAKI, H., MASUYA, M., SUZUKI, H., HOSHINO, N., MIYASHITA, H., NISHII, K., KAGEYAMA, S., MINAMI, N., & SHIKU,

H. (2001). Efficient ex vivo generation of human dendritic cells from mobilized CD34+ peripheral blood progenitors. *Int J Hematol*, **74**, 287–296.

OKAMURA, Y., WATARI, M., JERUD, E. S., YOUNG, D. W., ISHIZAKA, S. T., ROSE, J., CHOW, J. C., & STRAUSS, J. F. R. (2001). The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem*, **276**, 10229–10233.

O'NEIL, W. M., MACARTHUR, R. D., FARROUGH, M. J., DOLL, M. A., FRETLAND, A. J., HEIN, D. W., CRANE, L. R., & SVENSSON, C. K. (2002). Acetylator phenotype and genotype in HIV-infected patients with and without sulfonamide hypersensitivity. *J Clin Pharmacol*, **42**, 613–619.

ORTIZ DE MONTELLADO, P. R., MATHEWS, J. M., & LANGRY, K. C. (1984). Autocatalytic inactivation of cytochrome P-450 and chloroperoxidase by 1-aminobenzotriazole and other aryne precursors. *Tetrahedron*, **40**, 511–9.

ORTMANN, B., MARTIN, S., VON BONIN, A., SCHILTZ, E., HOSCHTZKY, H., & WELTZIEN, H. U. (1992). Synthetic peptides anchor t cell-specific tnp epitopes to mhc antigens. *J Immunol*, **148**, 1445–1450.

O'SULLIVAN, B. J. & THOMAS, R. (2002). CD40 ligation conditions dendritic cell antigen-presenting function through sustained activation of NF-kappaB. *J Immunol*, **168**, 5491–5498.

OUAAZ, F., ARRON, J., ZHENG, Y., CHOI, Y., & BEG, A. A. (2002). Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity*, **16**, 257–270.

PADOVAN, E., BAUER, T., TONGIO, M. M., KALBACHER, H., & WELTZIEN, H. U. (1997). Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *Eur J Immunol*, **27**, 1303–1307.

PADOVAN, E., MAURI-HELLWEG, D., PICHLER, W. J., & WELTZIEN, H. U. (1996). T cell recognition of penicillin G: structural features determining antigenic specificity. *Eur J Immunol*, **26**, 42–48.

PALMER, J. M., ROBE, A. J., BURT, A. D., KIRBY, J. A., & JONES, D. E. (2004). Covalent modification as a mechanism for the breakdown of immune tolerance to pyruvate dehydrogenase complex in the mouse. *Hepatology*, **39**, 1583–92.

PANINA-BORDIGNON, P., CORRADIN, G., ROOSNEK, E., SETTE, A., & LANZAVECCHIA, A. (1991). Recognition by class II alloreactive T cells of processed determinants from human serum proteins. *Science*, **252**, 1548–1550.

PARK, B. K., KITTERINGHAM, N. R., MAGGS, J. L., PIRMOHAMED, M., & WILLIAMS, D. P. (2005). The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol*, **45**, 177–202.

PARK, B. K., NAISBITT, D. J., GORDON, S. F., KITTERINGHAM, N. R., & PIRMOHAMED, M. (2001). Metabolic activation in drug allergies. *Toxicology*, **158**, 11–23.

PARK, B. K., PIRMOHAMED, M., & KITTERINGHAM, N. R. (1992). Idiosyncratic drug reactions: a mechanistic evaluation of risk factors. *Br J Clin Pharmacol*, **34**, 377–395.

PARK, B. K., PIRMOHAMED, M., & KITTERINGHAM, N. R. (1998). Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. *Chem Res Toxicol*, **11**, 969–88.

PARK, B. K., TINGLE, M. D., GRABOWSKI, P. S., COLEMAN, J. W., & KITTERINGHAM, N. R. (1987). Drug-protein conjugates-XI. Disposition and immunogenicity of dinitrofluorobenzene, a model compound for the investigation of drugs as haptens. *Biochem Pharmacol*, **36**, 591–9.

PARK, B. K. & WHITTAKER, A. D. (1978). An immunochemical study of the hapten formed from ethynyloestradiol and guinea pig liver microsomes. *FEBS Lett*, **91**, 273–275.

PARNES, O. (2004). From interception to incorporation: degeneracy and promiscuous recognition as precursors of a paradigm shift in immunology. *Mol Immunol*, **40**, 985–991.

PAUL, C., WOLKENSTEIN, P., ADLE, H., WECHSLER, J., GARCHON, H. J., REVUZ, J., & ROUJEAU, J. C. (1996). Apoptosis as a mechanism of keratinocyte death in toxic epidermal necrolysis. *Br J Dermatol*, **134**, 710–714.

PEARCE, R. E., UETRECHT, J. P., & LEEDER, J. S. (2005). Pathways of carbamazepine bioactivation in vitro: II. The role of human cytochrome P450 enzymes in the formation of 2-hydroxyiminostilbene. *Drug Metab Dispos*, **33**, 1819–1826.

PEARCE, R. E., VAKKALAGADDA, G. R., & LEEDER, J. S. (2002). Pathways of carbamazepine bioactivation in vitro I. Characterization of human cytochromes P450 responsible for the formation of 2- and 3-hydroxylated metabolites. *Drug Metab Dispos*, **30**, 1170–1179.

PHAM, N. H., BALDO, B. A., MANFREDI, M., & ZERBONI, R. (1996). Fine structural specificity differences of trimethoprim allergenic determinants. *Clin Exp Allergy*, **26**, 1155–1160.

