Mechanisms of antigen presentation to T-cells in *para*-phenylenediamine allergy.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Declaration

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

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Contents

Abbreviations

ACD	Allergic contact dermatitis
APC	Antigen presenting cells
BB	Bandrowski's base
BLCL	B lymphoblastoid cell line
BSA	Bovine Serum Albumin
CCL	Cys-Cys chemokine ligand
CCR	Cys-Cys chemokine receptor
CD	Cell surface molecules expressed on various cell types
CHS	Contact hypersensitivity
CLIP	Class-II-associated invariant chain peptide
CLP	Common lymphoid precursor (CLP)
cpm	Counts per minute
CSA	Cyclosporin A
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CTL	Cytotoxic T lymphocyte
CXCL	Cys-Cys chemokine with a single amino acid between the first two cysteines
CXCR	Cys-Cys chemokine with a single amino acid between the first two cysteines
DC	Dendritic cells
DMSO	Dimethyl sulphoxide
DNCB	2,4-dinitro-chlorobenzene
DNFB	2,4-dinitrofluorobenzene
EBV	Epstein-Barr virus
FACS	Fluorescence-activated cell sorter
FITC	Fluorescein isothiocyanate-conjugated
GM-CSF	Granulocyte macrophage colony stimulating factor
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HBSS	Hank's Buffered Salt Solution
HLA	Human leukocyte antigens
HPLC	High-performance liquid chromatography

HSC	Hematopoietic stem cells
ICAM-1	Intracellular adhesion molecule 1
ICOS	Inducible co-stimulator
IFN	Interferon
IL	Interleukin
LC	Langerhans' cell
LC-MS	Liquid chromatography-mass spectrometry
LLNA	Local lymph node assay
LMW	Low molecular weight
LPS	Lipopolysaccarides
LTT	Lymphocyte transformation test
M-DC	Myeloid-derived dendritic cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein-1
MLR	Mixed lymphocyte reaction
MoDC	Monocyte-derived dendritic cell
m/z	Mass:charge ratio
NAT	N-acetyltransferase
NF-κB	Nuclear factor-κB
Ni	Nickel
$NiCl_2$	Nickel chloride
NK	Natural killer cells
PAMP	Pathogen-associated molecular patterns
PAP	p-aminophenol
PBMC	Peripheral blood mononuclear cells
PBQ	p-benzoquinone
PBS	Phosphate buffer solution
P-DC	plasmacytoid dendritic cells
PE	Phycoerythrin
PHA	Phytohemagglutinin
PI	Pharmacological interaction
PKC	Protein kinase C
PPD	para-phenylenediamine

PRR	Pattern recognition receptors
rpm	Revolutions per minute
ROS	Reactive oxygen species
Rt	Retention time
SI	Stimulation index
SMX	Sulfamethoxazole
SMX-NO	Nitroso-sulfamethoxazole
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptors
TMA	Trimellitic anhydride
TNCB	Trinitrochlorobenzene
TNF	Tumor necrosis factor
T regs	Regulatory T cells
TR1	T regulatory type 1 cells
TT	Tetanus toxoid

Publications

- **Published papers**

  Coulter EM, Jenkinson C, Wu Y, Farrell J, Foster B, Smith A, McGuire C, Pease C, Basketter D, King C, Friedmann PS, Pirmohamed M, Park BK, Naisbitt DJ. Activation of T-cells from allergic patients and volunteers by p-phenylenediamine and Bandrowski's base. J Invest Dermatol. 2008 Apr;128(4):897-905.

  Farrell J, Jenkinson C, Lavergne SN, Maggs JL, Kevin Park B, Naisbitt DJ. Investigation of the immunogenicity of p-phenylenediamine and Bandrowski's base in the mouse. Toxicol Lett. 2009 Mar 28;185(3):153-9.

  Jenkinson C, Jenkins RE, Maggs JL, Kitteringham NR, Aleksic M, Park BK & Naisbitt DJ. A mechanistic investigation into the irreversible protein binding and antigenicity of p-phenylenediamine. Chem Res Toxicol. 2009 Jun;22(6):1172-80.

- **Published abstracts**

  Jenkinson C, Jenkins RE, Pease CK, Kitteringham NR, Aleksic M, Naisbitt DJ, Park BK A mechanistic investigation into the binding of PPD to a cysteine containing peptide and the model protein glutathione-S-transferase Pi. European Journal of Dermatology. 2008; 18(2): 253

  Jenkinson C, Jenkins RE, Pease CK, Kitteringham NR, Aleksic M, Pirmohamed M, Park BK, Naisbitt DJ. p-Phenylenediamine-bound irreversibly to cysteine residues on human serum albumin activates T-cells from hair dye allergic patients. European Journal of Dermatology. 2008; 18(2): 253

- **Manuscripts in press**

  Jenkinson C, Jenkins RE, Aleksic M, Pirmohamed M, Naisbitt DJ, Park BK Characterization of *p*-phenylenediamine-albumin binding sites and T-cell responses to hapten-modified protein. Submitted to the Journal of Investigative Dermatology

  Coulter EM, Jenkinson C, Farrell J, Pease CK, White A, Basketter D, Williams D, King C, Pirmohamed M, Park BK, Naisbitt DJ. Measurement of CD4+ and CD8+ T-lymphocyte cytokine secretion and gene expression changes from *p*-phenylenediamine allergic patients and tolerant individuals. Submitted to the Journal of Investigative Dermatology.

Abstract

The skin, as the outermost barrier of the body's defence system, is the first to encounter chemical factors from the environment. Many of the hundreds of exogenous compounds it comes into contact with are harmless, however in some instances chemical entities are known to act specifically as skin allergens. In these hypersensitive individuals this contact can elicit a reaction known as allergic contact dermatitis (ACD), which is a delayed-type antigen specific T cell mediated response resulting in skin erythema and oedma. Contact allergens are low weight molecular compounds that are incapable of initiating an immune response by themselves. They must bind to proteins to form stable hapten-protein complexes that prime the immune system to produce these antigen-specific T cells. The aromatic amine para-phenylenediamine (PPD) is an example of a chemical capable of initiating this immunological response. PPD is one of the most frequently encountered contact allergens, due to its common use in hair dye formulations, textile dyes and henna tattoos. The sensitising potential of PPD is thought to be due to the chemicals innate instability; it undergoes auto-oxidation to produce an electrophilic quinonediimine intermediate, which is susceptible to self-conjugation reactions to form the trimer Bandrowski's base (BB). Animal models have classed PPD and BB as potent sensitisers and human studies have indicated both have T-cell stimulatory capacity. However the antigen responsible remains undefined. The aim of this study was to investigate the antigenic factor involved in PPD-mediated ACD and examine in detail the mechanisms involved in the stimulation of a T-cell response.

PPD and BB were shown to directly stimulate proliferation of purified CD4+ and CD8+ lymphocytes from allergic patients. In contrast only CD4+, BB-specific T cells were characterised from blood of volunteers, however no evidence of BB-specific cells was found in cord blood. Their presence seems to reflect an acquired immune response not translated into an allergic reaction and represents an important discrimination between the two test groups. [^{14}C]PPD was found to bind irreversibly to both extracellular and intracellular protein; therefore, mass spectrometry was used to explore the binding of PPD to model peptides, the extracellular protein human serum albumin (HSA) and the intracellular protein glutathione-S-transferase Pi (GSTP). PPD selectively modified cysteine residues of the designer peptide DS3, GSTP (cys47) and HSA (cys34). PPD-modified HSA was shown to stimulate T-cells from allergic patient blood but not volunteers. Generation of PPD and PPD-modified HSA specific T cell clones allowed characterisation of mechanisms of antigen presentation. Three pathways of T cell activation by PPD were identified (1) classical hapten mechanism, involving irreversible protein adduct formation and processing, (2) direct irreversible binding of PPD to peptides embedded in the MHC groove or MHC itself, (3) p.i-mechanism involving a direct reversible interaction of PPD with MHC molecules. A comparison of the functional characteristics of T-cells from allergic patients and volunteers using Luminex methodology identified an important difference in cytokine secretion when patient and volunteer samples were compared. Specifically, stimulated cells from allergic patients secreted high levels of cytokines associated with a type 2 response.

In conclusion, we have identified the amino acid cysteine as a selective target for PPD modification in model peptides and proteins, and that a PPD hapten-protein complex

can stimulate T cells from allergic patients via a classical hapten mechanism. Furthermore these data reveal several important functional differences between T-cells from PPD allergic patients and volunteers. In particular, PPD and BB treatment of allergic patient samples was associated with (1) stimulation of CD4+ and CD8+ T-cells and (2) significantly higher levels of type 2 cytokine secretion, suggesting that the ability to produce cytokines such as IL-4, IL-5, IL-9 and IL-13 following antigen-stimulation may be an important factor contributing to the development of contact dermatitis.

CHAPTER 1

General Introduction

1.1 Introduction to the immune system

The human body is constantly exposed to microbes that can cause infectious diseases. To fight these invading microbes the immune system developed as the body's defence mechanism to detect, distinguish as non-self and eliminate these threats without damage to the host. These defence mechanisms comprise of physical or anatomical (skin, mucosal lining), mechanical (ciliated cells from respiratory tracts) and biochemical barriers (tears, saliva containing antimicrobial lysozymes) as well as two inducible defence systems; the innate and adaptive immune systems (Albiger *et al.*, 2007).

Innate immunity is made up of non-specific defence mechanisms that come into play immediately upon appearance of a pathogen in the body, where as the adaptive immune system is elicitated later (4-7 days post infection) and is a pathogen-specific response. Innate immunity relies upon mechanisms that exist before infection that are capable of a rapid response to microbes and act essentially in the same way to repeated infections (ie non clonal). The adaptive immune system involves greater variability and rearrangement of genes (clonal) and due to its ability to generate receptors; it can provide specific recognition of foreign antigens, immunological memory of infection and pathogen –specific adaptor proteins (Janeway *et al.*, 2002). The two systems work in tandem to protect the body from harm; the adaptive immune system being dependent on the innate to receive guidance on what to respond to and what to ignore (Janeway *et al.*, 2001). This is done via antigen presenting cells, more specifically dendritic cells that effectively link innate recognition of invading pathogens to the generation of appropriate types of adaptive immune responses (Lee and Iwasaki 2007).

Fundamental elements of innate immunity include physical and chemical barriers such as epithelial surfaces, phagocytic cells (neutrophils, monocytes, macrophages) and natural killer cells; blood proteins that include members of the complement system and other mediators of inflammation; and cytokines that regulate and coordinate many of activities of the cells of innate immunity (Abbas and Lichtman, 2005). The innate immune system works by recognising key molecular signatures borne by pathogens called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycans (PGNs) or viral RNA. It does this through a set of receptors known as pathogen recognition receptors (PRRs), that are expressed on cells such as macrophages, dendritic cells, mast cells, neutrophils, eosinophils and natural killer cells, that recognise the PAMPS (Martinon and Tschopp 2005). These cells become activated during infection and rapidly differentiate into effector cells whose main role is to get rid of infection. However in some cases the innate system is unable to deal with the infection, and so activation of the adaptive immune response becomes necessary. Specific PRRS known as the Toll-like receptors (TLRS) and the NOD-like receptors (NLRs) expressed as part of a cytoplasmic multi-protein complex known as the inflammasome aid this transition (section 1.5.2.2). Once a pathogen/ foreign antigen has activated dendritic cells they under go maturation and migrate to lymph nodes where they cause differentiation of antigen specific T cells.

DCs and T cells represent the two most important components of innate and adaptive immunity. Here I will describe (1) the activation of dendritic cells and how they process antigen and (2) the development of T cells in the thymus.

1.2 Dendritic cells

Dendritic cells (DCs) are potent antigen presenting cells that play a critical role in the induction of immunity and tolerance (Gad *et al.,* 2003). They are resident in most peripheral tissues, particularly those at the site of interface with the environment ie: skin and mucosa, representing 1-2% of total cell numbers (Guermonprez *et al.,* 2002). They act as sentinels of the immune system regulating the switch between innate and adaptive immunity.

In absence of inflammation and infectious stimuli, DCs are referred to as immature (Fujii *et al.,* 2004). In this form they express MHC molecules in their cytoplasm and express low levels of cell surface co-stimulatory molecules (Janeway 2001). In this immature form they exhibit endocytic activity via macropinocytosis, receptor mediated uptake by Fc receptors and macrophage mannose receptors (MMR) and phagocytose microbes and dying cells (Steinman, 2001). They are present in a tolergenic fashion, able to endocytose (Liu *et al.,* 2002; Iyoda *et al.*, 2002) and process antigens (Wilson *et al.*, 2003) but unable to initiate differentiation of effector T cells. Instead in this 'steady state' DCs elicit different types of peripheral tolerance such as deletion (Bonifaz *et al.*, 2002), anergy (Hawiger *et al.*, 2004) and expansion of T regulatory cells (Yamakaki *et al.*, 2007; see section). Induction of adaptive immunity is only enabled when DCs undergo maturation in response to danger or infectious non-self signals (Matzinger 2002; Medzhitov and Janeway 2002) and migrate to the draining lymph nodes to activate naive T-cells.

Migration of DCs to activate T-cells is dependent upon chemokine production and regulation of their receptors (Sallusto *et al.,* 1999). DCs are a heterogeneous population that possess unique homing abilities through the expression and regulation

of functional chemotactic receptors. Two known distinct subsets of DC are (1) myeloid –derived CD11c+ DC (M-DC) that produce high levels of IL-12 and migrate in response to a wide array of inflammatory chemotactic agonists produced at the peripheral sites of infection and immune reaction (Sozzani 2005) and (2) plasmacytoid CD123+ (P-DC) that produce high levels of the cytokine IFN and depend on IL-3 for their survival and differentiation into mature DC (Dzionek *et al.*, 2000). Although the pattern of chemokine receptors expressed by the two subsets is similar (high levels of CCR2 and CXCR4; low levels of CCR3, CCR4, CXCR1 and CXCR2), the capacity to migrate to chemotactic stimuli is very different. P-DCs display higher levels of CCR5, CCR7 and CXCR3 compared to M-DCs (Sozzani, 2005). This could explain the diversity in migration patterns of the two subsets with M-DCS responding to several inflammatory cytokines and P-DCS only responding to homeostatic chemokine CXCL12. This distinct migration is accompanied by a different profile of cytokine production – M-DCs producing homeostatic chemokines, where as P-DCS preferentially produce proinflammatory cytokines highlighting not only the different migratory properties of the subsets but also their ability to recruit different cell types at inflammation sites (Penna *et al.*, 2002).

1.2.1 Langerhans cells

As previously described, DCs are known to reside in sites of interface with the environment ie: skin and mucosa. An example of this is a set of specialised DC within the epidermis, termed as Langerhans cells (LCs). They are immature DC derived from bone marrow and constitute ~3% of all epidermal cells (Smith and Hotchkiss, 2001) Their role is to recognise, internalise and process antigen encountered in the skin, and

transport it to draining lymph nodes for presentation to responsive T cells (Cumberbatch *et al.*, 2000).

Migration of LCs from the epidermis to the skin draining lymph nodes is induced when the cell undergoes maturation, to form potent antigen presenting cells, resulting in the secretion of inflammatory cytokines and chemokines that regulate this migration. For example production of IL-1β and TNFα is upregulated during maturation and act in concert to supply LCs with two independent promotional signals (Cumberbatch *et al.*, 2000). IL-1β acts in an autocrine fashion on LC to deliver a signal for migration and a second signal is delivered in a paracrine fashion as IL-1β upregulates production of TNFα by keratinocytes, which in turn aids maturation of LCs (Kimber *et* al., 1998; Cumberbatch *et al.*, 2000; Kimber *et al.*, 2000). Major changes during this maturation of LCs include the altered expression of adhesion molecules (decrease in E-cadherin, increase in I-CAM) neccessary for release of LC from epidermis, expression of matrix metalloproteinases needed for passage across basal membrane and an increased expression of MHC class II determinants and co-stimulatory molecules (Cumberbatch *et al.*, 2003; Stoitzner *et al.*, 2002; Jakob *et al.*, 2001; Kimber *et al.*, 1999). Also chemokine receptors such as CCR1/2/4/5/6, responsible for keeping immature DC in peripheral tissues are down regulated during maturation, whereas CCR7 is upregulated, aiding migration to the lymph nodes (Sozzani, 2005). These changes result in the presentation of antigen to T-cells in the presence of co-stimulatory signals ie: - CD40, CD80 & CD86 (see section 1.5.3), initiating an antigen-specific immune response.

1.2.2 Antigen processing and presentation

Once dendritic cell maturation and migration has been initiated antigen must be presented to a T-cell in the context of a peptide bound to a major histocompatability complex (MHC) on the cell surface of the APC. This is a critical event in generating adaptive immune responses as this MHC-peptide complex interacts with the T-cell receptor (TCR) in a nanometer scale gap referred to as the immunological synapse (Bromley *et al.*, 2001; Abbas and Lichtman, 2005) and initiates appropriate immune action. Antigen processing of exogenous/endogenous proteins to produce the peptides that are consequently loaded onto the MHC molecules and presented to T-cells is the defining function of DCs as major antigen presenting cells and regulatory cells in the adaptive immune response (Robinson *et al.*, 2002).

Antigen processing involves breaking down complex proteins to simple peptides of 8-9 amino acids long for recognition by CD8+ cells and 12-20 amino acids long for recognition by CD4+ cells (Meuer *et al.*, 1982; Gonzalez *et al.*, 2007), which enables binding of this peptide to the MHC. Specific amino acids on the peptide interact with amino acid residues contained within the peptide-binding groove of the MHC. Initially interactions formed with the amino and carboxyl-terminal functional groups anchor the peptide to the ends of the groove via hydrophobic interactions. This binding then orientates the peptide correctly to enable additional hydrogen binding, van der Waals and hydrophobic interactions between the MHC and peptide (Park *et al.*, 1998). Polymorphisms within the MHC molecule contribute to the variability in the immune response as each MHC molecule can preferentially present different peptides to T-cells, however the T-cell receptor interacting with the MHC only

specifically recognise 1-2 amino acids on the peptide and a single amino acid change can prevent T cell receptor activation (Park *et al.*, 1998).

Peptides can be presented to T-cells either by MHC class I or MHC class II molecules and this distinction determines the type of T-cell response initiated. The MHC encodes two major classes of human leukocyte antigen (HLA) molecules that are structurally and functionally related genes encoded in the MHC (Krensky, 1997). MHC designated class I HLA – A/B/C are expressed on all nucleated cells, whereas MHC designated class II HLA- DR/DP/DQ expression is highly restricted and regulated (Kalish, 1995; Park *et al.*, 1998). In absence of stimuli/inflammation only professional antigen presenting cells (DCs, LCs, macrophages and B cells) can express MHC class II molecules (Kalish, 1995). Expression can be induced by IFNγ on keratinocytes and other endothelial cells, however the ability of these cells to present antigen is uncertain as they lack certain co-stimulatory molecules (Kalish 1995).

As previously discussed the purpose of the HLA class I and class II molecules is to present peptides, fragments of foreign peptides and self-peptide antigens to T-cells involved in immunity, suppression or effector mechanisms (Krensky, 1997). Recognition of a foreign antigen involves simultaneous recognition of a self-molecule as the T-cell receptor recognises the antigen bound to peptide. During T-cell development the immune system attempts to eliminate from its repertoire those T-cells that make active responses to self peptide-MHC complexes via a process known as negative selection (Janeway, 2001). However this is not always 100% successful and manifests in autoimmune diseases and allergy, where T-cells can respond to self

peptide-MHC complexes or to self-peptides modified by allergen, resulting in attack of tissues by the host (Klein and Kyweski, 2000).

The T-cell response to an antigen is governed by the source of the antigen and the way in which it is processed (Kalish, 1995) and the two MHC classes provide this discrimination. Processing via an endogenous (MHC I) or exogenous pathway (MHC II) is dependent on where the antigen originates from ie: intracellular/extracellular and therefore what response it will initiate (See section 1.2.3/1.2.4).

1.2.3 MHC class I molecules and endogenous antigen presentation

MHC class I molecules (e.g. HLA-A/B/C), expressed on all nucleated cells (Park *et al.*, 1998) present primarily endogenous antigens (viral, transplant and tumour-associated antigens) or intracellular antigens that are present in the cytosol leading to the activation of CD8+ T cells (Meuer *et al.*, 1982; Harding, 1996; Chung *et al.*, 2007). Endogenous antigens are generated from cytoplasmic proteins (Kalish, 1995; Krensky, 1997) that are degraded into peptides by the proteosome complex (Yang *et al.*, 1992). Once degraded, the peptides are transported from the cytoplasm to the lumen of the endoplasmic reticulum (ER) by TAP-related proteins to associate with newly synthesised MHC class I molecules (Cox *et al.*, 1990; Kalish, 1995). Assembly of the HLA class I complex occurs in the ER; class I heavy chains and β2 microglobulin are co-transitionally inserted into the ER membrane where the peptides transported by TAP are loaded onto the MHC. These peptide-MHC class I complexes are transported to the cell surface where they present antigen to CD8+ T cells (Cox *et al.*, 1990).

1.2.4 MHC class II molecules and exogenous antigen presentation

MHC class II molecules (HLA-DR/DP/DQ) carry out presentation of antigens that arise outside of the cell (extracellular bacteria, bacterial toxins, vaccines and allergens such as dust mites and pollen) via the exogenous pathway (Kalish, 1995) for recognition by CD4+ T-cells. These external antigens are internalised by the antigen-presenting cell by phagocytosis, and are taken to endosomal vacuoles that fuse with lysosomes. Acidic conditions and multiple degradative enzymes provided by this endosome/lysosome compartment enable breakdown of the proteins into peptides ready for interaction with MHC class II molecules. Class II molecules are assembled without peptide in an intermediate compartment within the ER and the cell surface. The class II α and β chains are co-transitionally inserted into the ER membrane where they associate with a chaperone invariant chain protein Ii. This functions to inhibit binding of peptides to the molecule until arrival in the endosome/lysosome compartment (Bodmer *et al.*, 1994; Krensky, 1997) Three α/β heterodimers and 3 Ii form a nonameric complex, which exits the ER and is transported to the endosome/lysosome compartment for peptide loading. Here aspartic and cysteine proteases progressively degrade Ii, leaving CLIP (class II associated invariant chain peptide) fragment of Ii in the middle of the peptide-binding cleft. CLIP is dislodged and exchanged for an antigenic peptide (Bryant and Ploegh, 2004). Finally the resulting peptide-MHC class II complex is transported to the cell surface for interaction with CD4+ T-cells.

It should be noted that segregation of the exogenous and endogenous pathways are not absolute; cross presentation ie: - exogenous antigen presentation by MHC class I and endogenous antigen presentation by MHC class II has been demonstrated

(Nuchtern *et al.*, 1990). However this is rare and the division of the immune response by the different antigen processing pathways has functional significance. CD4+ T-cells, initiated by the exogenous pathway, provide help for antibody production that is necessary to combat bacteria and bacterial toxins. Likewise CD8+ T-cells mediate cytotoxicity that is believed to have major role in resistance to viral infections, and are initiated via the endogenous pathway.

Although the way in which an antigen is processed is vital in the immune response, many factors such as cytokines and antigen presenting cells themselves play a role in generation of specific T-cell populations.