PICHLER, W. J. (2002). Pharmacological interaction of drugs with antigen-specific immune receptors: the p-i concept. *Curr Opin Allergy Clin Immunol*, **2**, 301–5.

PICHLER, W. J. (2003). Delayed drug hypersensitivity reactions. *Ann Intern Med*, **139**, 683–693.

PICHLER, W. J. (2005). Direct T-cell stimulations by drugs–bypassing the innate immune system. *Toxicology*, **209**, 95–100.

PICHLER, W. J. & TILCH, J. (2004). The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy*, **59**, 809–820.

PICHLER, W. J., ZANNI, M., VON GREYERZ, S., SCHNYDER, B., MAURI-HELLWEG, D., & WENDLAND, T. (1997). High IL-5 production by human drug-specific T cell clones. *Int Arch Allergy Immunol*, **113**, 177–180.

PICKARD, C., SMITH, A. M., COOPER, H., STRICKLAND, I., JACKSON, J., HEALY, E., & FRIEDMANN, P. S. (2007). Investigation of mechanisms underlying the T-cell response to the hapten 2,4-dinitrochlorobenzene. *J Invest Dermatol*, **127**, 630–7.

PICKL, W. F., MAJDIC, O., KOHL, P., STCKL, J., RIEDL, E., SCHEINECKER, C., BELLO-FERNANDEZ, C., & KNAPP, W. (1996). Molecular and functional characteristics of dendritic cells generated from highly purified CD14+ peripheral blood monocytes. *J Immunol*, **157**, 3850–3859.

PIRMOHAMED, M., ALFIREVIC, A., VILAR, J., STALFORD, A., WILKINS, E. G., SIM, E., & PARK, B. K. (2000). Association analysis of drug metabolizing enzyme gene polymorphisms in HIV-positive patients with co-trimoxazole hypersensitivity. *Pharmacogenetics*, **10**, 705–713.

PIRMOHAMED, M., GRAHAM, A., ROBERTS, P., SMITH, D., CHADWICK, D., BRECKENRIDGE, A. M., & PARK, B. K. (1991). Carbamazepine-hypersensitivity: assessment of clinical and in vitro chemical cross-reactivity with phenytoin and oxcarbazepine. *Br J Clin Pharmacol*, **32**, 741–749.

PIRMOHAMED, M., JAMES, S., MEAKIN, S., GREEN, C., SCOTT, A. K., WALLEY, T. J., FARRAR, K., PARK, B. K., & BRECKENRIDGE, A. M. (2004). Adverse drug reactions as cause of admission to hospital: prospective analysis of 18,820 patients. *BMJ*, **329**, 15–9.

PIRMOHAMED, M., KITTERINGHAM, N. R., GUENTHNER, T. M., BRECKENRIDGE, A. M., & PARK, B. K. (1992). An investigation of the formation of cytotoxic, protein-reactive and stable metabolites from carbamazepine in vitro. *Biochem Pharmacol*, **43**, 1675–82.

PIRMOHAMED, M., LIN, K., CHADWICK, D., & PARK, B. K. (2001). TNFalpha promoter region gene polymorphisms in carbamazepine-hypersensitive patients. *Neurology*, **56**, 890–896.

PIRMOHAMED, M., NAISBITT, D. J., GORDON, F., & PARK, B. K. (2002). The danger hypothesis–potential role in idiosyncratic drug reactions. *Toxicology*, **181-182**, 55–63.

PIRMOHAMED, M. & PARK, B. K. (2001). HIV and drug allergy. *Curr Opin Allergy Clin Immunol*, **1**, 311–316.

PISTOOR, F. H., KAPSENBERG, M. L., BOS, J. D., MEINARDI, M. M., VON BLOMBERG, M. E., & SCHEPER, R. J. (1995). Cross-reactivity of human nickel-reactive T-lymphocyte clones with copper and palladium. *J Invest Dermatol*, **105**, 92–95.

POLESE, L., ANGRIMAN, I., CECCHETTO, A., NORBERTO, L., SCARPA, M., RUFFOLO, C., BAROLLO, M., SOMMARIVA, A., & D'AMICO, D. F. (2002). The role of CD40 in ulcerative colitis: histochemical analysis and clinical correlation. *Eur J Gastroenterol Hepatol*, **14**, 237–41.

POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M. Y., VAN HUFFEL, C., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B., & BEUTLER, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, **282**, 2085–2088.

PRENDIVILLE, J. (2002). Stevens-Johnson syndrome and toxic epidermal necrolysis. *Adv Dermatol*, **18**, 151–173.

PULLEN, S. S., MILLER, H. G., EVERDEEN, D. S., DANG, T. T., CRUTE, J. J., & KEHRY, M. R. (1998). CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry*, **37**, 11836–11845.

PYTHON, F., GOEBEL, C., & AEBY, P. (2007). Assessment of the U937 cell line for the detection of contact allergens. *Toxicol Appl Pharmacol*, **220**, 113–124.

QUEZADA, S. A., JARVINEN, L. Z., LIND, E. F., & NOELLE, R. J. (2004). CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol*, **22**, 307–328.

RAMMENSEE, H. G. (1995). Chemistry of peptides associated with MHC class I and class II molecules. *Curr Opin Immunol*, **7**, 85–96.

RAWLINS, M. & THOMPSON, J. (1991). *Mechanisms of Adverse Drug Reactions*, pp. 18–45. Oxford University Press.