1.3 T cell development and activation

Lymphocytes are small cells found in the blood that circulate through tissues via the lymph that affectively 'police' the body for foreign material. They originate from pluripotent haematopoietic stem cells in the thymus and differentiate into different mature haematopoietic cells, with decreasing pluripotency and increasing commitment to a single differentiation pathway (Rolink *et al.*, 2006), depending upon expression of certain types of cell surface antigens. Although initial stages of lymphocyte development are not dependent on existence of an antigen, once the cells express a mature antigen receptor subsequent survival and differentiation is dependent on presence of an antigen.

Haematopoietic precursors define the lineage potential of a given lymphocyte (ie: T-cell, B-cell, natural killer cell etc). The common lymphoid precursor (CLP) gives rise to T and B-lymphocytes that differentiate into the two different subsets capable of specifically recognising and distinguishing between different antigenic determinants. These subsets of cells differ in terms of function and comprise the two major areas of adaptive immunity: - humoral by B-cells and cell-mediated by T-cells.

B-lymphocytes are derived from bone marrow and are a source of various antibodies (IgG, IgA, IgM, IgD and IgE). They recognise antigen in its whole natural state via generation of these antibody molecules that attach to the antigen, enabling macrophages and natural killer cells to target and attack easier.

T lymphocytes are mediators of cellular immunity and recognise antigen in the form of short peptide fragments resulting from intracellular processing of antigen by MHC molecules, which are subsequently presented to the T-cell receptor. Derived in the thymus from haematopoietic stem cells that have migrated there from bone marrow, they consist of 3 subsets (1) helper T-cells, (2) cytotoxic T-cells and (3) regulatory T-cells. Differentiation into the subsets occurs through positive selection ie: those capable of interacting with MHC and negative selection ie: selection against cells reacting with self-peptides presented by MHC and thymocytes that react too strongly with antigen. Early stages of T-cell development is characterised by differential expression of the markers CD25 and CD44 and lack of CD4 & 8 expression (referred to as double negative, DN, thymocytes) (Rolink *et al.*, 2006; Bhandoola & Sambandam 2006). These DN thymocytes can be divided into 4 populations, each with the ability of differentiating into different cells: - DN1 ($CD25^{-}CD44^{+}$), DN2 ($CD25^{+}CD44^{+}$), DN3 ($CD25^{+}CD44^{-}$) & DN4 ($CD25^{-}CD44^{-}$). Final T-cell lineage commitment occurs at DN3 stage when extensive rearrangement of TCRβ gives rise to an αβ linage of $CD4^{+}CD8^{+}$ double positive cells.

The first step of T-cell selection occurs in the thymic cortex, where these double positive ($CD4^{+}CD8^{+}$) cells showing no affinity for MHC-peptide complexes die of neglect. The neglected cells represent the great majority (~95%) of recruited cells. By contrast, the positively selected cells, which express TCR showing sufficient affinity

for an MHC-peptide, migrate towards the medullary areas, where they differentiate into single positive $CD4^+$ (so called helper T-cells) or $CD8^+$ (cytotoxic T-cells) thymocytes. As positive selection allows the survival of a pool of cells containing all possible T-cell clones, including not only those potentially able to recognize exogenous peptides that will be encountered in the course of life but also all those reactive with self-peptides, this process favours the survival of anti-self T-cells. However, a subsequent process, which is named as negative selection, promotes the elimination of the great majority of thymocytes that are able to interact with MHC-self peptide complexes (Romagnani, 2006).

1.3.1 Differentiation of CD4+ and CD8+ T-cells

CD4+ T-cells are activated by recognition of an antigen associated with MHC class II molecules (de Jong et al., 2006), whereas recognition of an antigen associated with MHC class I molecules activates CD8+ T cells. Upon activation CD4+/CD8+ T-cells differentiate into functional subsets called T helper type 1 (Th1)/ T cytotoxic type 1 (Tc1) and T helper type 2 (Th2)/ T cytotoxic type 2 (Tc2), depending a number of factors such as DC polarisation which will be discussed in more detail in section 1.5.4.

Th1/Tc1 cells secrete IFN-γ and are instrumental against intracellular pathogens, such as viruses; Th1 cells via promotion of cell-mediated immune responses and Tc1 via targeted killing. Th2/Tc2 cells secrete IL-4, IL-5, IL-10, IL-13 and TGF-β. Th2 cells are known to be important in sustaining humoral immune response, especially the defence against allergens, however the role of Tc2 cells in the immune response is

largely unknown, although their presence in chronic infections, cancer and autoimmune diseases is associated with severity and progression (Iezzi *et al.*, 2006).

Chronic immune reactions are dominated by these type 1 or type 2 secreting T-cell populations with relative proportions of these subsets induced during an immune response being major determinants of the protective functions and pathologic consequences of the response. Each subset amplifies itself and cross-regulates the reciprocal subset. For this reason, once an immune response develops along one pathway, it becomes increasingly polarised in that direction, and the most extreme polarisation is seen in chronic infections or in chronic exposure to environmental antigens, when immune stimulation is persistent. These T-cell populations show distinct patterns of migration to sites of infection as a result of differences in their binding to endothelial selectins and in their responsiveness to various chemokines (Kay, 2001). Th1 and Th2 have been shown to express different cell surface chemokine receptor and are therefore attracted by different chemokines (See Table 1.1; Sallusto *et* al., 1999; Sebastini *et al.*, 2002).

Table 1.1 T cell differentiation and associated cytokine and chemokine secretion

T cell	Type	Cytokines	Chemokines	Chemokine receptors
CD4+	Th1	IFNγ	ITAC, IP-10, MIG & RANTES	CXCR3, CCR5
	Th2	IL4, 5,10,13 & TGFβ	I-309, MDC, TARC & eotaxin	CCR3, 4, 5
CD8+	Tc1	IFNγ	ITAC, IP-10, MIG & RANTES	CXCR3, CCR5
	Tc2	IL4, 5,10,13 & TGFβ	I-309, MDC, TARC & eotaxin	CCR3, 4, 5

1.3.2 Regulatory cells

A third type of CD4+ T-cells can also be induced, these are known as Th3 or T – regulatory cells (Tr1) that secrete IL-10 or TGF-β. They are thought to be capable of suppressing type 1 and 2 responses and function in infection to limit pathogen-induced immunity. During the generation of effector cells in lymph nodes, several potentially hazardous self-reactive T-cells capable of causing particular autoimmune diseases are formed. Mechanisms including clonal deletion (through positive and negative selection) and anergy exist, that physically eliminates or functionally inactivates these potentially hazardous self-reactive lymphocytes. However there is accumulating evidence that regulatory T-cells (T regs) actively suppress the activation and expansion of self-reactive T-cells, thereby preventing autoimmune disease. Naturally occurring CD4+ T regs (the majority of which express CD25) are engaged in dominant control of self-reactive T-cells and contribute to the maintenance of immunologic self-tolerance. Their repertoire of antigen specificities is as broad as that of naive T-cells, and they are capable of recognizing both self and non-self antigens, thus enabling them to control various immune responses (Sakaguchi 2004). Foxp3 and CD25 are highly specific markers for natural T regs, the former being intracellular and the latter being expressed on the cell surface. The transcription factor Foxp3 is a key control molecule for the development and function of natural CD25+CD4+ Tregs as it is not expressed on CD25–CD4+ T-cells, Th1 cells, or Th2 cells. Disruption of the Foxp3 gene blocks the development of natural Tregs or produces dysfunctional Tregs, thus leading to hyper-activation of T-cells reactive with self-antigens causing autoimmune and allergic response (Sakaguchi et al., 2006). Recently CD127 expression has also been described as an excellent marker for T regs in human blood (Naisbitt *et al.*, 2007). It is a cell surface marker expressed at low

levels on an overwhelming majority of T reg cells and can distinguish 10% of CD4 population as regulatory (Liu *et al.*, 2006)

The majority, if not all, of such CD25+CD4+ regulatory T-cells are produced by the normal thymus as a functionally distinct and mature subpopulation of T-cells and can be polarised into a third Th subtype – adaptive T helper type 3 (Th3) and T regulatory type 1 (TR1) cells. Th3/Tr1 develop as a consequence of activation of mature T-cells under particular conditions of suboptimal antigen exposure and/or co-stimulation (Maggi et al., 2005). They have the ability to inhibit the proliferation of other cells mainly by the secretion of cytokines IL-10 and TGF-β.

1.4 Hypersensitivity reactions

Chemical allergy is a common and important occupational health issue. Such allergens have the potential to cause an adverse reaction resulting from previous sensitization to that chemical or to one that is structurally similar (Basketter et al., 1988; Nakagawa et al., 1996). They are defined as an immediate or delayed type inappropriate immune response to a chemical (or drug) that results in tissue damage. They are unpredictable reactions that chemical and cellular mechanisms associated with the development of this tissue damage and individual susceptibility factors are ill defined. Skin is the most frequent target for hypersensitivity reactions (Roychowdry & Svensson 2005) and although these skin reactions may be less common than dose-dependent reactions they tend to be proportionally more serious (Naisbitt *et al.*, 2000).

1.4.1 Classification of hypersensitivity reactions

These hypersensitivity reactions can be classified into 4 different groups according to the scheme of Gell and Coombs (1963).

Type I reaction – Anaphylaxis: Rapid and immediate reactions occur upon contact of antigen against which the host has pre-existing IgE antibody. Antigen cross links IgE bound to mast cells and basophils (via Fc receptors) inducing degranulation and release of preformed mediators (eg histidine) and synthesis of newly formed mediators (eg leukotrienes & cytokines) producing anaphylactic reactions.

Type II reaction – Cytotoxic: Caused by specific antibody binding to cellular or tissue antigens. Antibodies (IgM or IgG) direct the non-specific elements of the immune system resulting in the cell being destroyed by lysis or removed by phagocytosis.

Type III reaction – Complex-mediated: Mediated by immune complexes essentially of IgG antibodies with soluble antigens and occurs when these immune complexes are deposited in tissue. Activation of complement leads to release of chemoattractants from the site of deposition, leading to local tissue damage and inflammation. This can be exacerbated by 'frustrated phagocytosis' and release of reactive oxygen species.

Type IV reaction (Delayed type hypersensitivity) – cell mediated: Target cell presents an antigen in context of MHC molecule that is recognised by a specific T-cell. T-cells pre-primed to this antigen will release cytokines upon secondary contact resulting in inflammatory reactions and possible cell death. Reactions include drug hypersensitivity, contact sensitivity and graft rejection.

Type IV reactions lead to clinical manifestations in skin, including eczema and bullous, maculopapular, and pustular exanthema, however can be subdivided into a further four groups (Type IV a-d) based on the functionality and phenotype of responsive T-cells (Pichler 2003).

Type IV a and b reactions derive from a Th1 (IFN-γ) and Th2 (IL-4 and IL-5) polarised immune response activating monocytic and eosinophilic inflammation, respectively.

Type IV c reactions derive from a cytolytic T-cell response (perforin and granzyme B) resulting in CD4+- and CD8+T-cell-mediated killing of target cells (i.e. keratinocytes). Toxic epidermal necrolysis and Stevens-Johnson syndrome are two cases of a severe form of drug-induced bulolous exanthema that specifically manifest hypersensitivity though cytolytic CD8+ T-cell mechanisms. These diseases are characterised by the presence of a high percentage of perforin-positive CD8+ T-cells

in the dermis and epidermis, the expression of αβ T-cell receptors and kill through perforin and granzyme B (Pichler 2003), and are typical symptoms associated with anticonvulsant treatments such as carbamazepine (Wu et al., 2006).

Type IV d reaction is associated with the T-cell dependent production of IL-8 which in turn signals neutrophil recruitment and activation.

1.4.2 Terms of contact dermatitis

Before discussing in more detail the mechanisms involved in elicitation of these hypersensitivity reactions it would be useful to define some of the terms that will be used in this section ie: immunogen, hapten, antigen and contact sensitiser.

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

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

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

Contact sensitiser/allergen: A chemical that upon contact with skin can lead to the development of allergic reaction after repeated exposure in certain susceptible individuals

1.4.3 Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) represents a specific example of a delayed type hypersensitivity reaction. As the skin is the outermost barrier of the body's defence system it is first to encounter chemical and physical factors from the environment (Saint-Mezard, 2004). Out of the hundreds of exogenous compounds it comes into contact with, most do not present any toxicological risk (Smith-Pease, 2003). However in some instances chemical entities can act specifically as skin allergens resulting in a T cell mediated inflammatory reaction at the site of challenge in certain hypersensitive individuals causing skin erythema and oedema, which although are not life threatening are a significant cause of patient morbidity. Development of ACD can be divided into two stages defined as sensitisation and elicitation and is dependent on 3 factors: antigen presenting cells, hapten specific T-cells and the antigen itself (Krasteva *et al.*, 1999). Contact allergens are low weight molecular compounds (>1000Da) that are incapable of initiating an immune response by themselves, as they do not occupy a sufficient molecular volume. They are incomplete antigens that must bind to cellular and serum proteins to form stable associations known as hapten-protein complexes (Park *et* al., 1998) in order to initially sensitise an individual during primary exposure. This results in the immunological priming of the hosts cell mediated immune system by generating a circulating population of antigen-specific memory T-cells that can be activated upon subsequent exposure to the same chemical.

1.4.3.1 Sensitisation phase (afferent/induction phase)

This occurs upon first contact with the skin by an allergen. Although allergens do not have specific cellular receptors to interact with, (ie: - toll-like receptor mediated recognition of pathogens) they are able to diffuse into the skin due to their lipophilic

nature, and bind to a wide range of extracellular and cell-membrane associated proteins (Cavani *et al.*, 2007). The ability of this hapten-protein complex to induce sensitisation relies on its activation of innate immunity and through this the promotion of dendritic cell maturation and migration to the draining lymph nodes, where T-cell priming can occur. Human skin hosts two dendritic cell populations: (1) Langerhans cells (LCs - $CD207^{high}CD11b^{low}$ myeloid dendritic cells) are considered the major DC population in skin hypersensitivity & (2) dermal dendritic cells (dDCs – $Cd207^{low}CD11^{high}$). Interaction of these DCs with the antigen promotes migration to lymph nodes (Cavani *et al.*, 2007). During migration LCs and dDCs are induced to differentiate from immature antigen presenting cells to mature immunostimulatory cells that effectively present antigen to responsive T-cells (Kimber *et al.*, 2002). Conversion from this 'resting' to 'activated' state is facilitated by changes in chemokine receptor expression (Wang *et al.*, 1999) and inflammatory cytokines IL-1β, TNFα and IL-18 (Cavani *et al.*, 2007). DC then process and present the antigen as haptenated peptides on MHC class I and II molecules, which is recognised by specific naive CD8+ and CD4+ T-cells in the paracortex of lymph node. This results in the generation and proliferation of circulating memory T-cell populations with acquired memory for that specific antigen that will be activated upon subsequent rechallenge with the antigen (Figure 1.1). Allergen-induced ACD is considered to be primarily mediated by CD8+ T cytotoxic type 1 (Tc1) cells with IFNγ, the prototype Tc1 cytokine, implicated as the primary inflammatory cytokine for ACD in animals and man (Pickard *et* al., 2007; Martin *et al.*, 2004; He *et al.*, 2006; Vocanson, 2006). This sensitisation step lasts for 10-15 days and has no clinical consequences (Saint-Mezard, 2004).

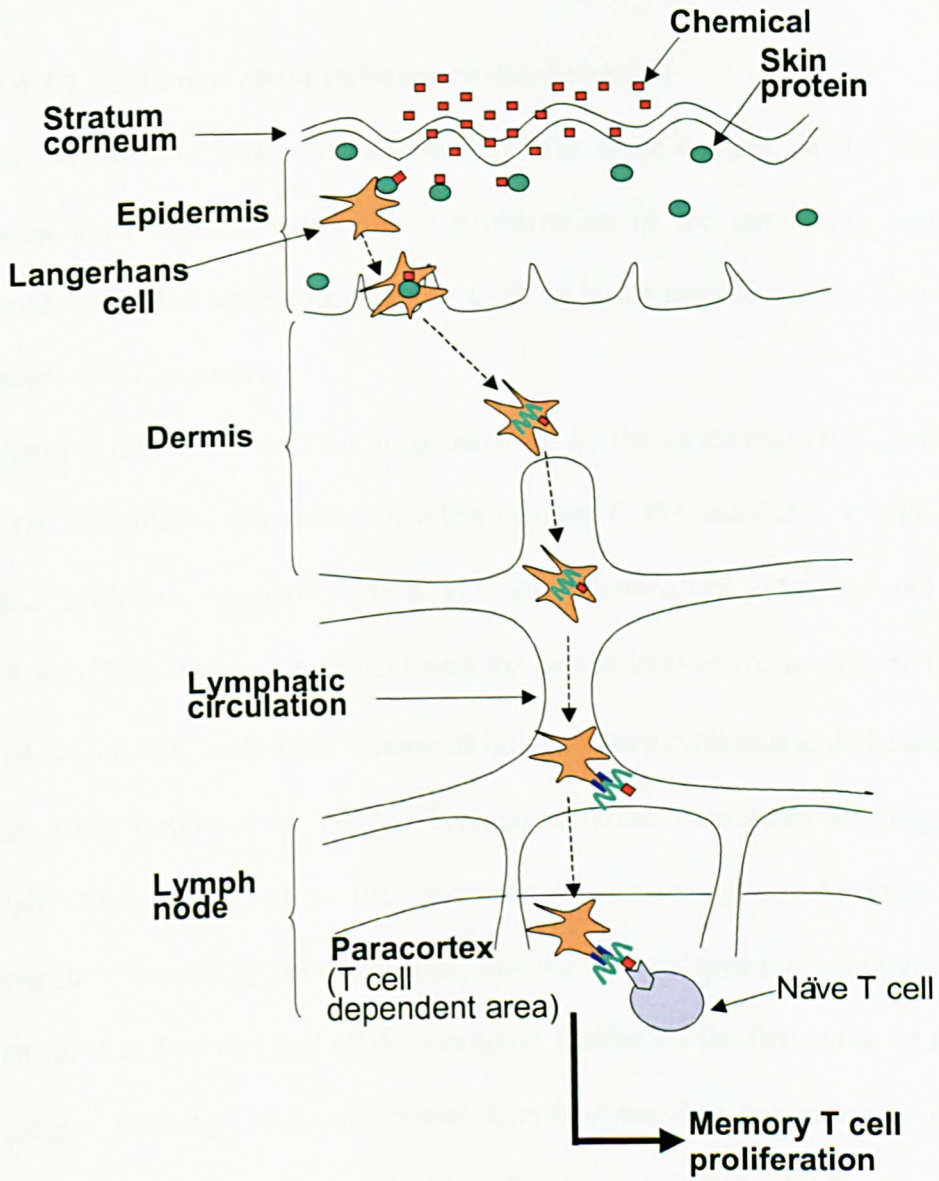


Figure 1.1. Induction phase of allergic contact dermatitis. During the induction phase the sensitising chemical (or hapten) penetrates the stratum corneum of the skin and binds to skin proteins/peptides to form a macromolecular immunogen which is recognised and internalised by Langerhans cells (LC). This recognition causes a maturation process in which the LC internalise and process the hapten-protein to a form that can be recognised by T-cells. These cells also migrate, from the epidermis via the dermis into the lymphatics to the lymph node(s) draining the exposure site. During this migration phase, the LC mature to become efficient antigen presenting DC, presenting the hapten in conjunction with MHC II, and thus recognised by naive CD4+ T-cells residing in the paracortex of the lymph node. This stimulates the proliferation and generation of a pool of allergen specific T-cells, some of which will form a population of memory T-cells that circulate and specifically recognise the same hapten upon subsequent exposure (figure taken from Smith Pease, 2003).

1.4.3.2 Elicitation phase (efferent/challenge phase)

Re-exposure of sensitised individuals to the same antigen, in the presence of co-stimulatory signals, will cause a proliferation of the circulating memory T-cells produced in the sensitisation phase resulting in the incidence of ACD within 24 - 72 hours of the exposure.

Antigen diffuses into the skin, is taken up by the epidermal DCs, forming antigen-MHC complexes that activate resident memory CD8+ and CD4+ T-cells in the dermis and epidermis, triggering the release of inflammatory cytokines and chemokines (Figure 1.2). Initial skin contact with the hapten induces the release of TNFα and IL-1β, which also leads to the release of inflammatory cytokines and chemokines (Enk *et al.*, 1992; Heufler *et al.*, 1992). This induces further recruitment of antigen specific T-cells from the blood to the epidermis by increasing blood vessel permeability therefore increasing fluid movement into the affected area (and through maturation of DCs). It is theorised that CD8+ cytotoxic T-cells are the first set to be recruited after antigen challenge and can activate keratinocytes that constitutively express MHC class I molecules (Cavani *et al.*, 2007; Traidi *et al.*, 2000). This T-cell activation leads to release of IFNγ, which increases expression of adhesion molecules (I-CAM) & MHC class II molecules and secretion of pro-inflammatory cytokines (IL-1β, IL-6 and GM-CSF) from the keratinocytes (Smith-Pease, 2003). This results in the recruitment of further T-cells (including the CD4+ subset), monocytes and T-lymphocytes not specific for the initial antigen, all of which are responsible for the clinical symptoms of erythema and oedema. After 72 hours the immune response is down regulated, possibly by a set of regulatory T-cells contained in the CD4+ subset known as CD4+CD25+ regulatory cells (Dubois *et al.*, 2003). They control expansion

and differentiation of CD8+ T-cells by secreting immunosuppressive cytokines such as IL-10 (Cavani *et al.*, 2001 & 2007) that inhibit IFN, IL-1, TNF, IL-6, IL-12 and GM-CSF. Prostaglandins produced by keratinocytes and macrophages can also inhibit production of IL-1 and IL-2.