REILLY, T. P., LASH, L. H., DOLL, M. A., HEIN, D. W., WOSTER, P. M., & SVENSSON, C. K. (2000). A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J Invest Dermatol*, **114**, 1164–73.

REILLY, T. P., MACARTHUR, R. D., FARROUGH, M. J., CRANE, L. R., WOSTER, P. M., & SVENSSON, C. K. (1999). Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in human immunodeficiency virus-infected individuals? *J Pharmacol Exp Ther*, **291**, 1356–1364. Clinical Trial.

RESCIGNO, M., MARTINO, M., SUTHERLAND, C. L., GOLD, M. R., & RICCIARDI-CASTAGNOLI, P. (1998). Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med*, **188**, 2175–80.

RIEDER, M. J., UETRECHT, J., SHEAR, N. H., CANNON, M., MILLER, M., & SPIELBERG, S. P. (1989). Diagnosis of sulfonamide hypersensitivity reactions by in-vitro "rechallenge" with hydroxylamine metabolites. *Ann Intern Med*, **110**, 286–289.

RIEDER, M. J., UETRECHT, J., SHEAR, N. H., & SPIELBERG, S. P. (1988). Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. *J Pharmacol Exp Ther*, **244**, 724–728.

ROBERTS, S. A., PRICE, V. F., & JOLLOW, D. J. (1990). Acetaminophen structure-toxicity studies: in vivo covalent binding of a nonhepatotoxic analog, 3-hydroxyacetanilide. *Toxicol Appl Pharmacol*, **105**, 195–208.

RODRIGUEZ-PENA, R., LOPEZ, S., MAYORGA, C., ANTUNEZ, C., FERNANDEZ, T. D., TORRES, M. J., & BLANCA, M. (2006). Potential involvement of dendritic cells in delayed-type hypersensitivity reactions to beta-lactams. *J Allergy Clin Immunol*, **118**, 949–956.

ROMANI, N., GRUNER, S., BRANG, D., KAMPGEN, E., LENZ, A., TROCKENBACHER, B., KONWALINKA, G., FRITSCH, P. O., STEINMAN, R. M., & SCHULER, G. (1994). Proliferating dendritic cell progenitors in human blood. *J Exp Med*, **180**, 83–93.

ROTH, R. A., LUYENDYK, J. P., MADDOX, J. F., & GANEY, P. E. (2003). Inflammation and drug idiosyncrasy–is there a connection? *J Pharmacol Exp Ther*, **307**, 1–8.

ROYCHOWDHURY, S., VYAS, P. M., REILLY, T. P., GASPARI, A. A., & SVENSSON, C. K. (2005). Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. *J Pharmacol Exp Ther*, **314**, 43–52.

ROYCHOWDHURY, S., VYAS, P. M., & SVENSSON, C. K. (2007). Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. *Drug Metab Dispos*, **35**, 676–681.

RUBIN, R. L. & KRETZ-ROMMEL, A. (2001). Phagocyte-mediated oxidation in idiosyncratic adverse drug reactions. *Curr Opin Hematol*, **8**, 34–40.

RUDOLPH, M. G. & WILSON, I. A. (2002). The specificity of TCR/pMHC interaction. *Curr Opin Immunol*, **14**, 52–65.

RUSTEMEYER, T., PREUSS, M., VON BLOMBERG, B. M. E., DAS, P. K., & SCHEPER, R. J. (2003). Comparison of two in vitro dendritic cell maturation models for screening contact sensitizers using a panel of methacrylates. *Exp Dermatol*, **12**, 682–691.

RUTAULT, K., ALDERMAN, C., CHAIN, B. M., & KATZ, D. R. (1999). Reactive oxygen species activate human peripheral blood dendritic cells. *Free Radic Biol Med*, **26**, 232–238.

SAEKI, M., SAITO, Y., NAGANO, M., TESHIMA, R., OZAWA, S., & SAWADA, J. (2002). mRNA expression of multiple cytochrome p450 isozymes in four types of cultured skin cells. *Int Arch Allergy Immunol*, **127**, 333–6.

SAITO, T. & YAMASAKI, S. (2003). Negative feedback of T cell activation through inhibitory adapters and costimulatory receptors. *Immunol Rev*, **192**, 143–160.

SAKAGUCHI, H., ASHIKAGA, T., MIYAZAWA, M., YOSHIDA, Y., ITO, Y., YONEYAMA, K., HIROTA, M., ITAGAKI, H., TOYODA, H., & SUZUKI, H. (2006). Development of

an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT. *Toxicol In Vitro*, **20**, 774–784.

SATO, K., YAMASHITA, N., & MATSUYAMA, T. (2002). Human peripheral blood monocyte-derived interleukin-10-induced semi-mature dendritic cells induce anergic CD4(+) and CD8(+) T cells via presentation of the internalized soluble antigen and cross-presentation of the phagocytosed necrotic cellular fragments. *Cell Immunol*, **215**, 186–194.

SAUTER, B., ALBERT, M. L., FRANCISCO, L., LARSSON, M., SOMERSAN, S., & BHARDWAJ, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med*, **191**, 423–434.

SCHEIBNER, K. A., LUTZ, M. A., BOODOO, S., FENTON, M. J., POWELL, J. D., & HORTON, M. R. (2006). Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol*, **177**, 1272–1281.

SCHEUERMAN, O., NOFECH-MOSES, Y., RACHMEL, A., & ASHKENAZI, S. (2001). Successful treatment of antiepileptic drug hypersensitivity syndrome with intravenous immune globulin. *Pediatrics*, **107**, E14.