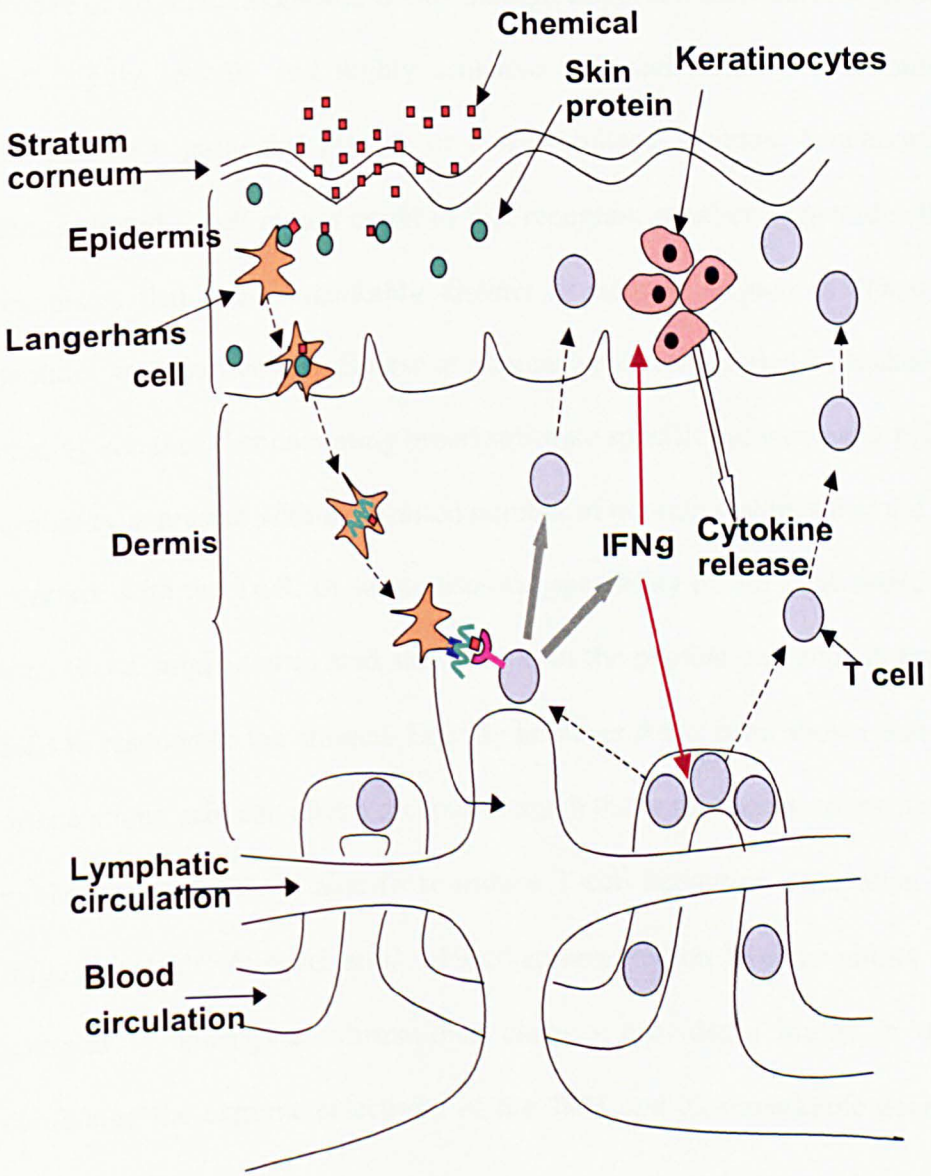


Figure 1.2. Elicitation phases of allergic contact dermatitis. The elicitation phase occurs locally in the skin upon subsequent exposure to sensitising chemical. The general principle is essentially similar to the sensitisation phase, except that the hapten is recognised by memory T-cells, of CD4+ and CD8+ subsets, which have been recruited to the site of inflammation. These T-cells release cytotoxic mediators which result in the local tissue inflammation characteristic of allergic contact dermatitis (Figure taken from Smith Pease, 2003).

1.5 Activation of the immune system by contact allergens

1.5.1 Signal one; Interaction of T-cells with MHC class I and II molecules;

The central interaction controlling the specificity of the adaptive immune response is that between a T-cell receptor (TCR) and antigen in the form of a peptide held in the groove of an MHC molecule. It was thought important that this recognition event was both highly specific and highly sensitive with individual T-cells only capable of recognising a particular peptide or closely related variants. Wucherpfennig (2004) demonstrated T-cell clones could in fact recognise number of peptides from different organisms that were remarkably distinct in primary sequences. They showed that peptides were particularly diverse at sequence positions buried in pockets of the MHC binding site thus demonstrating broad substrate specificity, whereas a higher degree of similarity is present within a limited number of peptide residues that are created at the interface with the TCR. In some cases the specificity of the TCR–MHC interaction is such that a single amino acid substitution in the peptide can abolish the ability of T-cells to respond to the antigen. Equally however it has been shown that a substitution of one amino acid can alter a receptor enough that a secondary amino acid substitution to the same peptide is able to re-induce T-cell activation equivalent to that of the original peptide. Ausubel *et al.*, (1996) commented on how the ability of TCR-MHC complex to undergo conformational changes provides a bridge in the paradox in combining the extreme selectivity of the TCR and its remarkable degree of receptor plasticity.

While the nature of protein antigen presentation to T-cells as been fully elucidated, the mechanism by which a chemical or drug antigen is presented is still subject to debate. As low weight molecular compounds are too small to act as an antigen by

themselves and are generally non-reactive, how they are recognised by T-cells is unclear. Three pathways have been described for chemical stimulation on T-cells.

The first is by means of the classical hapten mechanism, originally described by Landsteiner and Jacobs (1935) when they identified a strong correlation between the sensitisation potential in vivo and the protein reactivity in vitro. The hapten mechanism theorises that that a chemical or drug is recognised by specific T-cells only following haptenation to self-proteins (Park *et al.*, 2001). This leads to formation of a neoantigen that can be recognized by T-cells to override self-tolerance, and induction of a potentially pathogenic immune response. This mechanism has been well documented for powerful electrophilic contact sensitisers such as dinitrohalobenzenes (Cavani *et al.*, 1995; Park *et al.*, 1987; Weltzien *et al.*, 1996) that readily react with nucleophilic residues on proteins, particularly cysteines and lysines preferentially on cellular proteins (Hopkins *et al.*, 2005), and respiratory allergens such as trimellitic anhydride (Dearman *et al.*, 1991, 2000), which has been shown to bind to lysine residues on serum proteins (Hopkins *et al.*, 2005). Some low weight molecular compounds such as sulfamethoxazole (SMX) are classified as pro-haptens that must first become bioactivated by metabolising enzymes to reactive metabolites (nitroso-sulfamethoxazole in the case of SMX), which then binds to serum proteins and cellular membranes to be recognised by T-cells (Naisbitt *et al.*, 1999:2000; Hopkins *et al.*, 2005; Sanderson *et al.*, 2006). For all these chemicals, formation of a protein conjugate that is taken up by antigen presenting cells, processed into peptide fragments, which translocate to the cell surface in the context of MHC for presentation and recognition by specific T-cells is vital in overriding tolerance to

induce a potentially pathogenic immune response as shown by Pathway 1 in Figure 1.3 (Naisbitt *et al.*, 1999; 2001; Hopkins *et al.*, 2005; Sanderson *et al.*, 2006).

It has also been shown that chemicals and drug metabolites can stimulate T-cells through direct covalent binding to MHC molecules expressed on APC surface (Pathway 2 Figure 1.3), therefore bypassing the need for antigen processing (Schnyder *et al.*, 2000; Burkhart *et al.*, 2001). However more recently it has been suggested that not all chemicals or drugs necessarily require covalent interactions with MHC molecules (or a peptide embedded within) in order to trigger an immune response. This new hypothesis has been called the pharmacological interaction (p-i) concept, and postulates that chemicals and drugs can activate T-cells directly in the absence of metabolism, covalent binding, and antigen processing, (Pichler, 2002; 2003) through a reversible interaction between the TCR, MHC, and the chemical/drug (Pathway 3, Figure 1.3). Several groups have demonstrated evidence of this interaction: -

(1) 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 detectable metabolism (Naisbitt *et al.*, 2003a, 2005a; Schnyder *et al.*, 1997; Zanni *et al.*, 1997);

(2) Continual presence of free drug/chemical is needed for activation and washing away the drug from antigen presenting cells (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 for and in these T-cells it appears to act in a peptide-

independent superantigen-like fashion (Gamerdinger *et al.*, 2003; (Depta *et al.*, 2004; Schnyder *et al.*, 1997; Naisbitt *et al.*, 2003b);

(3) proliferation of T-cell clones is detectable after APC fixation with glutaraldehyde/paraformaldehyde (Naisbitt *et al.*, 2003b, 2005a; Schnyder *et al.*, 1997; Kalish *et al.*, 1994), although they are incapable of antigen processing (Schnyder *et al.*, 1997; Shimonkevitz *et al.*, 1983);

(4) 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 Ca2+ mobilisation (Zanni *et al.*, 1998), are too rapid to allow metabolism and antigen processing.

This non-conventional presentation pathway contradicts the original thought that the immune stimulatory capacity of most chemicals and drugs could be predicted by their protein reactivity and has been extended to other non reactive chemicals and drugs such para-phenylenediamine (Sieben *et al.*, 2002), lamotrigine (Naisbitt *et al.*, 2003) and lidocaine (Zanni *et al.*, 1998).

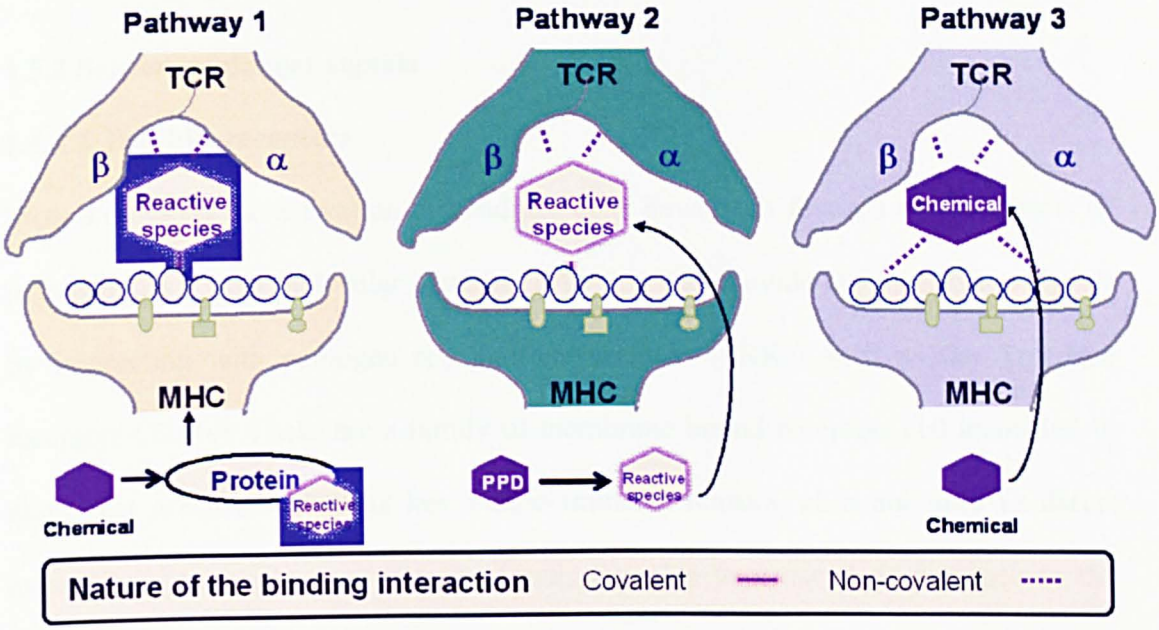


Figure 1.3. Possible pathways of drug/chemical presentation to T-cells

It has been suggested that interaction of the MHC-peptide complex with the TCR alone is insufficient to stimulate T-cell activation. Indeed Matzinger's (1994) danger hypothesis theorises that the non-self nature of the foreign antigen is not what induces the immune response; instead 'danger signals', such as cell damage or infection, that activate the immune system. Therefore it has been suggested that the T-cell receptor (TCR)- mediated recognition of the MHC-peptide complex is thought to be signal one and the effective activation of the adaptive immune response requires this and a secondary signal. Signal two is the engagement of co-stimulatory molecules present on professional APC's and their ligands on T-cells. Absence of signal two leads to tolerance, either by anergy (Gad *et al.*, 2003) or by apoptosis of responding T-cells (Appleman & Boussiotis, 2003). Upregulation of these co-stimulatory markers on APCs such as dendritic cells is triggered by danger signals working to enhance the signal two pathway.

1.5.2 Sensors of danger signals

1.5.2.1 Toll-like receptors

Most studies on the activation of dendritic cells have been focused on the effects of pathogen-associated molecular patterns (PAMPS) that provide DC maturation signals by interacting with pathogen recognition receptors (PRRs) such as the Toll like receptors (TLRs). These are a family of membrane bound receptors (10 identified in man) that are established as key innate immune sensors, able not only to detect extracellular pathogens and danger signals but also to sense their presence in the cytoplasm. TLRs are not only expressed on immunocompetent leukocytes such as monocytes, NK cells, mast cells, eosinophils and neutrophils, but are also expressed on endothelial cells and epithelial cells including keratinocytes. The most commonly studied PAMP is the bacterial cell wall component lipopolysaccharide (LPS), which is known to interact with TLR4 (Poltorak *et al.*, 1998). Upon recognition of ligands TLRs dimerise and initiate a signalling cascade that leads to the activation of pro-inflammatory response and induces cytokines (Rock *et al.*, 1998; Akira 2006). Whilst contact sensitisers do not have specific cellular receptors to interact with such as for LPS and TLR-4, it is believed they act through the ability to cause cell stress or death therefore providing maturation signals to dendritic cells. Initial studies revealed that cells killed necrotically, but not viable or apoptotic 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-signaling molecules, or 'endogenous adjuvants', and their ability to provide maturation signals to dendritic cells has been discussed in detail (Todryk *et al.*, 2000). Recently ground-breaking studies by Shi et al (2003; 2006) have defined uric acid crystals, released by injured or dying cells, as

potent messengers that can stimulate dendritic cell maturation and enhance CD8+ T-cell response *in vivo*.

1.5.2.2 The inflammasome

Newly described is the possibility that contact sensitisers can activate a cytosolic multiprotein complex known as the inflammasome (Figure 1.4) that comprises of a NACHT-, LRR-, and pyrin domain (NALP) family member, the protein apoptosis associated speck-like protein containing a CARD (ASC) and caspase-1 (Srinivasula *et al.*, 2002; Agostini *et al.*, 2004; Martinon and Tschopp, 2005). NALPs form a subfamily of the NACHTLRR family that includes nucleotide oligomerisation domain (NOD) proteins and are reminiscent of Toll-like receptors (Martinon and Tschopp, 2005). Upon exposure to danger signals, they induce ASC-mediated caspase-1 recruitment and activation, thereby triggering an inflammatory response (Kanneganti *et al.*, 2006; Mariathasan *et al.*, 2006; Martinon *et al.*, 2006).

Efficient LC migration and antigen presentation as well as the later recruitment of primed lymphocytes to the skin is dependent on the concomitant presence of pro-inflammatory cytokines in particular IL-1β and IL-18 induced by the contact sensitizers (CS) themselves (Grabbe *et al.*, 1996; Griffiths *et al.*, 2005). Both these cytokines are produced as inactive cytoplasmic precursors, which are processed into their active forms by caspase-1 (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Ghayur *et al.*, 1997; Dinarello, 1998).

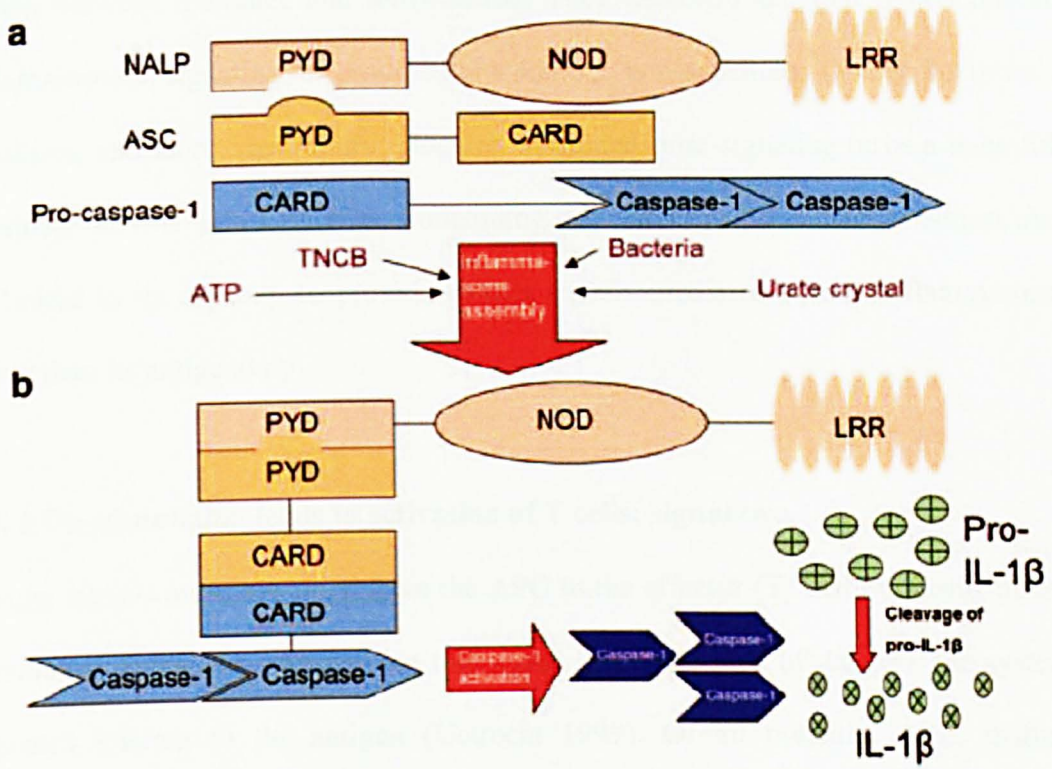


Figure 1.4. Structure and activation of the inflammasome. (**a**) NALP3 is the key central component with a pyrin domain (PYD) as its amino-terminal sequence, a nucleoside oligomerization domain (NOD), and a domain of leucine-rich repeats (LRR). Further components of the NALP3 inflammasome are the adaptor protein ASC (apoptosis-associated specklike protein containing a caspase-recruitment domain (CARD)) and pro-caspase-1. Upon activation (**b**) by "danger signals," such as urate crystals or bacteria, the inflammasome assembles and releases active caspase-1. Activated caspase-1 cleaves pro-interleukin-1β to active IL-1β, a potent mediator of inflammation. ATP, adenosine triphosphate; TNCB, trinitrochlorobenzene.

Watanabe *et al.*, (2007) demonstrated that an active inflammasome is present in the skin, specifically in keratinocytes and that some contact sensitisers, such as trinitrochlorobenzene (TNCB) can act as a direct trigger for the activation of caspase-1, IL-1β, and IL-18. Findings from ASC-/- and NALP3-/- mice showed they had an impaired early phase reaction during the challenge. These findings suggest that key components of the inflammasome may modulate the early effector phase of T-cell-mediated immune responses providing the pro-inflammatory signals necessary for T-cell priming. Furthermore they have shown that the inflammasome can act a master

switch between tolerance and sensitisation. They demonstrated that if concomitant inflammasome signaling is provided the known non-sensitiser DNTB becomes a sensitiser, and more importantly, blocking inflammasome-signaling turns a bona fide sensitiser, DNFB, into a tolerizer, concluding that potency of a contact sensitiser may be linked to its capacity to provide danger signals mediated by the inflammasome rather than its antigenicity.

1.5. 3 Co-stimulation leads to activation of T cells; signal two

Danger signals are transmitted from the APC to the effector (T) cell by means of co-stimulatory signalling, and without this signal 2 (i.e. absence of danger), the system becomes tolerant to the antigen (Uetrecht 1999). Of all potential APC, mature dendritic cells express the highest levels of co-stimulatory molecules and are therefore the most potent stimulators of naive T-cells (Reis & Sousa, 2006). A range of ligand-receptor interactions exist between the mature APC's and T-cells such as CD28/CTLA-4: B7-1 (CD80)/B7-2 (CD86) and CD40: CD40L interactions. The function of each of theses receptor-ligand interactions is discussed in detail below.

1.5.3.1 CD28:B7 interactions

Widely recognised as the prominent co-stimulatory signal for T-cells (Song *et al.*, 2008) is CD28, the constitutively expressed T-cell surface molecule. Its two cognate ligands B7-1 (CD80) and B7-2 (CD86) are present on activated APC. Engagement of CD28 with its ligands delivers signals that enhance T-cell proliferation by increasing expression and secretion of cytokines such as IL-2, TNFα, IFNγ and GM-CSF (Subudhi *et al.*, 2005) and ensuing differentiation into effector cells, promoting cell

cycle progression (Song *et* al., 2008) and enhancing memory T-cell development (Muller *et al.*, 2008). CD28 is also a potent activator of P13K, which binds to threonine protein kinases (PKB), ultimately activating NK-κB that is a key transducer of inflammatory signals (O'Sullivan & Thomas, 2002). Maturation of DC's also aids these processes as both B7-1 and B7-2 molecules are upregulated during maturation phase.

1.5.3.2 ICOS

CD28 is a member if the immunoglobulin superfamily (IgSF) also known as the B7 family of which the inducible co-stimulator (ICOS) receptor is also a member. Ligation of B7-H2 costimulatory molecule with ICOS enhances T-cell mediated proliferation. It is rapidly induced on T-cells following TCR engagement and CD28 signalling. ICOS and CD28 have unique and overlapping functions that synergise to activate and regulate CD4+ T-cell differentiation (Watanabe *et al.*, 2006) and augment cytokine production (IL-4, IL-5, IL-10, IFNγ and TNFα) which in turn provide critical signals for immunoglobulin production (Greenwald *et al.*, 2005; van Berkel *et al.*, 2005). Due to its inability to co-stimulate secretion of IL-2, ICOS is believed to be important during the effector stage of T-cell activation, particularly toward the Th2 phenotype (Watanabe *et al.*, 2006), whereas CD28 is important during the initiation of response (van Berkel *et al.*, 2005)

1.5.3.3 CD40:CD40 ligand

Another important co-stimulatory interaction is between the CD40 cell surface receptor (member of the tumour necrosis factor superfamily) expressed on professional APCs and its co-stimulatory molecule CD40 ligand (CD40L; member of

type II transmembrane glycoproteins with homology to TNFα) transiently expressed on activated T-cells. CD40 is upregulated during migration of DC from periphery to draining lymph nodes in response to a pathogen and ligation of CD40 by CD40L on the T-cell results in maturation of DC. CD40 signalling by the activated T-cells increases surface expression of MHC, co-stimulatory and adhesion molecules, therefore acting as a trigger for the expression of co-stimulatory molecules for efficient T-cell activation (O'Sullivan & Thomas, 2002). This interaction sustains the enhanced expression of B7-1 and B7-2 on APC, preventing anergy induction (Van Gool *et al.*, 1996). CD40 ligation can also induce high levels of inflammatory cytokines (IL-12, TNFα and IL-1β) that promote T-cell differentiation by polarising cells into Th1 or Th2 phenotypes, depending on the cytokine environment. Ie: - IL-12 polarises CD4+ T-cells towards a T helper (Th) 1 type thus enhancing proliferation of CD8+ and NK cells. Finally CD40-CD40L interaction has been shown to provide critical stimulation for sustained activation of NF-κB, which also plays a role in upregulating co-stimulatory molecules (CD40, CD80 & CD86) and immuno-stimulatory cytokines such as IL-12 & TNFα (Yoshimura *et al.*, 2001).

1.5.3.4 CTLA-4; co-inhibition

Many of the B7 superfamily were initially characterised as T-cell co-stimulatory molecules, however recently it has become clear that they can also co-inhibit T-cell responses. CTLA-4, a member of the CD28 family with receptors for B7-1 & B7-2 has been shown to provide an inhibitory signal on T-cell activation (Engelhardt *et al.*, 2006). It works as a negative regulator of T-cell activation, terminating T-cell responses and playing a key role in self-tolerance. Engelhardt *et al.*, (2006)

demonstrated that overexpression of CTLA-4 inhibits T-cell activation *in vivo* and *in vitro* and van Berkel *et al.,* (2005) noted CTLA-knockout mice showed spontaneous CD28-mediated CD4+ T-cell activation, expansion and differentiation. CTLA-4 expression is only upregulated upon T-cell activation induced by CD28:B7 interactions.