SCHEYTT, T., MERSMANN, P., LINDSTDT, R., & HEBERER, T. (2005). Determination of sorption coefficients of pharmaceutically active substances carbamazepine, diclofenac, and ibuprofen, in sandy sediments. *Chemosphere*, **60**, 245–253.

SCHNYDER, B., BURKHART, C., SCHNYDER-FRUTIG, K., VON GREYERZ, S., NAISBITT, D. J., PIRMOHAMED, M., PARK, B. K., & PICHLER, W. J. (2000). Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J Immunol*, **164**, 6647–6654.

SCHNYDER, B., MAURI-HELLWEG, D., ZANNI, M., BETTENS, F., & PICHLER, W. J. (1997). Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest*, **100**, 136–41.

SCHOLZ, W., PLATZER, B., SCHUMICH, A., HOCHER, B., FRITSCH, G., KNAPP, W., & STROBL, H. (2004). Initial human myeloid/dendritic cell progenitors identified by absence of myeloperoxidase protein expression. *Exp Hematol*, **32**, 270–6.

SCHWANDNER, R., DZIARSKI, R., WESCHE, H., ROTHE, M., & KIRSCHNING, C. J. (1999). Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem*, **274**, 17406–17409.

SEONG, S. Y. & MATZINGER, P. (2004). Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol*, **4**, 469–78.

SERRA, P., AMRANI, A., HAN, B., YAMANOUCHI, J., THIESSEN, S. J., & SANTA-MARIA, P. (2002). RAG-dependent peripheral T cell receptor diversification in CD8+ T lymphocytes. *Proc Natl Acad Sci U S A*, **99**, 15566–15571.

SHARMA, R. A., STEWARD, W. P., DAINES, C. A., KNIGHT, R. D., O'BYRNE, K. J., & DALGLEISH, A. G. (2006). Toxicity profile of the immunomodulatory thalidomide analogue, lenalidomide: phase I clinical trial of three dosing schedules in patients with solid malignancies. *Eur J Cancer*, **42**, 2318–2325.

SHI, Y., EVANS, J. E., & ROCK, K. L. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*, **425**, 516–21.

SHI, Y., GALUSHA, S. A., & ROCK, K. L. (2006). Cutting edge: elimination of an endogenous adjuvant reduces the activation of CD8 T lymphocytes to transplanted cells and in an autoimmune diabetes model. *J Immunol*, **176**, 3905–3908.

SHIELDS, P. L., MORLAND, C. M., SALMON, M., QIN, S., HUBSCHER, S. G., & ADAMS, D. H. (1999). Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J Immunol*, **163**, 6236–6243.

SHIMADA, T., GILLAM, E. M., SUTTER, T. R., STRICKLAND, P. T., GUENGERICH, F. P., & YAMAZAKI, H. (1997). Oxidation of xenobiotics by recombinant human cytochrome P450 1B1. *Drug Metab Dispos*, **25**, 617–22.

SHIMONKEVITZ, R., KAPPLER, J., MARRACK, P., & GREY, H. (1983). Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J Exp Med*, **158**, 303–316.

SIEBEN, S., BARON, J. M., BLOMEKE, B., & MERK, H. F. (1999). Multiple cytochrome P450-isoenzymes mRNA are expressed in dendritic cells. *Int Arch Allergy Immunol*, **118**, 358–61.

SINIGAGLIA, F., SCHEIDEGGER, D., GAROTTA, G., SCHEPER, R., PLETSCHER, M., & LANZAVECCHIA, A. (1985). Isolation and characterization of Ni-specific T cell clones from patients with Ni-contact dermatitis. *J Immunol*, **135**, 3929–3932.

SKOV, S., BONYHADI, M., ODUM, N., & LEDBETTER, J. A. (2000). IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. *J Immunol*, **164**, 3500–3505.

SMILEY, S. T., KING, J. A., & HANCOCK, W. W. (2001). Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol*, **167**, 2887–2894.

SOEBERG, B., SUMERSKA, T., BINNS, R. M., & BALFOUR, B. M. (1978a). Contact sensitivity in the pig. II. Induction by intralymphatic infusion of DNP conjugated cells. *Int Arch Allergy Appl Immunol*, **57**, 114–25.

SOEBERG, B., SUMERSKA, T., BINNS, R. M., & BALFOUR, B. M. (1978b). Contact sensitivity in the pig. III. Induction by intralymphatic infusion of DNP-conjugated cell membranes and soluble proteins, free DNFB and some small molecular weight derivatives. *Int Arch Allergy Appl Immunol*, **57**, 481–7.

SOLER, D., HUMPHREYS, T. L., SPINOLA, S. M., & CAMPBELL, J. J. (2003). CCR4 versus CCR10 in human cutaneous TH lymphocyte trafficking. *Blood*, **101**, 1677–1682.

SPEIR, J. A., STEVENS, J., JOLY, E., BUTCHER, G. W., & WILSON, I. A. (2001). Two different, highly exposed, bulged structures for an unusually long peptide bound to rat MHC class I RT1-Aa. *Immunity*, **14**, 81–92.

STAQUET, M. J., SPORTOUCH, M., JACQUET, C., SCHMITT, D., GUESNET, J., & PEGUET-NAVARRO, J. (2004). Moderate skin sensitizers can induce phenotypic changes on in vitro generated dendritic cells. *Toxicol In Vitro*, **18**, 493–500.

STARKEL, P., SEMPOUX, C., VAN DEN BERGE, V., STEVENS, M., DE SAEGER, C., DESAGER, J. P., & HORSMANS, Y. (1999). CYP 3A proteins are expressed in human neutrophils and lymphocytes but are not induced by rifampicin. *Life Sci*, **64**, 643–53.