A wide variety of contact sensitisers have been found to partly mature LC/DC's by provoking signal 2, as measured by upregulation of cell surface markers such as CD80, CD86, CD40, HLA-DR (Iijma *et al.*, 2003; Yoshida *et al.*, 2003; Aiba *et al.*, 1997; Coutant *et al.*, 1999) or activation of signal transduction pathways (Arrighi *et al.*, 2001; Aiba *et al.*, 2003). However these maturation effects cannot be separated from danger signals as a result of cell death, as many contact sensitisers are often toxic to immune cells due to their intrinsic protein reactivity. Recently Hulette et al. (2005) confirmed that a range of chemical sensitizers, including the allergen para-phenylenediamine (PPD) (a compound which is the focus of my thesis) caused significant DC maturation, however at concentrations that induced slight to moderate cytotoxicity. It is likely additional signals from the skin ie:- cytokines are required for full maturation.

1.5.4 Signal three: Cytokine release determines Th polarisation

As discussed above migrating DCs provide naive T-cells in the draining lymph nodes with two signals (1) antigen recognition via interaction with TCR and (2) co-stimulation for activation of T-cells via co-stimulatory molecules present on APC and T cell. Upon presentation of antigen and upregulation of co-stimulatory receptors, antigen-specific T-cells can be activated. DCs are able to influence CD4+ or CD8+ T-

cell polarisation through the secretion of cytokines specific for the phenotype of cell ie: - IL-1 secretion drives CD4+ T-cell polarisation and IL-12 secretion for CD8+ polarisation (Kapsenberg *et al.*, 1999; Curtsinger *et al.*, 1999:2003; de Jong *et al.*, 2002). IL-1 can contribute directly to activation through either direct interaction with the T-cell or induction of CD40 expression on dendritic cells (Curtsinger *et al.*, 1999), enabling CD4+ T-cells to regulate/maintain their own expansion. CD8+ cells need IL-2 provided by CD4+ T-cells, in addition to IL-12 produced by dendritic cells to elicit strong expansion and development of effector and memory functions (Mescher *et al.*, 2006).

DC's ability to carry a third signal to provide the CD4+/CD8+ T-cells with a polarising signal of Type 1 or Type 2 enabling rapid selection of appropriate effector mechanisms to ensure an optimal T-cell response. This leads to subsets of specific T-cells that either secret type 1 (Th1/Tc1) effector associated cytokines (IFN-γ and TNF-β) or type 2 (Th2/Tc2) associated cytokines (IL-4, IL-5, IL-9, IL-13) (Kapsenberg *et al.*, 1999; Nierkens *et al.*, 2002). DCs can deliver this third signal and influence polarisation of naive T-cells as they are able to adopt a type 1 or 2-promoting effector function depending on the context of their activation ie: - pathogen, tissue (Kalinski *et al.*, 1999). An essential discriminatory factor appears to be the level of IL-12 produced by the DC (Snijders *et al.*, 1998). Type 1 polarising DCs have been characterised by the production of IL-12, IL-1, IL-18, TNF and CXCL-10 (Toebak *et al.*, 2006; Kalinski *et al.*, 1999), whereas type 2 DCs produces IL-10 (de Jong *et al.*, 2002) The ability of DCs to adopt a type 1 or type 2 effector function allows the efficient rapid induction of T-cells with a cytokine profile to initiate an appropriate immune response, without the need for separate type 1 or 2-driving APC lineages (Kalinski *et al.*, 1999). Clinical and experimental studies have

shown that contact sensitisers display remarkable intrinsic differences in their ability to induce type 1 or 2 effector cells (Kimber *et al.*, 2003). Contact allergens such as DNCB and oxazolone have been shown to induce a strict type 1 polarisation, respiratory allergen TMA induces a type 2 polarisation and interestingly nickel can induce type 1 and type 2 polarisation; characterised by TNF and CXCL10 production and decreased IL-12 (Toebak *et al.*, 2006). Figure 1.5 summarise the three signals that influence T-cell activation.

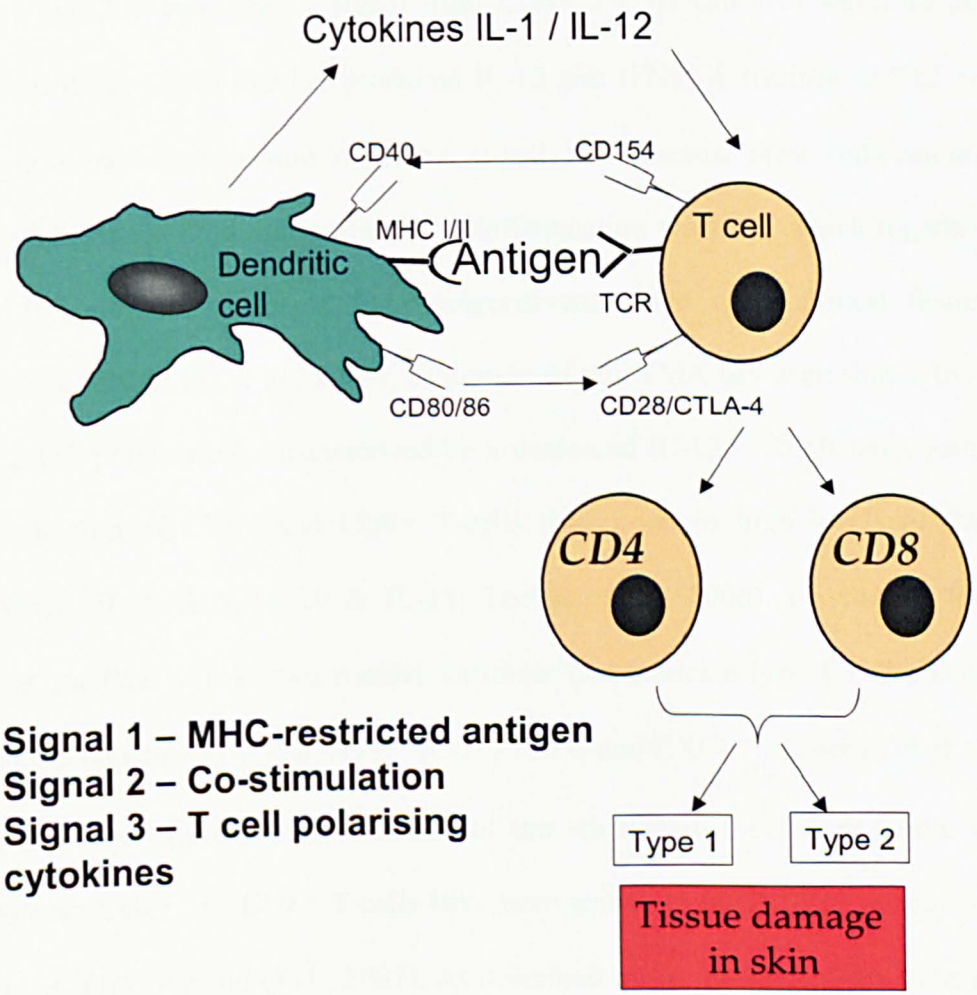


Figure 1.5. A three-cell model for the initiation of CD4+ and CD8+ T cell immune responses. It is thought that three signals are required to produce an immune response to an antigen (Curtsinger et al., 1999). The first represents the interaction between the MHC-restricted antigen and the T-cell receptor. The second signal, provided by the DC are the co-stimulatory molecules (that are upregulated in the presence of the antigen, activating pathways via linkage with T-cell receptors) and a series of pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ that act indirectly on APC to upregulate the expression of these co-stimulatory molecules. The third signal represents polarising cytokines that act directly on T-cells, and lead to either CD4+ (Th1 or Th2) or CD8+ immune responses

As well as the polarisation of activating DC, the cytokines IL-12 and IL-4 influence the processes leading to the differentiation of CD4+ T-cells into Th1 or Th2 respectively. They do this through specific activation of STAT 4 and 6 respectively, which is a major effector mechanism of the immune response regulating cytokine signalling (Nasta *et al.,* 2006). IFNγ and IL-12, which are known to augment cytotoxic effects (Mescher *et al.,* 2006) can cause polarisation of CD8+ T-cells into type 1 and 2 phenotypes. A signal from CD4+ T-cells can also works to polarise CD8+ cells as CD40 ligation produced IL-12 and IFNγ. Activation of Th2 cells is thought to have a major role in allergic sensitisation because these cells can mediate IgE synthesis via IL-4 and eosinophilic inflammation via IL-5, which together with IL- 13, contribute to airway hyper-responsiveness and other clinical features of allergic disease (Ling *et al.,* 2004). In support of this TMA has been shown to induce type 2 DC polarisation, characterised by a decreased IL-12 – IL-10 ratio, leading to the induction of CD4+ and CD8+ T-cells that produces high levels of Th2/Tc2 cytokines (IL-4, IL-5, IL-10 & IL-13; Toebak et al., 2006). In contrast however DNCB, another well known contact sensitiser that causes a type 1 DC polarisation causing production of IL-1β, IL-12, IL-18, TNFα and CXCL10 (Toebak et al., 2006), which in turn affects the polarisation of the stimulated T-cell population. DNCB responsive CD4+ and CD8+ T-cells have been shown to be Th1/Tc1 producing high levels of IFNγ (Pickard et al., 2007). As described earlier nickel appears to be able to induce both type 1 and type 2 DC polarisation (Toebak *et al.*, 2006). Clinical studies have shown that both type 1 and type 2 nickel-specific T-cells play a role in nickel allergy and tolerance: - Tc1 CD8+ cells (high IFNγ: low IL-10) were detected in the patients and Th2 CD4+ cells (low IFNγ: high IL-10) have been detected in cells from

volunteers (Cavani *et al.*, 1998; 2000). This suggests that Tc1 CD8+ T-cells are responsible for the initiation of allergic contact dermatitis, whereas Th2 CD4+ T-cells are acting to prevent this outcome, as characterised by the high levels of IL-10.

There is an ongoing debate as to whether $CD4^+$ or $CD8^+$ T-cells are the effector cells in delayed type hypersensitivity reactions. Originally contact hypersensitivity was thought of as a purely Th1 mediated $CD4^+$ T-cell response (Fong *et al.*, 1989). However reports have shown that CD8+ T-cells are the main effectors of CHS and can be induced by a number of haptens ie: - DNFB, urishol, nickel and TNP (Bour *et al.*, 1995; Xu *et al.*, 1996; Kalish *et al.*, 1995; Cavani *et al.*, 1998; Martin *et al.*, 2004). Saint-Mezard *et al.*, (2004) suggests that perhaps CD8+ cytotoxic T-cells are the first set to be recruited after antigen challenge due to constitutively expressed MHC class I molecules (Cavani *et al.*, 2007; Traidi *et al.*, 2000) on cells such as keratinocytes. Data from Akiba *et al.*, (2002) adds weight to this theory as they demonstrated CD8+ T cells could infiltrate challenged skin as early as 9 hours and was associated with IFN-γ production. The release of IFNγ, which increases expression of adhesion molecules (I-CAM) & MHC class II molecules, results in the later recruitment of CD4+ T-cells that possess down regulatory capabilities as seen by Cavani *et al.*, (1998:2003) with nickel data. Particularly for this metal the distinct differences in pattern of cytokine release (e.g. augmenting production of IL-10) by CD4+ T-cells from patients and volunteers suggests this could be an important element in determining whether an antigen induces allergy or tolerance (Cavani et al., 1998). I.e.: - allergic disease could result from an inappropriate balance between allergen activation of regulatory CD4+CD25+ T-cells and effector cells.

However it should also be noted that, in the absence of a functioning CD8+ T-cell population, CD4+ T-cells can act as the effector cells of CHS (Saint-Mezard *et al.*, 2004).

1.6 para-phenylenediamine

p-Phenylenediamine (PPD) is one of the most frequently encountered contact sensitisers and is the main focus of my PhD research. It is widely used as an intermediate in permanent hair dye formulations (Corbett *et al.*, 1973), in printing ink & photo development (Chung *et al.*, 1993) and more recently in henna tattoos (Le Coz *et al.*, 2000; Mautlich & Sullivan 2005)

Due to the expression of cell-mediated immunity on skin, contact with PPD can cause T-cell-mediated severe ACD (McFadden *et al.*, 2007; Coulter *et al.*, 2007; Picardo *et al.*, 1990), resulting in irritation, erythema, edema, keratoconjunctivitis, conjunctival swelling and eczema of the eyelids (Smith Pease, 2003; Basketter *et al.*, 1988; Mathur *et al.*, 1990). Reports of PPD-associated ACD have increased over recent years due to the broad-spectrum and increased use of PPD-containing products (McFadden *et al.*, 2007) ie: - in hairdyes and henna tattoos.

1.6.1 Sensitising potential

PPD-induced ACD is believed to be associated with PPD being intrinsically unstable and its ability to undergo auto-oxidation either in solution or on the skin. This leads to the formation of an electrophilic primary quinonediimine intermediate, which is susceptible to sequential self-conjugation reactions and further oxidation, resulting in the formation of Bandrowski's base – a rearrangement product of these reactions (Figure 1.6; Picardo *et al.*, 1990; Coulter *et al.*, 2007).

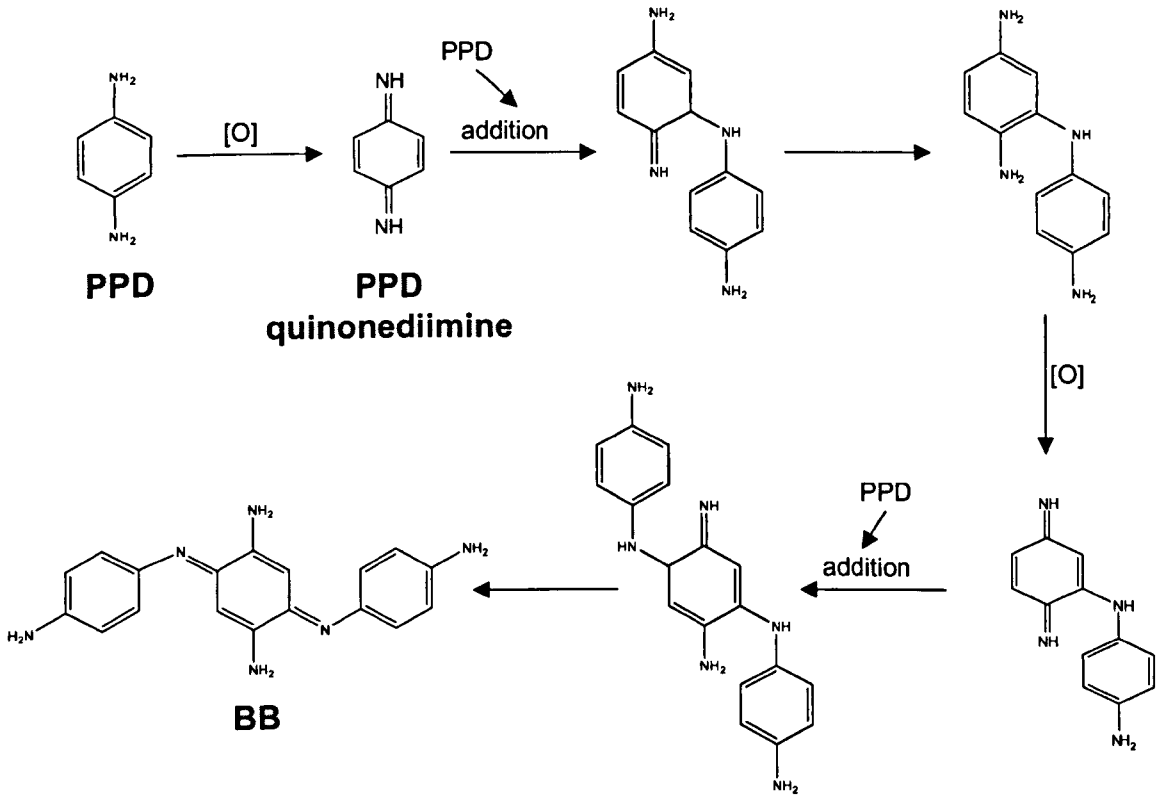


Figure 1.6 Proposed oxidation of PPD. PPD is rapidly oxidised to a quinonediimine intermediate, which is susceptible to nucleophilic addition through dimerisation with a second PPD molecule. This new intermediate can then undergo further oxidation and nucleophilic addition with a third PPD molecule. The unstable product rearranges to yield a trinuclear dye called Bandrowski's base (BB).

The sensitising potential of PPD its oxidation product Bandrowski's base (BB) has been well established using animal models with the two compounds being classed as strong and extreme sensitisers respectively, based on their ability to stimulate proliferative responses in lymph nodes of topically exposed mice (Warbrick *et al.*, 1999; White *et al.*, 2006). In these model systems, the immuno-stimulatory effects of PPD are thought to be dependent on cutaneous oxidation and the generation of BB (Farrell *et al.*, 2008). The same could be said for the effects of PPD in humans as in conditions of prolonged exposure to PPD ie: henna tattoos, LLNA or human patch testing, a substantial amount of PPD will become air-oxidized on the skin surface

forming possible sensitising auto-oxidation products ie: BB (Goux *et al.*, 2007). However patch-testing studies on humans suggest that the nature of the antigenic determinant is different in allergic patients. Patel *et al.*, (2007) described a high frequency of patch test reactivity amongst PPD-allergic individuals, and whilst the patch test is time-dependent (Basketter *et al.*, 2006) indicating that oxidation is important for immune reactivity, only 16% of PPD patch test positive patients are responsive to BB (White *et al.*, 2006). Thus, primary PPD oxidation products are thought to be major antigenic determinants in human subjects. These data suggest that exposure to PPD and BB might stimulate a T-cell response; however the chemical entity that interacts with immune cells has not been fully elucidated.

1.6.2 Detoxification pathway and Genetic polymorphisms

PPD is a small molecule therefore it needs to bind to protein to form hapten-protein complexes in order to be recognised by the immune system. As PPD is protein unreactive it has to undergo some form of activation (ie: oxidation to its dimers and trimers) to enable it to bind to protein, therefore a balance between activation and detoxification processes is critical for immunological potential of a compound. It is believed that PPD may lose its reactivity potential through N-acetylation, which acts as a detoxification step to prevent contact dermatitis (Kawakubo *et al.*, 1997). N-acetylation is carried out by 2 isoenzymes NAT-1 and NAT-2 (Meyer & Zanger, 1997) both of which are synthesised in human skin, keratinocytes and Langerhans cells (Kawakubo *et al.*, 2000; Westphal *et al.*, 2000). PPD is acetylated to monoacetyl-PPD (MAPPD), which in turn is acetylated to N,N'-diacetyl-PPD (DAPPD; Kawakubo *et al.*, 2000:1990; Garrigue *et al.*, 2006). Blomeke *et al.*, (2008) have recently confirmed that acetylation of PPD can be regarded as a detoxification

step as no patch tests are positive to the acetylation products MAPPD and DAPPD. This pathway also prevents BB formation thereby reducing the amount available for activation if this is indeed the immunogenic component.

However it has since been suggested that genetic polymorphisms related to this enzymatic detoxification may explain individual susceptibility. It is well known that genetic factors can play a major role in sensitisation of an individual. For example functional data showing a restriction of nickel-specific T-cell responses to certain MHC class II molecules support the concept of a role of allelic HLA variability in susceptibility to metal sensitivity (Emtestam *et al.*, 1996). Chessman *et al.*, (2008) recently showed a link between HLA-B*5701 expression and abacavir hypersensitivity when skin patch testing combined with clinical diagnosis showed the sensitivity of HLA-B*5701 in predicting hypersensitivity was ~100%. They also showed that abacavir was highly immunogenic and specific in activation of CD8+ T-cells not only in patients with a history of AHS but in healthy abacavir-naive HLA-B*5701-positive donors.

Likewise genetic polymorphisms may contribute to allergic contact dermatitis to PPD. Polymorphisms affecting enzyme functions have been elucidated for genes encoding NAT-1 and NAT-2 (Grant *et al.*, 1997). Westphal *et al.*, (2000) showed a slight increase in of the NAT1*10 allele which encodes for a rapid acetylator type in PPD sensitive patients compared to controls. A second rapid acetylator polymorphism was found to be associated with NAT2 genes – NAT2*4 wild type allele (Westphal *et al.*, 2000; Nacak *et al.*, 2004) and was also significantly increased in the patient group. Susceptibility to contact allergy appears to be associated, at least in part with a linked

NAT2*4/NAT1*10 haplotype, either via direct enhancement or relation to an unknown susceptibility factor.

Recently an association between a TNFA-308 G/A polymorphism and sensitisation to PPD has been described (Blomeke *et al.*, 2008). TNFα plays an important role in development of an allergic reaction by providing chemokine and cytokine signals that enable activation and migration of antigen-loaded dendritic cells. A single nucleotide polymorphism (SNP; guanine to adenine, TNFA-308 G/A), located at nucleotide-308 upstream of the TNFA transcription start site, is known for its strong influence on the promoter activity of the TNFA gene. Previous studies indicate that the adenine nucleotide at position -308 in the promoter region of the TNFA gene is associated with an increased production of TNF-α (Heeson *et al.*, 2003; Louis *et al.*, 1998; Bouma *et al.*, 1996) and has been associated with various inflammatory disorders including allergic and irritant contact dermatitis (Witte *et al.*, 2002; Allen *et al.*, 2000; Westphal *et al.*, 2003). Blomeke et al (2008) found a genotype containing the TNFA A allele was significantly more common in individuals with sensitization to PPD than in healthy control subjects, suggesting that individuals who have the genetic capacity to produce higher levels of TNF-α after encounter with a chemical, such as PPD, may have an increased susceptibility for sensitization and ACD.

1.6.3 Ability of PPD to provide signal one for T-cell activation

Although a variety of factors including skin penetration, cell stress, cytotoxicity and dendritic and T-cell activation determine the sensitizing potential of low molecular weight chemicals such as PPD, formation of a protein adduct is believed to be the primary event required to initiate an immune response (Eilstein *et al.*, 2006; 2007). The electrophilicity and therefore protein reactivity of a chemical is thought to relate

closely to its ability to promote an immune reaction (Eilstein *et al.*, 2008; Warbrick *et al.*, 1999). PPD itself is protein unreactive and requires activation to become immunogenic (Aeby *et al.*, 2008). Since the capacity for auto-oxidation plays a key role in inducing sensitising effects of PPD (Picardo *et al.*, 1990), it was always assumed that PPD was present as a pro-hapten, activated through oxidation to BB, the 'true immunogenic hapten' (Krasteva *et al.*, 1993; Basketter *et al.*, 1992). In this respect studies by Krasteva *et al.*, (1993) have shown that BB, but not PPD or primary oxidation products, stimulate T-cells from allergic patients. However, more recent work with cloned T-cells from PPD allergic individuals provides evidence to suggest that PPD and BB might actually stimulate proliferation of T-cells via two independent mechanisms (Sieben *et al.*, 2002). PPD associated non-covalently with MHC molecules was found to stimulate certain T-cells directly without a requirement for processing, while BB stimulated T-cells via a classical hapten mechanism involving both irreversible binding and processing. Little is known about the nature of PPD-specific protein modifications if in fact it can covalently modify protein and how this relates to the initiation of an immune response. The scarcity of data in this respect is largely due to the number of potential target proteins for reactive chemicals (2000 have been separated from skin; Basketter *et al.*, 2006), the abundance of amino acid binding sites on any given protein candidate and the accessibility of binding sites for reactive chemicals. In this respect Eilstein *et al.*, (2006; 2007) have used p-benzoquinonediimines to show that they can react with multiple nucleophilic amino acid residues through a set of complex mechanisms, indicating a potential route to protein haptenation. Furthermore, Eilstein *et al.*, (2008), have shown the selectivity of 2,5-dimethyl-p-benzoquinonediimine binding to lysine in a model peptide containing potentially reactive amino acids (except cysteine). 2,5-dimethyl-p-

benzoquinonediimine reacts with lysine via Schiff base formation and an oxido-reduction process giving rise to an aldehyde intermediate and peptide cyclization, demonstrating that sensitising potential may relates to its chemical reactivity towards a critical amino acid *in situ*. Work in this thesis will examine more closely the ability of PPD to provide signal one to activate T-cells by investigating its reactivity towards protein and how PPD protein complexes are presented to the T-cells.

1.6.4 Activation of DCs by PPD provides signal two

Ability of PPD or an oxidation product to overcome the skin barrier and penetrate the epidermis to activate skin dendritic cells (DC's) is required for the induction of skin sensitization (Aeby *et al.*, 2008). Previous work using DC's has found that exposure to PPD and BB can provide signal two to the cells, resulting in functional maturation, as measured by increased expression of cell surface receptors ie: CD86 (Rougier *et al.*, 2000; Ashikaga *et al.*, 2002; Aeby *et al.*, 2004, 2008) with BB needing much lower concentrations to induce a response. Hulette *et al.*, (2005) have shown that PPD concentrations (above 2.5 mM) that are associated with significant cell death stimulate dendritic cell maturation, as assessed by up-regulation of CD86 expression; presumably via a classical "danger" response (Matzinger, 1994). Moreover, Toebak *et al.*, (2006) have shown that PPD concentrations of 200 μM and above stimulate significant secretion of CXCL8, a chemokine involved in recruitment of cells expressing CXCR1 and CXCR2. Recently, Coulter *et al.*, (2007) have shown that exposure of dendritic cells to lower concentration of PPD (5-50μM) is associated with an increase in CD40 and MHC class II expression. They proposed the following concentration dependent effects of PPD on DC function ie: low concentrations (<50μM) induce CD40 and MHC class II expression; medium concentrations

(<200μM) induce CXCL8 secretion and high concentration (<2500μM) lead to CD86 expression and strong cytotoxicity. These results confirm the theory that receptor binding alone is insufficient to initiate an immune response and that some form of cytotoxicity/danger signal is also required.

1.6.5 T-cell differentiation and cytokine secretion

Our understanding of the cellular basis of contact dermatitis is ill-defined therefore functional studies, assessing the secretion of cytokines following hapten stimulation, are important as cytokines are known to directly relate to the induction of specific chemicals manifestations of the disease (Pichler, 2003). For example known contact sensitisers DNCB and nickel have been shown to secrete predominantly Th1 cytokines (Pickard *et al.*, 2007; Lecart *et al.*, 2001; Cavani *et al.*, 2000). In contrast a murine model of PPD-induced contact sensitisation found Th2 cytokines, IgE antibodies and mast cells are crucial in the development of tissue pathology (Yokozeki *et al.*, 2003). In human studies stimulation of patient lymphocytes with PPD and BB were associated with significantly higher levels of Th2 cytokine secretion and increased gene expression (Coulter *et al.*, in press) suggesting the ability to produce cytokines such as IL-4, IL-5 and IL-13 may contribute to the development of contact dermatitis.

PPD will be used in this thesis to explore the ability of a chemical to produce appropriate antigenic signals to activate T-cells, how this antigen can be presented and what cytokines are secreted.

1.7 Proteomic approaches for identification of allergens

Potential of a chemical to cause skin sensitisation and possibly allergic contact dermatitis is a critical toxicological end point evaluated for all new chemicals developed for consumer/occupational use (Gerberick *et al.*, 2007). Until recently the local lymph node assay was viewed as the most appropriate method to assess a chemicals potential to cause skin sensitisation. The LLNA is based upon characterisation of induced proliferative responses in draining lymph nodes following topical exposure of mice to chemicals and has been a vital tool in predicting skin sensitising potential of many chemicals ie: PPD (White *et al.*, 2006), DNCB (Loveless *et al.*, 1996), cinnamic aldehyde (Basketter *et al.*, 2001) and benzyl benzoate (Smith & Hotchkiss, 2001).

However, due to the forth-coming European Union ban on *in vivo* testing of cosmetics and toiletry ingredients there is an need to develop non-animal based methods to assess sensitising potential of new chemicals. Human cell based methods are already being employed such as DC activation, T-cell proliferation assays and development of DC-like cells lines is proving a useful tool in the assessment of sensitising potential.

In addition, mass spectrometry methods are now being developed to give a more in-depth understanding of chemical mechanisms of protein complex formation. It is well documented that covalent modification of proteins by chemicals is one of the determining events in skin sensitisation (Aleksic *et al.*, 2008) and the correlation between protein reactivity forming a potential allergen and skin sensitisation is well established (Landsteiner & Jacobs, 1935; Dupuis & Benezra, 1982). Most chemical allergens are thought to be electrophilic compounds that react with nucleophilic side

chains of proteins and peptides. The electrophilicity and therefore protein reactivity of a chemical is thought to relate closely to its ability to promote an immune reaction (Ashby *et al.*, 1995; Basketter *et al.*, 1988) It is hoped that understanding this mechanism of binding and identifying possible target amino acids for sensitisers will be a useful diagnostic tool in the future.

Due to the complexity of the skin proteome and the lack of knowledge about specific protein targets of sensitising chemicals, investigations into their reactions with proteins are largely focused on isolated models. Several groups have already developed chemical reactivity screening methods to assess a chemicals skin sensitisation potential (Aptula *et al.*, 2006; Divkovic *et al.*, 2005; Gerberick *et al.*, 2004; Kato *et al.*, 2003). These methods involve measuring a chemicals' reactivity with single nucleophile-containing peptides. Peptide reactivity with the test chemicals is reported as percent peptide depletion and data from these assays are proving useful indicators of contact sensitisers (data is in agreement with previously known LLNA data).

More advance mass spectrometry techniques are being used for analysis of more complex peptides and proteins to identify specific amino acid targets of chemical sensitisers. In these analyses the identification of proteins by MS uses tryptic digests of the proteins with two main approaches being used. The first is peptide mass fingerprinting performed with MALDI-TOF MS and the second is peptide sequencing using tandem MS (MS/MS). Mass fingerprinting involves measuring the molecular masses of all the tryptic products. The sample is mixed with a UV absorbing matrix (e.g. a-cyano-4-hydroxycinnamic acid) that acts as a proton donor so that irradiation

with a UV laser leads to generation of ions that travel up the time of flight tube at different speeds depending on their mass-to-charge ratio. This generates molecular masses that are matched with the theoretical sizes of the trypsin fragments from known protein sequences, producing a peptide mass fingerprint, enabling protein identification (Figure 1.7).

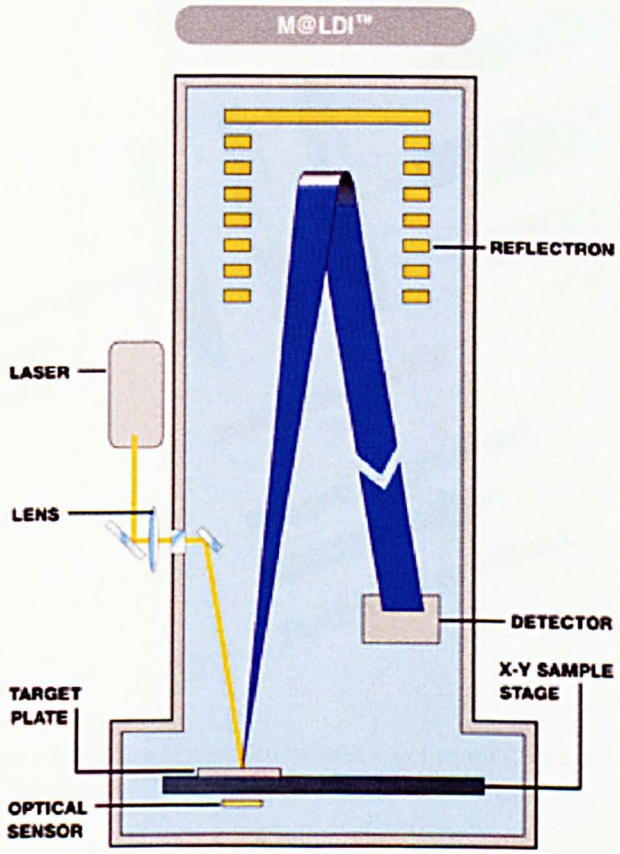


Figure 1.7 Schematic of matrix-associated laser desorption ionisation. Applied Biosystems, Voyager-DE PRO manual, Warrington, UK.

Peptide sequencing by MS uses two mass spectrometers connected in series. After ionisation, the mixture of charged peptides enters the first mass spectrometer, where the peptides are separated according to their mass/charge ratios. The instrument ie: quadrupole, is adjusted so that only a specific species is directed into a collision cell, where the peptide is broken down into a series of fragments, resulting from the sequential removal of individual amino acids from the end of the peptide ion. The

fragments are separated in the second mass spectrometer. The differences in molecular weight between successive fragments, which differ by the mass of one amino acid, identify the peptide sequence (Figure 1.8).

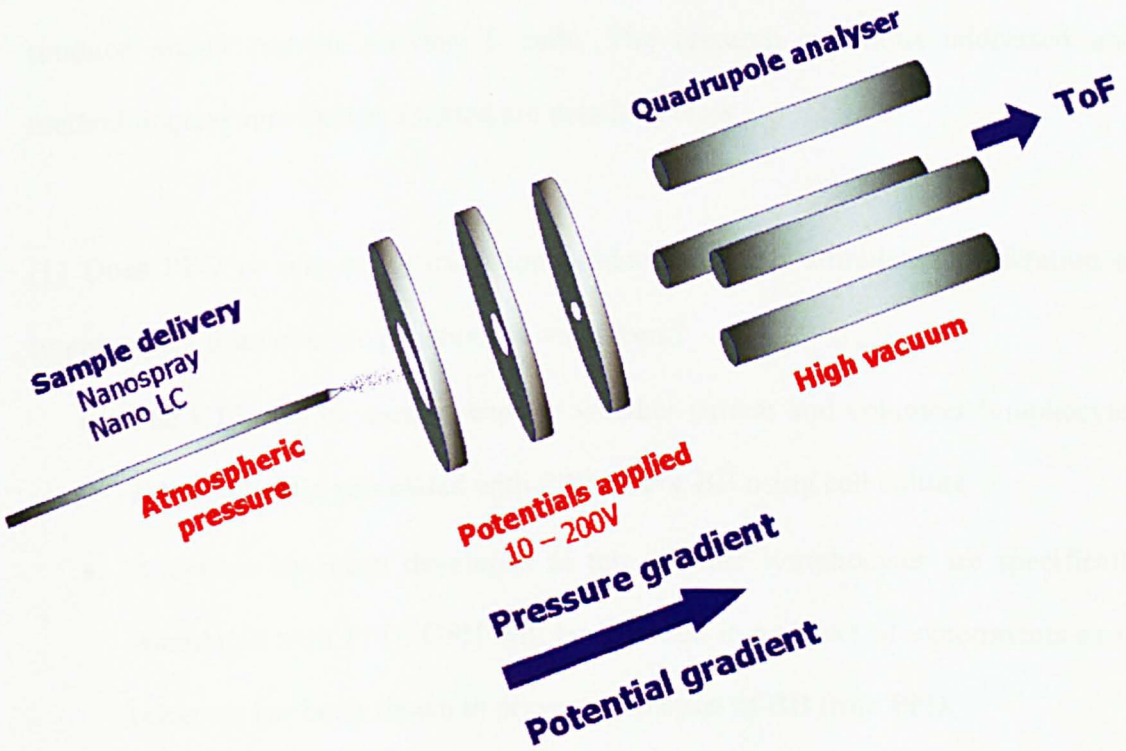


Figure 1.8. Schematic of electrospray ionisation mass spectrometry; Applied Biosystems, API QSTAR PULSARi manual, Warrington, UK

These methods can be used to compare masses of peptides from control and chemical containing incubations of proteins to identify specific amino acid modifications. For example, work done with DNCB on human serum albumin (Aleksic *et al.*, 2007) and model skin proteins cytokeratin and cofilin (Aleksic *et al.*, 2008) has identified cysteine, lysine, tyrosine and histidine as potential targets for modification.

I intend to utilize all of the above approached as part of my analysis to determine the target(s) for PPD protein modification.

1.8 Aims of thesis

The aims of these studies were to investigate the chemical and cellular basis of PPD hypersensitivity, more specifically identifying the mechanisms in which PPD can produce signal one to activate T cells. The research questions addressed and methodological approached adopted are detailed below.

(1) Does PPD or one of its oxidation products i.e.: BB stimulate proliferation of lymphocytes from allergic patients and volunteers?

- The LTT will be used to explore whether patient and volunteer lymphocytes are specifically stimulated with PPD and/or BB using cell culture
- A system has been developed to test whether lymphocytes are specifically stimulated with PPD. GSH will be included in a subset of experiments as its presence has been shown to prevent formation of BB from PPD.

(2) Do PPD/BB have the capacity to form stable adducts?

- The aim is to use model peptides and proteins glutathione-*S*-transferase pi (GSTP) & human serum albumin (HSA) to investigate binding capacity of PPD using solvent extraction, gel electrophoresis and mass spectrometry methods
- GSTP & HSA were selected as model proteins as they represent a major intracellular and a major extracellular protein; their 3D structures are available; full M/S coverage of protein is possible; knowledge of reactivity with amino acids with a range of other haptens is available for comparison

(3) Can this PPD protein complexes cause proliferation of lymphocytes from allergic patients and volunteers?

- The LTT will be used to explore whether patient and volunteer lymphocytes are specifically stimulated with PPD-modified HSA
- Cytokine secretion in response to PPD, BB and PPD-modified HSA stimulation will also be measured to give an insight into cellular mechanisms involved

(4) Which pathways are involved in PPD presentation to specific T-cells?

- The aim is to generate PPD/PPD-HSA specific T-cell clones from allergic patient cells to investigate mechanisms involved in T-cell activation using established cloning methods in the lab
- Previous work done with other contact sensitisers has highlighted 3 different mechanisms by which T-cells can be activated: -
    - Classical hapten mechanism with a covalently modified protein conjugate (Pickard *et al.*, 2007)
    - Direct chemical modification of peptide presented in MHC groove in which irreversibly binding is neccessary but processing of antigenic protein is avoided (Zanni *et al.*, 1997).
    - P.i-mechanism involving a direct reversible interaction of PPD with MHC molecules (Pichler, 2003).

CHAPTER 2

Activation of T-cells from allergic patients and volunteers by p-phenylenediamine and Bandrowski's base

2.1 Introduction

Skin sensitization is the process that follows the epicutaneous application of an allergen to the skin, which results in an immunological response specific for this allergen such as the aromatic amines *p*-phenylenediamine (PPD). PPD is one of the most widely encountered primary intermediates in hair dye formulations (Corbett *et al.*, 1973) and henna tattoos (Le Coz *et al.*, 2000, Mautlich and Sullivan 2005). On contact with skin PPD can cause allergic contact dermatitis, a delayed cell-mediated immune response that leads to erythema and edema (Smith Pease 2003). Recently Rastogi et al., (2006) demonstrated that there is 1.1% unconsumed PPD in hair dye formulations after colour development, showing that hair dying exposes individuals to PPD for prolonged periods.

PPD itself is believed to be protein unreactive (White *et al.*, 2006) and requires activation by oxidation to become immunogenic (Aeby *et al.*, 2004). Auto oxidation leads to the formation of an electrophilic primary quinonediimine intermediate, which is susceptible to sequential self-conjugation reactions and further oxidation, resulting in the formation of Bandrowski's base – a rearrangement product of these reactions (Picardo *et al.*, 1990).

PPD and BB are classed as strong and extreme sensitisers respectively, based on their ability to stimulate proliferative responses in lymph nodes of topically exposed mice (Warbrick *et al.*, 1999; White *et al.*, 2006). In humans, 50% treated with topical solution of PPD at concentrations of 10% and above showed evidence of sensitisation (Marzulli and Maibach 1974) and Patel et al., (2007) described a high frequency of patch test reactivity amongst PPD-allergic individuals.

In conditions of prolonged exposure to PPD ie: henna tattoos, LLNA or human patch testing, a substantial amount of PPD will become air-oxidized on the skin surface

forming possible sensitising auto-oxidation products i.e.: BB (Goux *et al.*, 2007). Data suggests that PPD and BB are immunogenic but the chemical entity that interacts with immune cells has not been fully elucidated.

PPD or an oxidation product must overcome the skin barrier and penetrate the epidermis to activate skin dendritic cells (DC's) and induce skin sensitization (Aeby *et al.*, 2008). Previous work using DC's have found that exposure to PPD and BB resulted in functional maturation, as measured by increased expression of cell surface receptors i.e.: CD86 (Rougier *et al.*, 2000; Ashikaga *et al.*, 2002; Aeby *et al.*, 2004) with BB needing much lower concentrations to induce a response. Hulette et al., (2005) have shown that PPD concentrations (above 2.5 mM) that are associated with significant cell death stimulate dendritic cell maturation, as assessed by up-regulation of CD86 expression; presumably via a classical "danger" response (Matzinger, 1994) Moreover, Toebak et al., (2006) have shown that PPD concentrations of 200 μM and above stimulate significant secretion of CXCL8, a chemokine involved in recruitment of cells expressing CXCR1 and CXCR2. Recently Coulter et al., (2007) have proposed concentration dependent effects of PPD on DC function ie: low concentrations (<50μM) induce CD40 and MHCII expression, medium concentrations (<200μM) induce CXCL8 secretion and high concentration (<2500μM) lead to CD86 expression and strong cytotoxicity.

Studies by Krasteva et al., (1993) have shown that BB, but not PPD or primary oxidation products, stimulate T-cells from allergic patients. However, more recent work with cloned T-cells from PPD allergic individuals provides evidence to suggest that PPD and BB might actually stimulate proliferation of T-cells via two independent mechanisms (Sieben *et al.*, 2002). PPD associated non-covalently with MHC molecules was found to stimulate certain T-cells directly without a requirement for

processing, while BB stimulated T-cells via a classical hapten mechanism involving both irreversible binding and processing. Importantly, in both studies, neither PPD nor BB stimulated proliferation of lymphocytes from non-allergic individuals. In view of the fact that different groups have generated apparently conflicting results, it was decided to assess the ability of PPD and BB to stimulate T-cells and analyze in parallel the degradation and protein reactivity of each compound in solution.

Data presented within shows that circulating T-cells from allergic patients were stimulated with both PPD and BB; moreover, in contrast to previous reports, T-cells from most non-allergic volunteers were stimulated, but only with BB.

These data show that BB is antigenic even in individuals that have never dyed their hair, suggesting possible sensitization through other routes such as black and dark blue clothing dyes that contain PPD. However, since PPD-specific T-cell responses can be demonstrated additionally in allergic patients, this suggests that PPD, but not BB, plays an important role in sensitization.

2.2 Methods

2.2.1. Donor Characteristics

Peripheral blood mononuclear cells were obtained from venous blood obtained from 8 PPD allergic, patch test positive patients and 16 healthy volunteers. Approval for the study was obtained from Liverpool local research ethics committee and informed consent was obtained. In addition, peripheral blood mononuclear cells were isolated from 6 cord blood samples taken after the cords had been clamped, approval and informed consent for this collaborative project with Professor Peter Friedmann (Professor of Dermatology, University of Southampton) was granted by the Southampton and South West Hants ethics committee. The details of the patients and volunteers are shown in table 2.I.

Table 2.1. Clinical details of patients and volunteers

	Age	Sex[a]	Previous hair dye use	PPD allergy	Other allergy	Months since reaction	Patch test result
1	59	F	Yes	Yes		18	+
2	66	F	Yes	Yes		Unknown	+
3	65	F	Yes	Yes		25	+
4	63	F	Yes	Yes		60	+
5	52	M	Yes	Yes	Red, orange & blue dye, potassium dichromate	24	+
6	41	F	Yes	Yes		28	+
7	65	F	Yes	Yes	Nickel, orange dye	72	+
8	45	F	Yes	Yes		22	+
9	25	F	Yes	No			Np[b]
10	26	F	Yes	No	Septrin		Np
11	49	F	Yes	No			Np
12	26	F	Yes	No			Np
13	51	F	Yes	No			Np
14	34	F	Yes	No			Np
15	30	F	Yes	No			Np
16	34	M	Yes	No			Np
17	27	M	No	No			Np
18	23	M	No	No			Np
19	28	M	No	No			Np
20	65	M	No	No			Np
21	34	M	No	No			Np
22	45	M	No	No			Np
23	38	M	No	No			Np
24	33	M	Yes	No	Tetracycline		Np

[a]M, male; F, female
[b]np, not performed

2.2.2. Cell culture medium and chemicals

Cell culture medium consisted of RPMI-1640 supplemented with pooled heat inactivated human AB serum (10%, v/v), HEPES (25 mM), L-glutamine (transferrin (25 μg ml-1), streptomycin (100 μg ml-1) and penicillin (100 U ml- reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

PPD was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Bandrowski's base was obtained ICN Biomedicals Inc. (Aurora, Ohio, U.S.A).

[14C]PPD (specific activity, 60 mCi/mmol) and [14C] BB (specific activity, 60mCi/mmol; radiochemically homogeneous as determined by HPLC) was synthesized by Amersham Biosciences UK Limited (Bucks, UK). Stock solutions (1mg/ml) were dissolved in cell culture medium:dimethyl sulphoxide (4:1, v/v) (Sigma Chemical Co. Poole, Dorset, U.K.) and diluted as required. Chromatographygrade solvents were products of Fisher Scientific (Loughborough, Leicestershire,U.K.).

Cell culture media used to generate EBV transformed B-cell lines was supplemented with pooled heat-inactivated fetal calf serum (10%, v/v) (as opposed to human AB serum).

2.2.3. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized freshly drawn blood from the 8 healthy volunteers by single step density centrifugation using Lymphoprep (Nycomed, Birmingham, UK). Whole blood (60 ml) was layered on the surface of Lymphoprep (2 x 10 ml) and then centrifuged at 2000 rpm for 20 minutes. The PBMCs formed a distinct cloudy band between the blood plasma and red blood cells, and were removed using a sterile Pasteur pipette (Figure 2.1.), washed twice in

HBSS (pH 7.4), and centrifuged at 1800 rpm and 1500 rpm, respectively. PBMCs were re-suspended in 5 ml HBSS and cell viability was then assessed by trypan blue dye exclusion; cells found to be greater than 95% viable were re-suspended in culture medium in the required concentration.