STEVENS, R. C., LAIZURE, S. C., WILLIAMS, C. L., & STEIN, D. S. (1991). Pharmacokinetics and adverse effects of 20-mg/kg/day trimethoprim and 100-mg/kg/day sulfamethoxazole in healthy adult subjects. *Antimicrob Agents Chemother*, **35**, 1884–1890.

STRICKLAND, I., FITZGERALD, D., & FRIEDMANN, P. S. (1996). Immune responses to DNCB and carbamazepine are dependent on intermediate metabolism. *J Invest Dermatol*, **107**, 499.

STUPP, Y., PAUL, W. E., & BENACERRAF, B. (1971). Structural control of immunogenicity. II. Antibody synthesis and cellular immunity in response to immunization with mono-epsilon-oligo-L-lysines. *Immunology*, **21**, 583–594.

SUBUDHI, S. K., ALEGRE, M.-L., & FU, Y.-X. (2005). The balance of immune responses: costimulation verse coinhibition. *J Mol Med*, **83**, 193–202.

SWANSON, H. I. (2004). Cytochrome P450 expression in human keratinocytes: an aryl hydrocarbon receptor perspective. *Chem Biol Interact*, **149**, 69–79.

SYKULEV, Y., JOO, M., VTURINA, I., TSOMIDES, T. J., & EISEN, H. N. (1996). Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity*, **4**, 565–571.

SYME, R., BAJWA, R., ROBERTSON, L., STEWART, D., & GLUCK, S. (2005). Comparison of CD34 and monocyte-derived dendritic cells from mobilized peripheral blood from cancer patients. *Stem Cells*, **23**, 74–81.

TAKEDA, K., TAKEUCHI, O., & AKIRA, S. (2002). Recognition of lipopeptides by Toll-like receptors. *J Endotoxin Res*, **8**, 459–463.

TAN, P. H., SAGOO, P., CHAN, C., YATES, J. B., CAMPBELL, J., BEUTELSPACHER, S. C., FOXWELL, B. M. J., LOMBARDI, G., & GEORGE, A. J. T. (2005). Inhibition of NF-kappa B and oxidative pathways in human dendritic cells by antioxidative vitamins generates regulatory T cells. *J Immunol*, **174**, 7633–7644.

TAPIA, B., PADIAL, A., SNCHEZ-SABAT, E., ALVAREZ-FERREIRA, J., MOREL, E., BLANCA, M., & BELLN, T. (2004). Involvement of CCL27-CCR10 interactions in drug-induced cutaneous reactions. *J Allergy Clin Immunol*, **114**, 335–340.

TERMEER, C., BENEDIX, F., SLEEMAN, J., FIEBER, C., VOITH, U., AHRENS, T., MIYAKE, K., FREUDENBERG, M., GALANOS, C., & SIMON, J. C. (2002). Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med*, **195**, 99–111.

THEISOHN, M. & HEIMANN, G. (1982). Disposition of the antiepileptic oxcarbazepine and its metabolites in healthy volunteers. *Eur J Clin Pharmacol*, **22**, 545–551.

THOMPSON, C. B., LINDSTEN, T., LEDBETTER, J. A., KUNKEL, S. L., YOUNG, H. A., EMERSON, S. G., LEIDEN, J. M., & JUNE, C. H. (1989). CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci U S A*, **86**, 1333–1337.

TINGLE, M. D., CLARKE, J. B., KITTERINGHAM, N. R., & PARK, B. K. (1990). Influence of glutathione conjugation on the immunogenicity of dinitrophenyl derivatives in the rat. *Int Arch Allergy Appl Immunol*, **91**, 160–5.

TODRYK, S. M., MELCHER, A. A., DALGLEISH, A. G., & VILE, R. G. (2000). Heat shock proteins refine the danger theory. *Immunology*, **99**, 334–7.

TOEBAK, M. J., MOED, H., VON BLOMBERG, M. B. E., BRUYNZEEL, D. P., GIBBS, S., SCHEPER, R. J., & RUSTEMEYER, T. (2006). Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by dendritic cells. *Contact Dermatitis*, **55**, 238–245.

TOMSON, T., TYBRING, G., BERTILSSON, L., EKBOM, K., & RANE, A. (1980). Carbamazepine therapy in trigeminal neuralgia: clinical effects in relation to plasma concentration. *Arch Neurol*, **37**, 699–703.

TREPANIER, L. A., YODER, A. R., BAJAD, S., BECKWITH, M. D., BELLEHUMEUR, J. L., & GRAZIANO, F. M. (2004). Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J Acquir Immune Defic Syndr*, **36**, 1041–1050.

TROOST, R. J., PARYS, J. A. V., HOOIJKAAS, H., VAN JOOST, T., BENNER, R., & PRENS, E. P. (1996). Allergy to carbamazepine: parallel in vivo and in vitro detection. *Epilepsia*, **37**, 1093–1099.

TSUBERY, H., OFEK, I., COHEN, S., & FRIDKIN, M. (2000). The functional association of polymyxin B with bacterial lipopolysaccharide is stereospecific: studies on polymyxin B nonapeptide. *Biochemistry*, **39**, 11837–11844.

TUSCHL, H., KOVAC, R., & WEBER, E. (2000). The expression of surface markers on dendritic cells as indicators for the sensitizing potential of chemicals. *Toxicol In Vitro*, **14**, 541–9.

UDENFRIEND, S., CLARK, C. T., AXELROD, J., & BRODIE, B. B. (1954). Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. *J Biol Chem*, **208**, 731–739.