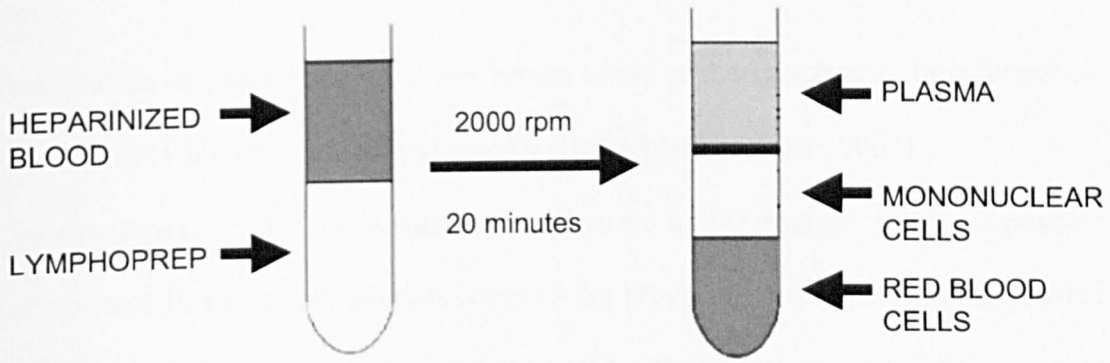


Figure 2.1. Isolation of blood lymphocytes by single step density centrifugation.

2.2.4. Generation of EBV-transformed B-lymphoblastic cell lines

In vitro infection of human lymphocytes by Epstein-Barr virus (EBV) results in the growth transformation and establishment of immortalized B lymphoblastoid cell lines (BLCLs). EBV-transformed B-cell lines (referred to as antigen presenting cells) were generated from PPD-allergic patients and healthy volunteers by the transformation of freshly isolated lymphocytes with supernatant from the EBV-producing cell line B9-58. Recipient lymphocytes ($2x10^6$) (isolated from PPD-allergic patients and healthy volunteers as described above) were incubated with the supernatant (5 ml) from the EBV-producing cell line B9-58 (37°C, 5% CO_2) supplemented with cyclosporin A (CSA; 1 μg/ml) to prevent T-cell mediated suppression of B-cell infection. Cells were dispensed into 24-well cell culture plates (1 ml per well). One-half of the culture

medium was changed twice a week until cells were confluent and transferred to flasks.

2.2.5. Determination of patient and volunteer lymphocyte proliferation with para-phenylenediamine and Bandrowski's base; the lymphocyte transformation test

Proliferation of patient and volunteer lymphocytes in the lymphocyte transformation test with PPD and BB as described previously (Pichler and Tilch, 2004). Lymphocytes (1.5×10^5; total volume 0.2 ml) from 8 PPD allergic, patch test positive patients and 16 non-sensitised volunteers (6 not previously exposed to hair dye) were isolated on at least two occasions, and incubated with the antigens (37°C, 5% CO_2). Cell cultures containing lymphocytes in the absence of PPD or BB were taken as a negative control and lymphocyte incubations with tetanus toxoid (TT) (5 μg/ml) were taken as a positive control. Final concentrations of PPD and BB ranged from 0.1-500 μM. All stock solutions (10mg/ml) were prepared (10% DMSO; 90% cell culture media), diluted and added to lymphocytes without delay. After 6 days proliferation was measured by the addition of [^{3}H] thymidine (0.5μCi) for the final 16 hours (h). Proliferative responses were calculated as mean stimulation index (SI; cpm in drug-treated cultures / cpm in cultures with DMSO alone). An SI of 2.0 or above was considered a positive response.

2.2.6. Determination of the stability of p-phenylenediamine and Bandrowski's base in culture

[^{14}C]PPD/BB (50 mM [0.2 mCi]; ± glutathione [1 mM]) was incubated with lymphocytes (1.5×10^6; total volume 1ml) in cell culture medium at 37°C for 144h

(duration of the lymphocyte transformation test). At 0, 2, 24, 72 and 130h, 100 µl aliquots were taken and analyzed by radiometric HPLC. Samples were eluted from a Zorbax SB-C18 column (250 mm × 46 mm id; Phenomenex, Macclesfield, Cheshire, UK) at room temperature with a gradient of methanol (5 % for 5 min; 5 – 60 % over 15 min) in 10mM ammonium acetate (pH 6.9); the flow rate was 0.9 ml/min. Mobile phase was delivered by a Kontron 325 pump (Watford, Herts., U.K.). Analytes in the eluate were monitored with a Spectra Physics UV1000 spectraphotometer (254nm; Hemel Hempstead, Herts.,U.K.) and radiolabelled analytes were quantified using a Canberra-Packard Radiometric Flo-One/β radioactivity detector. The eluate was mixed with Ultima-Flo AP scintillant at a rate of 1 ml/min.

To determine whether PPD/BB binds covalently to protein, irreversible binding of radioactive compound to cellular or serum protein was determined by exhaustive solvent extraction (method adapted from Kitterringham *et al.,* 1988; Pirmohammed *et al.,* 1995). Cellular and serum protein were precipitated and separated by centrifugation at 0, 2, 24, 72 and 130h and subsequently extracted using 100% acetonitrile (rapid addition of 2 ml x 1 wash, i.e. 2 volumes of acetonitrile, for 10 minutes at 4000 rpm; and 1 ml x 3 washes, equal volumes, for 10 minutes at 4000 rpm). Protein was dissolved in sodium hydroxide (0.2 M; 1 ml) and aliquots (100 µl) were taken from each wash stage for quantification of bound radioactivity; samples were analysed in by LSC using a Packard 1500 Tri-carb liquid scintillation counter (Canberra-Pangbourne, Berkshire, UK). Protein concentration was measured according method of Bradford (1976). Briefly, a standard curve was constructed in duplicate (protein / BSA concentrations: 0, 0.25, 0.5, 1, 2, 3, 4, 5 mg /ml) to which several PPD sample dilutions were compared (1 in 5, 1 in 10, 1 in 20, and 1 in 50). 10 µL of the diluted sample combined with 10 µL of distilled H_2O was added to sample

wells; 20 μL of standard sample was added to the standards wells. 200 μL of a 1 in 5 dilution Bradford reagent (used to determine the concentration of proteins in solution, a procedure based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution causing a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present) (Sigma Chemical Co. Poole, Dorset, U.K.) was added to all the wells, and the absorbance read at 570 nm.

Exhaustive solvent extraction was also used to determine whether there is any difference in binding of PPD to cellular and serum protein between allergic patient and volunteers' lymphocytes.

2.2.7. Investigation of the mechanism of PPD-mediated lymphocyte proliferation

To confirm that lymphocytes from allergic patients were actually stimulated with PPD or a primary oxidation product, in addition to BB, it was essential to develop an *in vitro* system in which BB formation was inhibited. In this respect, lymphocytes were incubated with PPD (5 μM) in the presence or absence of glutathione (1 mM), which prevents BB formation, but not irreversible binding of PPD-derived material to cells/serum (see results). After 4 and 16h, lymphocytes were washed repeatedly, to remove soluble PPD, suspended in fresh medium and cultured at 37 °C for the remainder of the proliferation assay (section 4.2.4). Overview of the cellular and mechanistic methods used so far is shown in Figure 2.2.

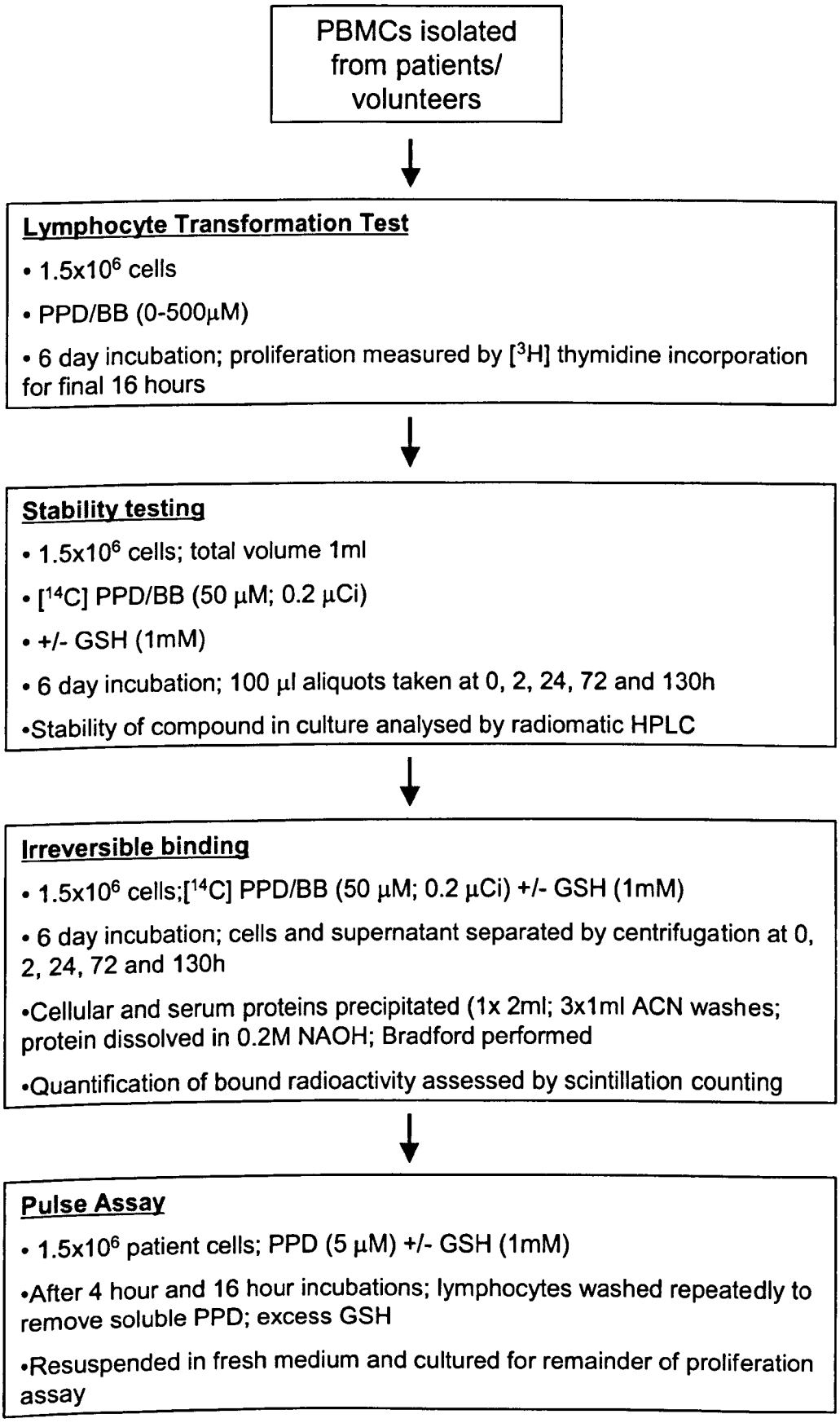


Figure 2.2. Overview of methods used

2.2.8. Generation of T cell clones

PPD and BB stimulated lymphocytes from allergic patients and volunteers were cloned by limiting dilution using previously described methodology (Schnyder *et al.*, 1997; Wu *et al.*, 2006). Bulk cultures were generated by stimulation of freshly isolated lymphocytes (2 x 10^6; total volume 1 ml) from volunteers and allergic patients, with PPD and BB (5 – 20 μM); on day 6 and 9, IL-2 (60 U/ml) was added to maintain antigen-specific proliferation. After 14 days, T cells were cloned by serial dilution; cells were plated at 0.3, 1 and 3 cells per well and re-stimulated with irradiated (60 Gy) lymphocytes (50,000 per well / 5x10^6 per plate), IL-2 (60 U/ml) and phytohemagglutinin (PHA) (1 μg/ml) (total volume 200 μL). IL-2 was again added to maintain antigen-specific proliferation on alternate days until the clones were ready to test for the specificity (28 days after serial dilution). T-cells (0.5 x 10^5) were incubated with irradiated (60 Gy) autologous EBV-transformed B-cells as antigen presenting cells (0.1 x 10^5) and the compound in which the lymphocytes were initially stimulated with. After 48h, [3H] thymidine was added and proliferation determined 16h later by scintillation counting. T-cell clones with a SI of greater than or equal to 2.0 were re-stimulated with PHA (1 μg/ml) as described above and expanded in IL-2 containing medium (Figure 2.3).

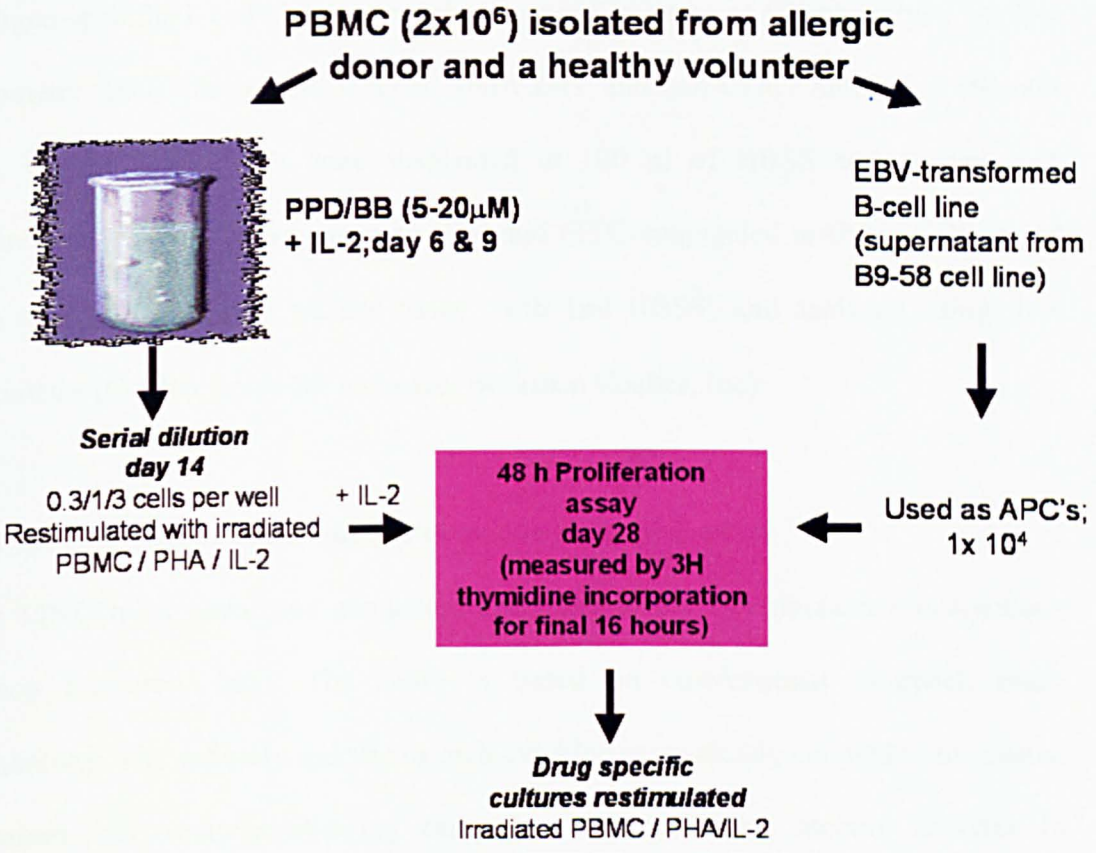


Figure 2.3 Scheme of methods used for generation of PPD/BB-specific T cell clones

2.2.9. Specificity and phenotype of the T-cell Clones and analysis of cytokine / chemokine production

Irradiated EBV / antigen presenting cells (0.1 x 10^5) and T-cell clones (0.5 x 10^5) were incubated with PPD or BB (both 5 - 50 mM). Proliferation, reflected by incorporation of [^{3}H] thymidine, was quantified. Supernatants were collected prior to the addition of [^{3}H] thymidine, pooled for each condition and stored at -80°C.

Concentrations of IL-1α, IL-1β, IL-4, IL-5, IL-6, TGFα, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IFNγ, TNFα, eotaxin, MCP-1, sCD40L, MIP-1α, MIP-1β, IP-10 and RANTES were measured in supernatants using the human cytokine / chemokine LINCOplex multiplex assay kit (manufactured by LINCO Research Inc., Hampshire, UK). Cytokine content was measured using a Liquichip 100 workstation (Qiagen Ltd, West Sussex, UK) with LiquiChip IS 2.3 software.

Antigen-specific T cell clones were characterised in terms of CD phenotype by flow cytometry using fluorescent labelled anti-CD4+ and anti-CD8+ antibodies (Serotec Ltd, Oxford UK). Cells were suspended in 100 µl of HBSS and stained with fluorescent PE-conjugated mAbs to CD4 and FITC-conjugated mAbs to CD8 for 30 min at 4°C. Cells were washed twice (with 1ml HBSS) and analyzed using flow cytometry (Coulter Epics XL software; Beckman Coulter, Inc).

2.2.9.1. LINCOplex multi-analyte detection assay procedure

The LINCOplex assay was conducted according to the manufacturer's instructions (Linco Research, Inc.). The assay is based on conventional sandwich assay technology. The antibody specific to each cytokine is covalently coupled to the coated Luminex microspheres allowing capture and detection of specific analytes in supernatant samples; each antibody is coupled to a different microsphere uniquely labelled with a fluorescent dye mixture (figure 3.2.a.). Assays are carried out in a 96-well plate format, with up to 100 tests per well. High-tech fluidics based on the principles of flow cytometry causes the stream of suspended microspheres to line up in a single file prior to passing through the detection chamber of the Luminex 100 compact analyzer which allows the particles to be measured discretely. Within the analyzer, lasers excite the internal dyes that identify each microsphere particle; a red laser excites both the internal red and infrared dyes, allowing the proper classification of the microsphere to one of the 100 sets (i.e. classification channel reading), whereas the green laser excites any orange fluorescence associated with the binding of your analyte (i.e. reporter channel reading) (figure 3.2.b.).

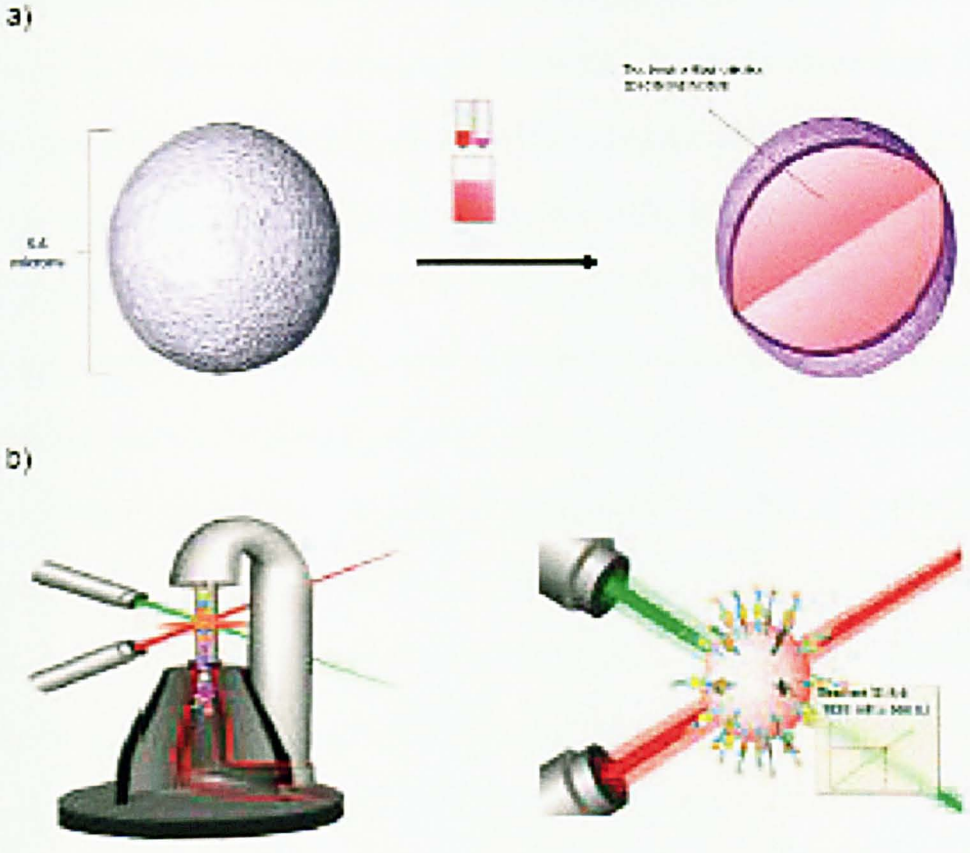


Figure 2.4. Luminex® system color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. By using different intensities of the two dyes each microsphere has a unique spectral signature (i.e. determined by the internal red/infrared fluorophore mixture) (a), which when passed through the detection chamber (b) allows the particles to be measured discretely. A red laser excites both the internal red and infrared dyes, allowing the proper classification of the microsphere to one of the 100 sets (i.e. classification channel reading) and a green laser excites any orange fluorescence associated with the binding of the analyte (i.e. reporterchannel reading). (Figures taken from the Luminex website: www.luminexcorp.com/)

All reagent dilutions (beads, cytokine standards, cytokine controls, biotinylated detection antibody, etc.) were prepared according to the manufacturer's instructions using a LINCOplex kit. Cytokine standard provided as a lyophilized cocktail was reconstituted with 250 µl de-ionized water to give a stock concentration of 10,000 pg/ml of each cytokine. Standard concentrations of 5000, 2000, 666, 222, 75, 25, 8.3 and 2.76 pg/ml were prepared by diluting the 10,000 pg/ml stock standard with assay buffer. The 0 pg/ml standard was buffer alone. Each of the two vials of lyophilized cytokine controls provided in the kit was reconstituted with 250 µl of de-ionized

water. Firstly, the filter plate was blocked by adding 200 µl assay buffer (50 mM PBS with 25 mM EDTA, 0.08% Sodium Azide, 0.05% Tween 20, and 1% BSA, pH 7.4) into each well of the microtiter plate, which was sealed and mixed on a plate shaker for 10 minutes at room temperature. The assay buffer was removed by vacuum, and 25 µl of assay buffer was added to the background wells, sample wells and the quality control wells. Then cell culture media was added to the background and control wells, followed by 25 µl of samples into the appropriate wells. To each well, 25 µL of the mixed colour coded beads was added ensuring that the bead mix was thoroughly shaken to avoid the beads settling. The plate was sealed and covered with aluminium foil then incubated with agitation on a plate shaker for 1 hour at room temperature. After the incubation period, the fluid was removed by vacuum, and the plate washed twice with 200 µL/well of wash buffer (10X Wash Buffer 1:10 dilution required with deionized water to give 10 mM PBS with 0.05% Proclin, and 0.05% Tween 20, pH 7.4), removing wash buffer by vacuum filtration between each wash to which 25 µL of detection antibody cocktail was added to each well. The plate was then sealed, covered and incubated with agitation on a plate shaker for 30 minutes at room temperature. After incubation, 25 µL Streptavidin-Phycoerythrin was added to each well containing the 25 µL of detection antibody cocktail, the plate was resealed, covered, and incubated with agitation on a plate shaker for a further 30 minutes at room temperature. The contents of the plate were again removed by vacuum, the plate was washed twice with 200 µL/well wash buffer, removing wash buffer by vacuum filtration between each wash. Finally, 150 µL sheath fluid was added to all wells, the plate was covered and the bead mix re-suspend on a plate shaker for 5 minutes. Cytokine content was measured using a Liquichip 100 workstation (Qiagen Ltd, West Sussex, UK) with LiquiChip IS 2.3 software. Samples were analyzed per well using a

minimum of 50 beads per region. The raw data (mean fluorescence intensity, MFI) were captured using LiquiChip IS 2.3 software; for data analysis, a 5-parameter logistic (5-PL) or curve-fitting method for calculating cytokine/chemokine concentrations in samples was applied to each standard curve and sample concentrations were interpolated from the standard curve. The gating specifications / settings ranged from 8,060 to 13,000 with the limit of detection (LOD), defined as the lowest concentration of analyte that can be detected, was 3.2 pg/ml. A standard curve (range of detectability between 3 and 10,000 pg/ml) was generated from a single mixed standard and the concentration of each analyte in the sample was determined automatically and data is presented as pg/ml.

2.2.10. Statistical Analysis

Statistical analyses were performed on SPSS 13.0; averaged data is presented as mean ± standard error of the mean (SEM). Increases in proliferation, cytokine secretion, and distribution of antigen formation towards cellular or serum proteins as a result of application of the test chemicals PPD and BB were compared to the appropriate vehicle control, using the Mann-Whitney test for non-parametric / non-normally distributed data. $P < 0.05$ was considered to be significant.

2.3 Results

2.3.1 Lymphocyte proliferation with p-phenylenediamine and Bandrowski's base

Lymphocyte proliferation associated with both PPD and BB exposure was concentration dependent, with PPD and BB concentrations between 0.1-50μM stimulating a significant proliferative response in patients and patients and volunteers cells respectively (Figure 2.5). After culture of patient lymphocytes with PPD (5μM) mean SI was 17.9±8.9, and with BB (5μM) 20.9±7.3 Volunteer lymphocyte mean SI with BB was 19.1±4.5. Table 2.2 shows full range of patient and volunteer responses.

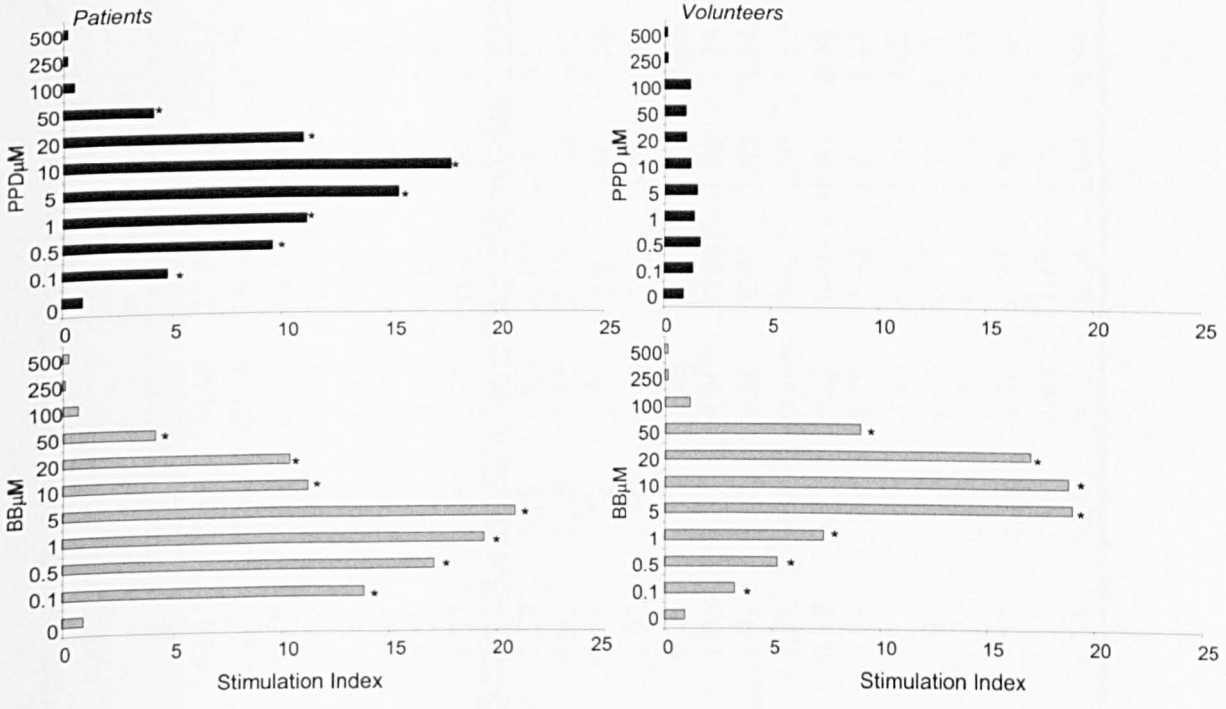


Figure 2.5. Antigen-specific stimulation of lymphocytes from allergic patients and volunteers. Mean proliferative response of lymphocytes from 8 allergic patients and 16 volunteers, stimulated with PPD and BB. Lymphocytes from 7/8 patients proliferated in a concentration dependent manner with PPD and BB, whereas most of the volunteers proliferated only in response to BB. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16h of the experiment. Results are expressed as mean SI of triplicate culture. Statistical analysis was performed by comparing incubations in the presence and absence of drug (*$P<0.05$). Coefficient of variation was consistently less than 20%. The counts per minute (cpm) in control incubations were less than 1500.

Table 2.2 LTT data for all patients and volunteers

	Stimulation Index (SI)													
	PPD (μM)							BB (μM)						
	0	0.1	0.5	1	5	10	20	0	0.1	0.5	1	5	10	20
Allergic Patients														
1	1.00	12.52	34.42	42.19	54.16	74.09	37.85	1.00	20.37	13.03	12.69	19.67	1.52	1.36
2	1.00	15.47	25.12	31.27	36.10	29.36	20.87	1.00	12.48	20.85	24.54	29.40	31.54	27.95
3	1.00	0.71	2.46	6.56	14.00	22.61	10.13	1.00	5.76	7.17	7.37	11.06	13.51	14.66
4	1.00	3.29	5.89	7.62	11.09	6.39	3.23	1.00	4.31	7.29	15.61	19.32	4.75	3.91
5	1.00	1.42	2.04	1.77	3.39	1.61	0.73	1.00	1.26	1.82	2.15	7.47	4.23	15.72
6	1.00	1.01	1.28	1.91	3.16	10.82	6.05	1.00	54.62	70.87	84.83	67.49	7.92	2.72
7	1.00	1.07	0.91	1.08	3.93	0.68	0.65	1.00	1.26	4.78	3.41	10.63	13.80	8.96
8	1.00	1.49	1.11	1.33	2.96	0.73	0.63	1.00	1.30	1.45	1.25	2.25	6.24	1.41
Volunteers														
9	1.00	0.59	1.58	1.50	0.70	0.88	1.07	1.00	0.63	1.60	4.81	9.06	11.42	10.64
10	1.00	1.41	1.22	0.92	0.88	0.15	0.23	1.00	6.52	7.06	7.94	8.23	0.85	1.59
11	1.00	0.79	0.75	0.91	0.72	0.85	0.94	1.00	7.06	13.36	15.03	38.66	26.50	25.30
12	1.00	0.93	1.19	1.92	1.26	1.10	1.43	1.00	1.33	1.26	3.38	15.01	24.30	41.28
13	1.00	1.90	1.05	0.70	1.30	1.46	1.11	1.00	0.64	11.04	18.55	15.50	15.39	6.54
14	1.00	1.77	1.68	1.86	1.57	0.90	0.68	1.00	1.38	1.31	2.11	11.62	14.66	3.92
15	1.00	1.59	1.46	1.02	1.02	0.86	0.92	1.00	1.54	3.15	7.43	33.90	30.75	70.65
16	1.00	1.83	4.37	1.78	0.63	0.94	0.53	1.00	5.60	7.91	18.99	37.06	41.61	40.59
17	1.00	0.40	0.50	0.38	0.37	0.27	0.40	1.00	0.43	0.40	1.62	6.83	7.71	7.12
18	1.00	1.90	1.06	1.03	1.40	1.19	1.32	1.00	1.94	10.90	14.03	66.96	40.65	22.27
19	1.00	1.29	1.45	1.35	0.61	0.38	0.23	1.00	4.23	8.90	10.92	13.06	3.85	0.27
20	1.00	1.69	1.91	1.03	1.17	1.64	0.75	1.00	16.07	10.99	3.37	33.72	37.41	51.48
21	1.00	1.43	1.75	1.16	0.37	0.15	0.16	1.00	2.35	10.81	6.87	2.15	0.14	0.21
22	1.00	1.72	1.36	1.53	1.78	1.78	0.27	1.00	1.36	4.11	8.26	12.13	25.50	1.36
23	1.00	0.93	1.77	1.31	1.35	1.44	1.79	1.00	1.14	1.02	1.13	1.86	1.81	1.22
24	1.00	1.06	1.01	0.85	0.50	0.17	0.24	1.00	0.54	0.67	0.78	1.62	1.32	1.49

Lymphocytes from allergic patients (8/8) and healthy volunteers (14/16) proliferated *in vitro* in response to BB. Only allergic patients lymphocytes (7/8) proliferated in response to PPD stimulation. Figure 2.6 characterizes concentration dependent proliferation from 3 representative patients and volunteers represented as cpm ±SD to demonstrate inter-variability between different individuals.

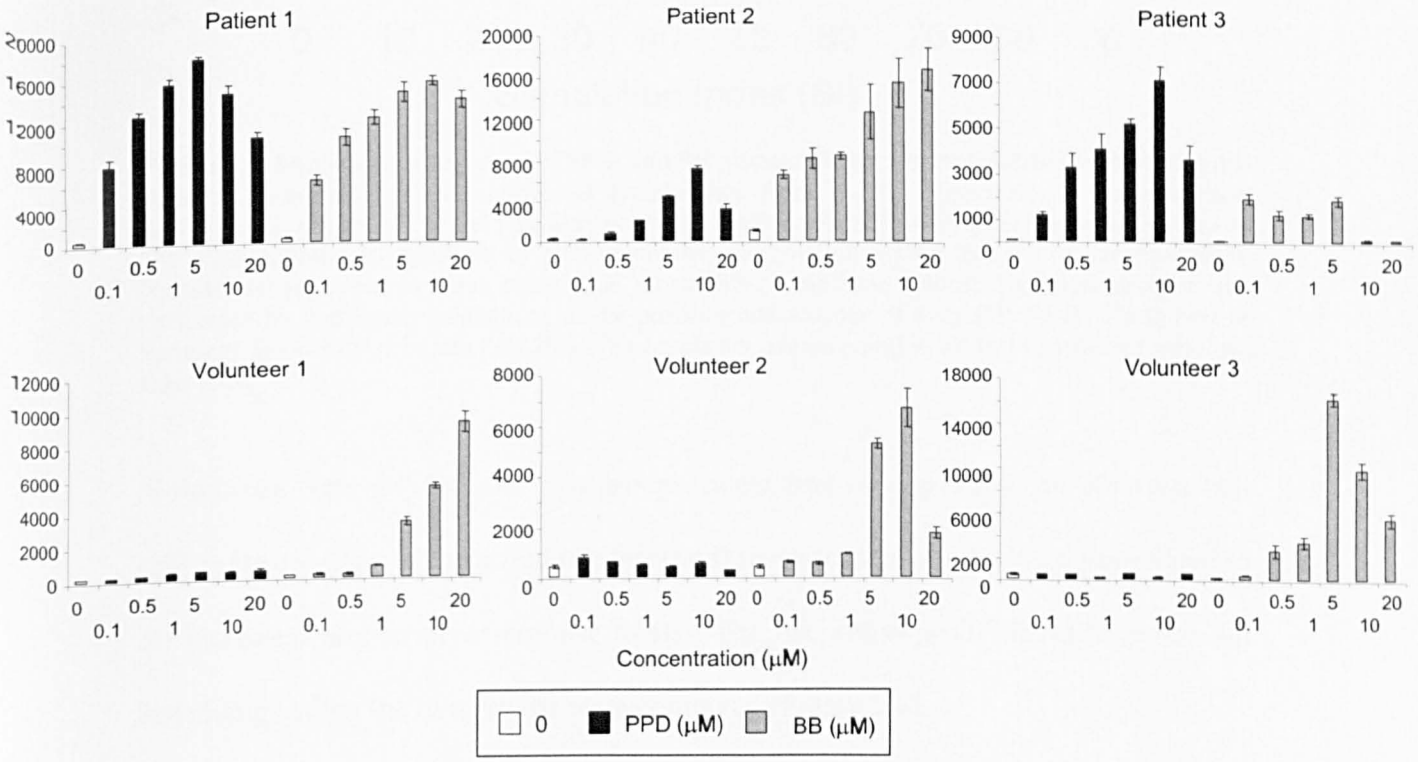


Figure 2.6. Lymphocytes from 3 representative patients proliferate with PPD and BB, whereas the volunteers proliferated only in response to BB. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16h of the experiment. Results are expressed as mean cpm of triplicate culture ± SD.

Figure 2.7 shows the maximum stimulation of patient and volunteer lymphocytes when exposed to PPD/BB. Although large inter-individual variations were seen with both experimental groups, data shows consistent stimulation of patient and volunteer cells to BB, and only patient cell stimulation to PPD.

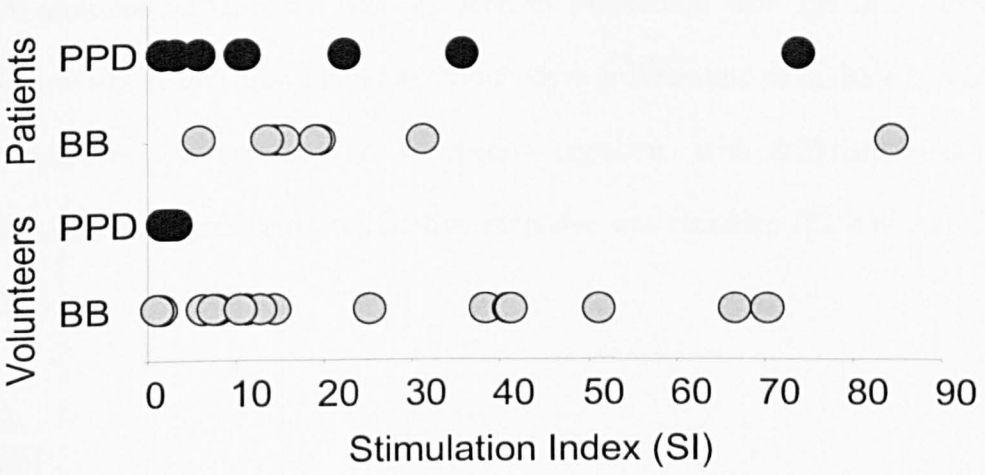


Figure 2.7. Maximum proliferation of PPD- and BB-stimulated lymphocytes isolated from patient and volunteer cohorts; combined results of lymphocytes from 8 PPD hypersensitive patients show proliferation with both PPD and BB, whereas the 16 healthy volunteers only proliferated in response to BB. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16h of the experiment. Results are expressed as mean stimulation index (SI) of triplicate culture. Statistical analysis was performed by comparing incubations in the presence and absence of drug (*P<0.05). Coefficient of variation was consistently less than 20%. The counts per minute (cpm) in control incubations were less than 1500.

Volunteers were divided into two groups, depending on depending on previous hair dye exposure (hair dye exposed/non exposed) to determine whether this was related to an increased proliferative response to BB. The maximum proliferative response did not change when the two groups were compared (Figure 2.8).

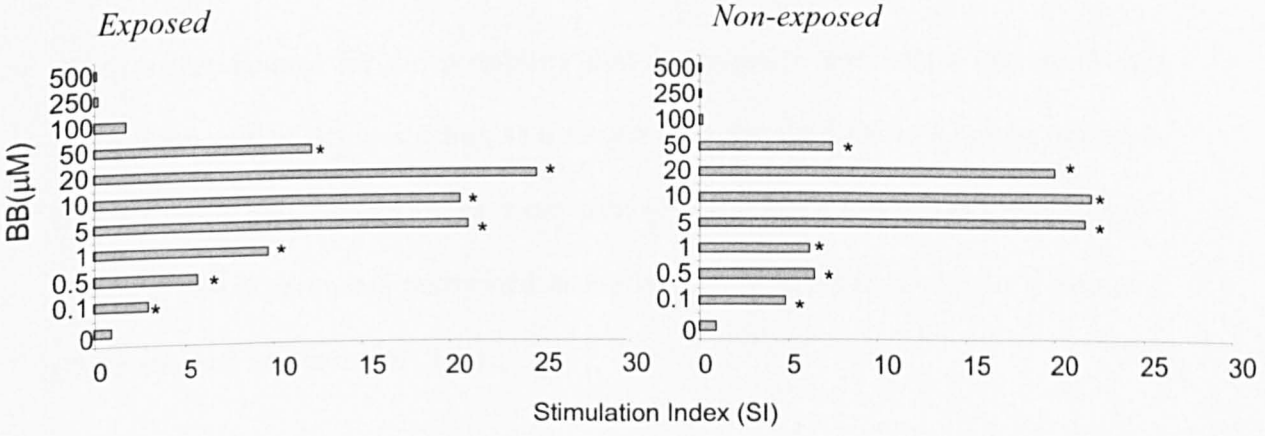


Figure 2.8. Mean proliferative response of lymphocytes from hair dye exposed (n=9) and not hair dye exposed volunteers (n=7), stimulated with PPD and BB. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16h of the experiment. Results are expressed as mean stimulation index (SI) of triplicate culture. Statistical analysis was performed by comparing incubations in the presence and absence of drug (*P<0.05). Co-efficient of variation was consistently less than 20%.

A proliferative response was not seen to stimulation with BB in 2 out of the 16 volunteers (Table 2.2). Therefore lymphocyte proliferation from the 2 non-responding volunteers was assessed on 4 separate occasions with different batches of BB, however no significant proliferative response was recorded (SI less than 2) (Figure 2.9)

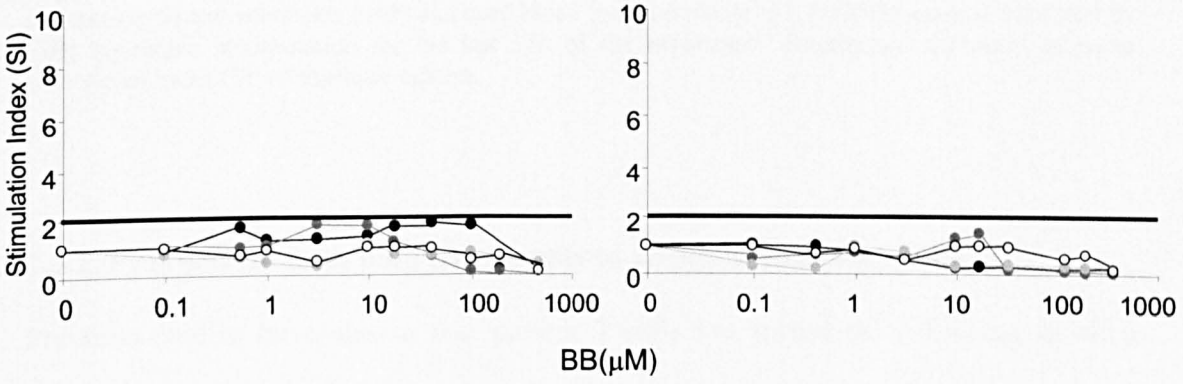


Figure 2.9. Two out of the sixteen volunteers did not response to PPD or BB when tested on 4 separate occasions. Cells were responsive and stimulated in the presence to the positive control Tetanus toxoid (5 μg/ml). Proliferation was measured by [^{3}H] thymidine incorporation for the last 16h of the experiment. Results are expressed as) mean stimulation index (SI) of triplicate culture. Statistical analysis was performed by comparing incubations in the presence and absence of drug (*P<0.05). Coefficient of variation was consistently less than 20%. The counts per minute (cpm) in control incubations were less than 1500.

As a further control for the possibility that responses to BB reflect specific T-cell recognition or that BB was acting as a simple mitogen, cord blood lymphocytes were cultured with BB. No responses were seen (Figure2.10) Cord blood samples were obtained and experiments performed in the laboratory of Professor Peter Friedmann (University of Southampton, UK).

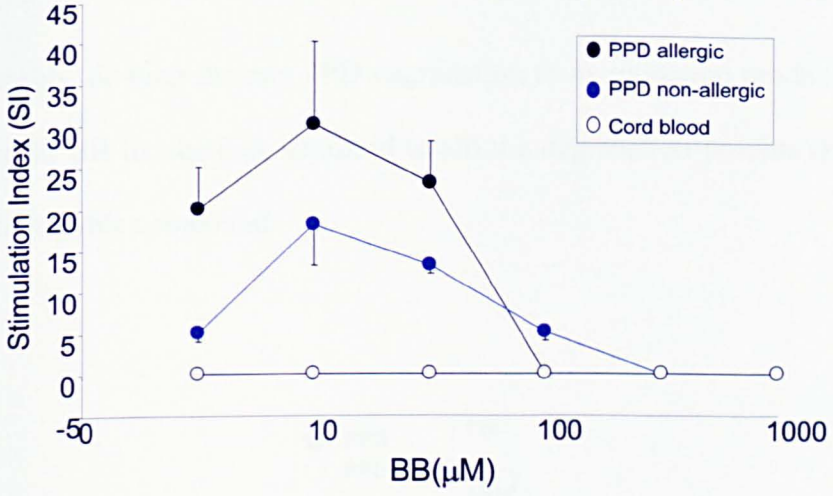


Figure 2.10. Comparison of BB-specific proliferation of peripheral blood lymphocytes from allergic patients (n=6) and volunteers (n=9) and cord blood lymphocytes (n=6). Proliferation was measured by [3H] thymidine incorporation for the last 16h of the experiment. Results are expressed as mean stimulation index (SI) of triplicate culture.

2.3.2. PPD and BB both bind irreversibly to serum and cellular proteins

Previous studies have shown that patient T-cells are stimulated following in vitro exposure with PPD (Sieben *et al.*, 2002). However, PPD oxidation, self conjugation, protein binding and BB formation in culture was not controlled for and as such the observed T-cell response may represent antigen stimulation with a PPD related product, not PPD itself. To determine whether PPD specifically stimulates allergic patient and healthy volunteer T-cells, the stability of PPD and formation of PPD oxidation products were measured.

Stability of PPD and BB was examined for the duration of the LTT using radiolabelled HPLC techniques to determine if PPD or one of its oxidation products is responsible for the observed proliferative response. PPD was found to rapidly degrade in solution in the presence of lymphocytes; after 24h the formation of Bandrowski's base was detected, and increased over the 130h period (Figure 2.11, 2.12). Bandrowski's base is more stable than PPD, although slight degradation was detected (Figure2.11, 2.13) GSH had opposing effects of the degradation of the chemicals,

considerably slowing the rate PPD degradation to its oxidation products, whereas its presence in BB incubations appeared to aid the degradation process (Figure 2.11) to an unidentifiable compound.