UETRECHT, J. & ZAHID, N. (1988). N-chlorination of phenytoin by myeloperoxidase to a reactive metabolite. *Chem Res Toxicol*, **1**, 148–151.

UETRECHT, J. P., SHEAR, N. H., & ZAHID, N. (1993). N-chlorination of sulfamethoxazole and dapsone by the myeloperoxidase system. *Drug Metab Dispos*, **21**, 830–4.

VALENTINIS, B., BIANCHI, A., ZHOU, D., CIPPONI, A., CATALANOTTI, F., RUSSO, V., & TRAVERSARI, C. (2005). Direct effects of polymyxin B on human dendritic cells maturation. The role of IkappaB-alpha/NF-kappaB and ERK1/2 pathways and adhesion. *J Biol Chem*, **280**, 14264–14271.

VALITUTTI, S., MLLER, S., CELLA, M., PADOVAN, E., & LANZAVECCHIA, A. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*, **375**, 148–151.

VAN DER HOOFT, C. S., STURKENBOOM, M. C. J. M., VAN GROOTHEEST, K., KINGMA, H. J., & STRICKER, B. H. C. (2006). Adverse drug reaction-related hospitalisations: a nationwide study in The Netherlands. *Drug Saf*, **29**, 161–168.

VAN DER MERWE, P. A. & DAVIS, S. J. (2003). Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol*, **21**, 659–684.

VAN DER VEN, A. J., MANTEL, M. A., VREE, T. B., KOOPMANS, P. P., & VAN DER MEER, J. W. (1994). Formation and elimination of sulphamethoxazole hydroxy-lamine after oral administration of sulphamethoxazole. *Br J Clin Pharmacol*, **38**, 147–150. Clinical Trial.

VAN 'T KLOOSTER, G. A., KOLKER, H. J., VAN NIJNANTEN, F. M. W., NOORD-HOEK, J., & VAN MIERT, A. S. (1992). Determination of trimethoprim and its oxidative metabolites in cell culture media and microsomal incubation mixtures by high-performance liquid chromatography. *J Chromatogr*, **579**, 354–360.

VANDEPUTTE, C., GUIZON, I., GENESTIE-DENIS, I., VANNIER, B., & LORENZON, G. (1994). A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol Toxicol*, **10**, 415–21.

VERGINIS, P., LI, H. S., & CARAYANNIOTIS, G. (2005). Tolerogenic semimature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4+CD25+ T cells. *J Immunol*, **174**, 7433–9.

VERHASSELT, V., BERGHE, W. V., VANDERHEYDE, N., WILLEMS, F., HAEGEMAN, G., & GOLDMAN, M. (1999). N-acetyl-L-cysteine inhibits primary human T cell responses at the dendritic cell level: association with NF-kappaB inhibition. *J Immunol*, **162**, 2569–2574.

VERHASSELT, V., BUELENS, C., WILLEMS, F., GROOTE, D. D., HAEFFNER-CAVAILLON, N., & GOLDMAN, M. (1997). Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. *J Immunol*, **158**, 2919–2925.

VERHEYEN, G. R., SCHOETERS, E., NUIJTEN, J.-M., HEUVEL, R. L. V. D., NELISSEN, I., WITTERS, H., TENDELOO, V. F. I. V., BERNEMAN, Z. N., & SCHOETERS, G. E. R. (2005). Cytokine transcript profiling in CD34+-progenitor derived dendritic cells exposed to contact allergens and irritants. *Toxicol Lett*, **155**, 187–194.

VERMES, I., HAANEN, C., STEFFENS-NAKKEN, H., & REUTELINGSPERGER, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*, **184**, 39–51.

VOGEL, C. (2000). Prostaglandin H synthases and their importance in chemical toxicity. *Curr Drug Metab*, **1**, 391–404.

VON BOEHMER, H. (2005). Mechanisms of suppression by suppressor T cells. *Nat Immunol*, **6**, 338–344.

VON BONIN, A., ORTMANN, B., MARTIN, S., & WELTZIEN, H. U. (1992). Peptide-conjugated hapten groups are the major antigenic determinants for trinitrophenyl-specific cytotoxic t cells. *Int Immunol*, **4**, 869–874.

VREE, T. B., VAN DER VEN, A. J., VAN WISSEN, C. P. V., VAN EWIJK-BENEKEN KOLMER, E. W., SWOLFS, A. E., VAN GALEN, P. M., & AMATDJAIS-GROENEN, H. (1994). Isolation, identification and determination of sulfamethoxazole and its known metabolites in human plasma and urine by high-performance liquid chromatography. *J Chromatogr B Biomed Appl*, **658**, 327–340.

VYAS, P. M., ROYCHOWDHURY, S., KHAN, F. D., PRISINZANO, T. E., LAMBA, J., SCHUETZ, E. G., BLAISDELL, J., GOLDSTEIN, J. A., MUNSON, K. L., HINES, R. N., & SVENSSON, C. K. (2006a). Enzyme-mediated protein haptenation of

dapsone and sulfamethoxazole in human keratinocytes: I. Expression and role of cytochromes P450. *J Pharmacol Exp Ther*, **319**, 488–496.

VYAS, P. M., ROYCHOWDHURY, S., KOUKOURITAKI, S. B., HINES, R. N., KRUEGER, S. K., WILLIAMS, D. E., NAUSEEF, W. M., & SVENSSON, C. K. (2006b). Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes: II. Expression and role of flavin-containing monooxygenases and peroxidases. *J Pharmacol Exp Ther*, **319**, 497–505.

VYAS, P. M., ROYCHOWDHURY, S., & SVENSSON, C. K. (2006c). Role of human cyclooxygenase-2 in the bioactivation of dapsone and sulfamethoxazole. *Drug Metab Dispos*, **34**, 16–18.

VYAS, P. M., ROYCHOWDHURY, S., WOSTER, P. M., & SVENSSON, C. K. (2005). Reactive oxygen species generation and its role in the differential cytotoxicity of the arylhydroxylamine metabolites of sulfamethoxazole and dapsone in normal human epidermal keratinocytes. *Biochem Pharmacol*, **70**, 275–286.

WALMSLEY, S. L., KHORASHEH, S., SINGER, J., & DJURDJEV, O. (1998). A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). Canadian HIV Trials Network 057 Study Group. *J Acquir Immune Defic Syndr Hum Retrovirol*, **19**, 498–505. Clinical Trial.

WALMSLEY, S. L., WINN, L. M., HARRISON, M. L., UETRECHT, J. P., & WELLS, P. G. (1997). Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: toxicological and pathological implications. *Aids*, **11**, 1689–97.

WALSH, J. S., REESE, M. J., & THURMOND, L. M. (2002). The metabolic activation of abacavir by human liver cytosol and expressed human alcohol dehydrogenase isozymes. *Chem Biol Interact*, **142**, 135–54.

WALUNAS, T. L., BAKKER, C. Y., & BLUESTONE, J. A. (1996). CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med*, **183**, 2541–2550.

WANG, C. Y., MAZER, S. P., MINAMOTO, K., TAKUMA, S., HOMMA, S., YELLIN, M., CHESS, L., FARD, A., KALLED, S. L., OZ, M. C., & PINSKY, D. J. (2002). Suppression of murine cardiac allograft arteriopathy by long-term blockade of CD40-CD154 interactions. *Circulation*, **105**, 1609–14.

WATANABE, J., SHIMADA, T., GILLAM, E. M., IKUTA, T., SUEMASU, K., HIGASHI, Y., GOTOH, O., & KAWAJIRI, K. (2000). Association of CYP1B1 genetic polymorphism with incidence to breast and lung cancer. *Pharmacogenetics*, **10**, 25–33.

WELTZIEN, H. U., MOULON, C., MARTIN, S., PADOVAN, E., HARTMANN, U., & KOHLER, J. (1996). T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. *Toxicology*, **107**, 141–51.

WHO (1972). International drug monitoring: the role of national centres. Tech. Rep. 498, Tech Rep Ser WHO.

WIFFEN, P., GILL, M., EDWARDS, J., & A.MOORE (2002). Adverse drug reactions in hospital patients. A systematic review of the prospective and retrospective studies. *Bandolier Extra*, **June**, 1–16.

WIJSMAN, J. A., DEKABAN, G. A., & RIEDER, M. J. (2005). Differential toxicity of reactive metabolites of clindamycin and sulfonamides in HIV-infected cells: influence of HIV infection on clindamycin toxicity in vitro. *J Clin Pharmacol*, **45**, 346–51.

WILLIAMS, D. P. & PARK, B. K. (2003). Idiosyncratic toxicity: the role of toxicophores and bioactivation. *Drug Discov Today*, **8**, 1044–1050.

WILLIAMS, D. P., PIRMOHAMED, M., NAISBITT, D. J., MAGGS, J. L., & PARK, B. K. (1997). Neutrophil cytotoxicity of the chemically reactive metabolite(s) of clozapine: possible role in agranulocytosis. *J Pharmacol Exp Ther*, **283**, 1375–1382.

WILLIAMS, D. P., PIRMOHAMED, M., NAISBITT, D. J., UETRECHT, J. P., & PARK, B. K. (2000). Induction of metabolism-dependent and -independent neutrophil apoptosis by clozapine. *Mol Pharmacol*, **58**, 207–216.

WILLIAMS, R. (1959). *Detoxication Mechanisms*, chap. The metabolism of foreign compounds, pp. 717–740. 2nd edn. John Wiley, New York.

WOLKENSTEIN, P., LORIOT, M.-A., FLAHAULT, A., CADILHAC, M., CAUMES, E., ELIASZEWICZ, M., BEAUNE, P., ROUJEAU, J.-C., CHOSIDOW, O., & GROUP, T. E. (2005). Association analysis of drug metabolizing enzyme gene polymorphisms in AIDS patients with cutaneous reactions to sulfonamides. *J Invest Dermatol*, **125**, 1080–1082.

WU, Y. (2006). *Characterization of the chemical and cellular basis of carbamazepine hypersensitivity*. Ph.D. thesis, University of Liverpool.

WU, Y., FARRELL, J., PIRMOHAMED, M., PARK, B. K., & NAISBITT, D. J. (2007). Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity. *J Allergy Clin Immunol*, **119**, 973–981.

WU, Y., SANDERSON, J. P., FARRELL, J., DRUMMOND, N. S., HANSON, A., BOWKETT, E., BERRY, N., STACHULSKI, A. V., CLARKE, S. E., PICHLER, W. J., PIRMOHAMED, M., PARK, B. K., & NAISBITT, D. J. (2006). Activation of T cells by carbamazepine and carbamazepine metabolites. *J Allergy Clin Immunol*, **118**, 233–241.

WUCHERPFENNIG, K. W. (2004). T cell receptor crossreactivity as a general property of T cell recognition. *Mol Immunol*, **40**, 1009–1017.

YAMADA, H., ARAI, T., ENDO, N., YAMASHITA, K., FUKUDA, K., SASADA, M., & UCHIYAMA, T. (2006). LPS-induced ROS generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells. *Life Sci*, **78**, 926–933.

YANG, C.-W. O., HUNG, S.-I., JUO, C.-G., LIN, Y.-P., FANG, W.-H., LU, I.-H., CHEN, S.-T., & CHEN, Y.-T. (2007). HLA-B *1502-bound peptides: Implications for the pathogenesis of carbamazepine-induced Stevens-Johnson syndrome. *J Allergy Clin Immunol*.

YAWALKAR, N., SHRIKHANDE, M., HARI, Y., NIEVERGELT, H., BRAATHEN, L. R., & PICHLER, W. J. (2000). Evidence for a role for IL-5 and eotaxin in activating and

BIBLIOGRAPHY

recruiting eosinophils in drug-induced cutaneous eruptions. *J Allergy Clin Immunol*, **106**, 1171–1176.

YENGI, L. G., XIANG, Q., PAN, J., SCATINA, J., KAO, J., BALL, S. E., FRUNCILLO, R., FERRON, G., & ROLAND WOLF, C. (2003). Quantitation of cytochrome P450 mRNA levels in human skin. *Anal Biochem*, **316**, 103–10.

YI, A. K., KLINMAN, D. M., MARTIN, T. L., MATSON, S., & KRIEG, A. M. (1996). Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol*, **157**, 5394–5402.

ZAKRZEWSKA, J. M. & IVANYI, L. (1988). In vitro lymphocyte proliferation by carbamazepine, carbamazepine-10, 11-epoxide, and oxcarbazepine in the diagnosis of drug-induced hypersensitivity. *J Allergy Clin Immunol*, **82**, 110–115.

ZANNI, M. P., MAURI-HELLWEG, D., BRANDER, C., WENDLAND, T., SCHNYDER, B., FREI, E., VON GREYERZ, S., BIRCHER, A., & PICHLER, W. J. (1997). Characterization of lidocaine-specific T cells. *J Immunol*, **158**, 1139–48.

ZANNI, M. P., VON GREYERZ, S., SCHNYDER, B., BRANDER, K. A., FRUTIG, K., HARI, Y., VALITUTTI, S., & PICHLER, W. J. (1998). HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *J Clin Invest*, **102**, 1591–8.

ZHANG, Y. & WILCOX, D. E. (2002). Thermodynamic and spectroscopic study of Cu(II) and Ni(II) binding to bovine serum albumin. *J Biol Inorg Chem*, **7**, 327–337.

Index

INDEX

halothane, 16–17
hapten hypothesis, 33–35, 113, 171
high-performance liquid chromatography,
55, 80, 91, 102–105

lipopolysaccharide, 25, 56, 59, 61, 73, 81,
91
contamination, 27, **63**, 68, 72
lymphocyte transformation test, 115, 122,
123–126, 136, 150–151, **155**, 154–
156, 162–163

major histocompatibility complex, 31–32
mass spectrometry, 104
molecular interactions, **140**, 142–143, 165
myeloperoxidase, 12–13, 18, 45, 87, 102,
147, 169
expression, 19, **87**, 87–89, **90**, 96–98,
169
in agranulocytosis, 98
primer and probe design, **80**

nickel, 24, 36–37
nuclear magnetic resonance, 55, 58

oxcarbazepine, 113

p-i concept, 35–36, 43, 113, 139, 160, 165,
171–172, 175
penicillin, 39–42, 73
peptides
as antigens, 22, 31–33
peripheral blood mononuclear cells
isolation, 55–56
polymerase chain reaction, 80
primer and probe design, 77–80
polymyxin B, 59, **63**, 68
proliferation assay, 58, 119, 127, **128**, 151,
156, **158**
combined pulse, 121, 132, **134**, 139
fixation, 35, 120, 132, **133**, 138–139,
154, 160, **162**, 165
pulses, 35, 120, 127, **129**, 137, 153,
156–160, **162**, 165

reactive oxygen species, 14, 30, 99
and dendritic cell activation, 30, 73

serial triggering, 33
stimulation index, 58

sulfamethoxazole, 36, 42–43, 50–110, 167–
170
adduct detection, 95, 99
ELISA, 76–77, 82, **88**
flow cytometry, 76, 82, **85–86**
animal models, 57, 61, **64**, 68–69
hydroxyl derivative, 101–110
chromatography, **106**, 110
fragmentation, **107**
generation, 103, 108, **108**
hypersensitivity, 51–52
animal models, 42–43
as antigen, 35–36, 43, 51, 144, 170
as danger signal, 51–52, 61–66, 168
genetic associations, 34
primary stimulation, 36, 57–58, 61,
65, 69
metabolism, 16, 18–19, **40**, 42, 80, 87,
87, 101–102, 169
nitroso, 114
reactions, 42
synthesis, **54**, 53–55, 59, 74
toxicity, 15, 82, 99, 168

T-cell
activation, 21–22, 31–37
cloning, 112, 117, 122, 137, 143, 151,
156, 163
cytotoxicity, 23, 152
effector mechanisms, 22–24
receptor, 21, 31–33, 43, 113–114, 143,
165, 171–173
internalisation, 35, 121, 132, **135**,
139
regulatory, 24
toll-like receptors, *see also* danger signals,
25, **26**, 27, 29, 72, 95
trimethoprim, 146–166
hypersensitivity, 147, 149–150, 162
as antigen, **155**, **157**, **162**, 171
metabolism, 19, 147, **148**, 163

Udenfriend's reaction, 108

224