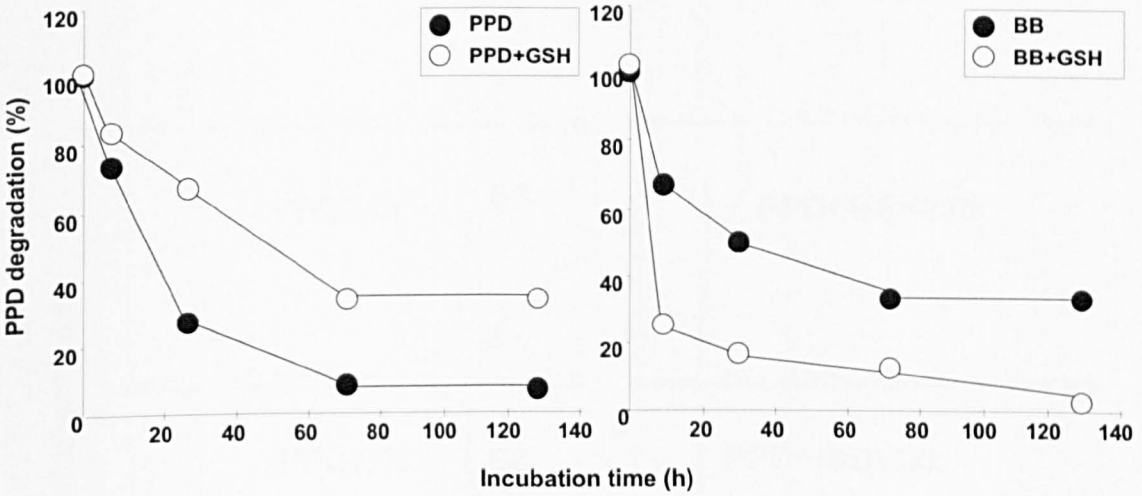


Figure 2.11. Rate of disappearance of a) PPD and b) BB in the presence and absence of glutathione. [^{14}C]PPD/BB (50 mM [0.2 mCi] ± glutathione [1 mM]) was incubated lymphocytes (1.5 x 10^6;total volume 1 ml) in cell culture medium at 37 °C for 144h (duration of the lymphocyte transformation test). At 0, 4, 24, 48, 72 and 130h, 100 ml aliquots were taken and analyzed by radiometric HPLC.

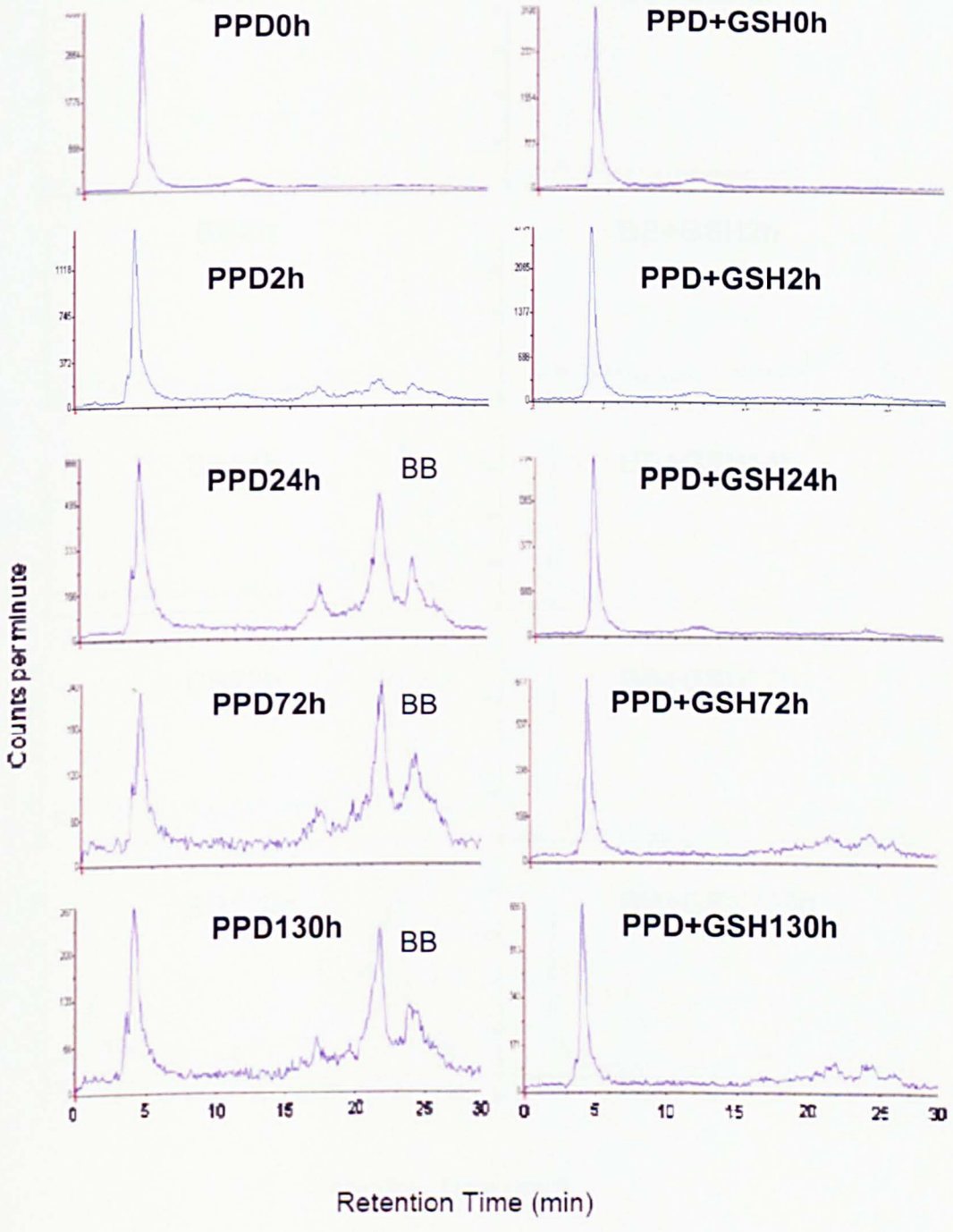


Figure 2.12. HPLC radio-chromatographs showing the degradation of PPD. [^{14}C]PPD (50 mM [0.2 mCi]; ± glutathione [1 mM]) was incubated with lymphocytes (1.5 x 10^6; total volume 1 ml) in cell culture medium (37°C, 5% CO_2) for the duration of the LTT. At 0, 2, 24, 48, 72 and 130h, 100 µl aliquots of supernatant were taken and analyzed by radiometric HPLC. A 24-h incubation yielded significant quantities of BB whereas PPD was the only detectable radiolabelled peak, in the presence of glutathione at the same time point.

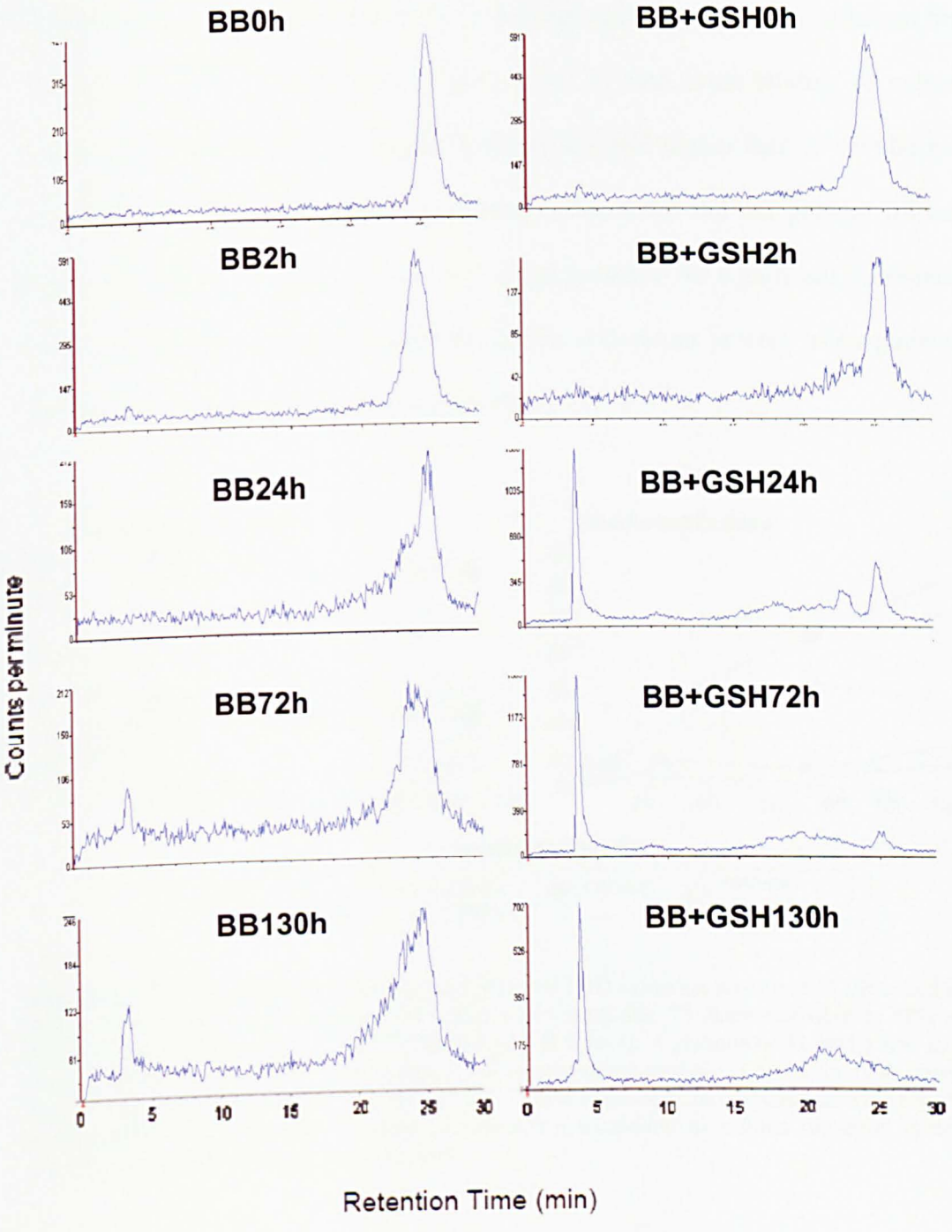


Figure 2.13. HPLC radio-chromatographs showing the degradation of BB. [^{14}C]PPD (50 mM [0.2 mCi]; ± glutathione [1 mM]) was incubated with lymphocytes (1.5 x 10^6; total volume 1 ml) in cell culture medium (37°C, 5% CO_2) for the duration of the LTT. At 0, 2, 24, 48, 72 and 130h, 100 µl aliquots of supernatant were taken and analyzed by radiometric HPLC. BB is relatively stable for the 130h incubation, whereas GSH appears to aid degradation process to an unidentified compound

PPD and BB conjugated irreversibly to cellular and serum proteins, although binding of BB occurred at a lower rate (Figure 2.14). In both cases binding of radioactive material to serum protein was approximately 2-3 fold higher than to cellular protein. Addition of glutathione to the incubations lowered but did not prevent irreversible binding of PPD and BB to cellular and serum proteins. No significant difference was observed between binding of PPD to cellular and serum protein when patients and volunteers were compared (data not shown).

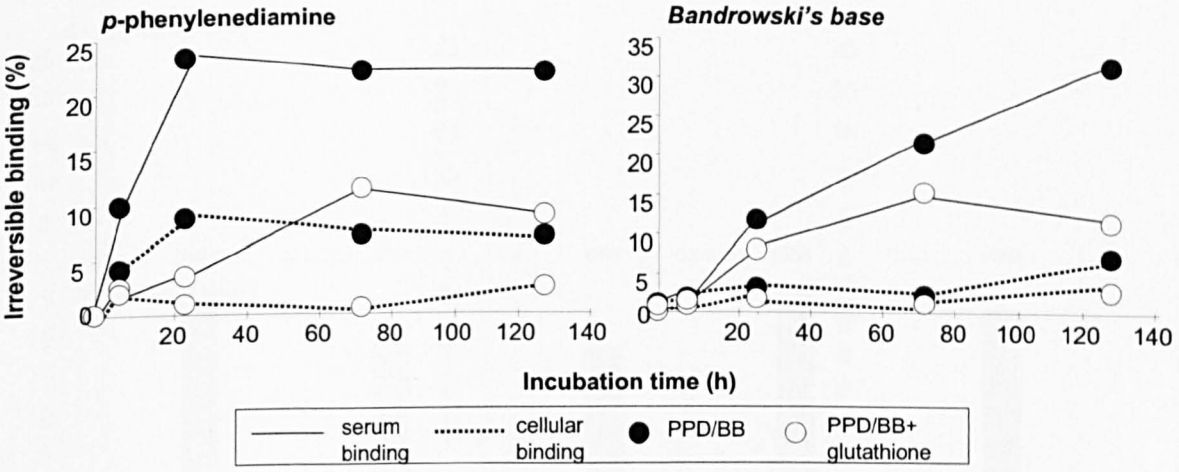


Figure 2.14. Comparison of the irreversible binding of PPD oxidation products and BB to cellular and extracellular protein in the presence and absence of glutathione. To determine whether PPD and BB binds covalently to protein, [^{14}C]PPD/BB (50 mM [0.2 mCi]; ± glutathione [1 mM]) was incubated with lymphocytes (1.5 x 10^6; total volume 1 ml) in cell culture medium at 37 °C for 144h (duration of the lymphocyte transformation test). At 0, 2, 24, 72 and 130h, cells and supernatant were separated by centrifugation and irreversible binding of radioactive compound to cellular or serum protein was determined by exhaustive solvent extraction.

2.3.3. Patient lymphocytes are specifically stimulated with PPD

To evaluate whether patient cells are specifically stimulated with PPD, lymphocytes were incubated with PPD for 4 and 16h in the presence or absence, of glutathione. Cells were washed to remove unbound PPD and PPD degradation products and suspended in fresh medium for the remainder of the incubation. Importantly, in the

presence of glutathione, lymphocytes were not exposed to BB. Incubation of lymphocytes with PPD (+/- glutathione [1 mM]) for 4h did not stimulate a significant proliferative response. In contrast, 16h incubation with PPD was associated with significant proliferation (figure 2.15). The levels of PPD-specific proliferation in the presence and absence of glutathione were comparable (i.e. glutathione did not inhibit the response).

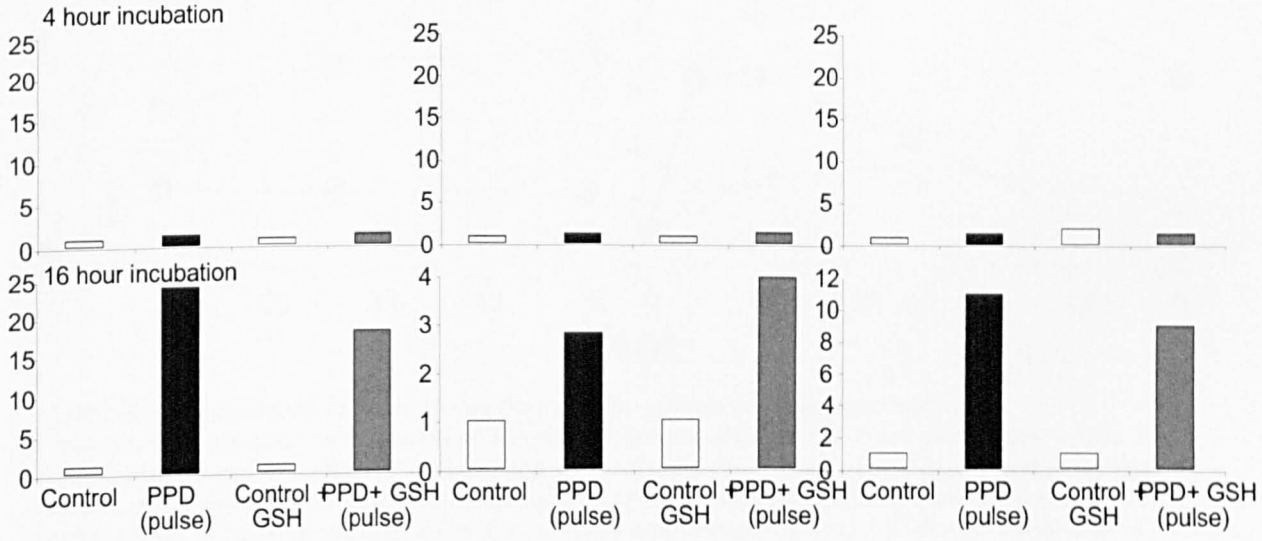


Figure 2.15 Proliferation of lymphocytes from 3 allergic patients pulsed with PPD in the presence and absence of glutathione for 4 h and 16 h. To evaluate whether patient cells are specifically stimulated with PPD, lymphocytes were incubated with PPD for 4 and 16h in the presence (to ensure no exposure to BB) or absence of glutathione. Cells were washed to remove unbound PPD and PPD degradation products and suspended in fresh medium for the remainder of the incubation. No significant proliferation was detected after the 4h incubation; in contrast the 16h incubation with PPD was associated with significant proliferation with comparable levels of PPD-specific proliferation in the presence and absence of glutathione.

2.3.4. Generation of antigen-specific T-cell clones from patients and volunteers and analysis of cytokine / chemokine secretion

Following limiting dilution and clonal expansion, low numbers (less than 1 in 50 seed

T-cells from polyclonal cultures) of BB-specific CD4+ T-cell clones were generated from allergic patients (no BB: 2333.1 cpm; 5μM: 5993.9 cpm; 10μM: 6075.4 cpm; n=30) and volunteers (no BB: 2286.4 cpm; 5μM: 10708.8 cpm; 10μM: 8608.0 cpm; n=44). Proliferation of T-cell clones following BB stimulation was concentration dependent for both patients and volunteers (figure 2.14a; n = 3 clones); however no specific stimulation was observed with PPD.

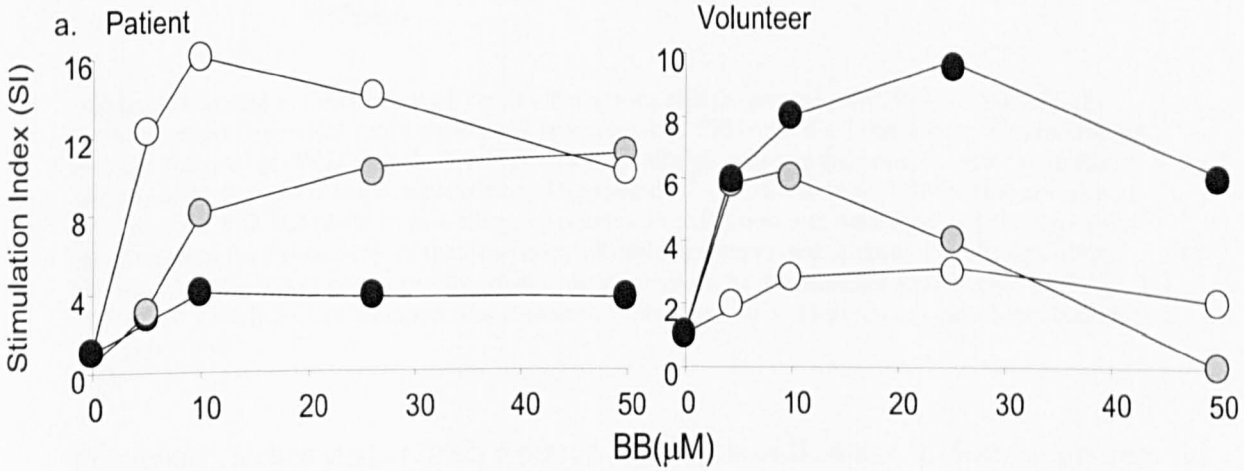


Figure 2.16a. Stimulation of T-cell clones from allergic patients and volunteers with BB. Concentration-dependent proliferation of 3 representative and BB-specific T-cell clones derive from 2 allergic patients and 2 volunteers. Proliferation was measured by [3H] thymidine incorporation for the last 16h of the experiment. Results are expressed as cpm of triplicate culture. Statistical analysis was performed by comparing incubations in the presence and absence of drug (*P<0.05). Coefficient of variation was consistently less than 20%. The cpm in control incubations was less than 1500.

PPD-specific CD4+ T-cell clones were also generated, but only using lymphocytes from allergic patients (0: 2333.1 cpm; 5μM: 5993.9 cpm; 10μM: 6075.4 cpm; n=30) (figure 2.15b.). PPD-specific clones proliferated only in the presence of PPD (i.e. no response was seen with BB; figure 2.16c.), providing conclusive evidence that T-cells from allergic patients are stimulated with PPD or a PPD degradation product.

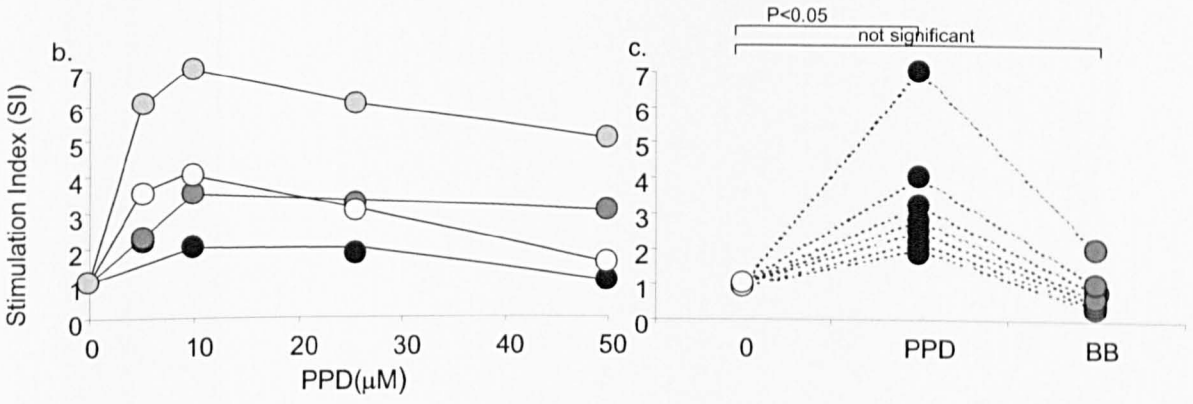


Figure 2.16b and c. Stimulation of T-cell clones from allergic patients with PPD and/or BB. (b) Concentration-dependent proliferation of 4 representative PPD-specific T-cell clones derived from 4 allergic patients. (c) PPD specific T-cell clones from allergic patients did not cross react with BB. Graph shows the maximum stimulation of PDD-specific T-cell clones with PPD and the absence of proliferation with BB (n=8; from 4 allergic patients). Proliferation was measured by [3H] thymidine incorporation for the last 16h of the experiment. Results are expressed as cpm of triplicate culture. Statistical analysis was performed by comparing incubations in the presence and absence of drug (*P<0.05). Coefficient of variation was consistently less than 20%. The cpm in control incubations was less than 1500.

Previously, Sieben et al., (2002) reported high levels of IL-4 and IL-5 secretion from PPD or BB stimulated T-cells from allergic patients. To obtain a global analysis of cytokine / chemokine secretion and to explore possible difference between patient and volunteer clones, cytokine levels were measured in supernatants following antigen-stimulation, using Luminex technology. The mean standard curve for two representative cytokines, IL-4 and TNF-α, is shown in figure 2.17.

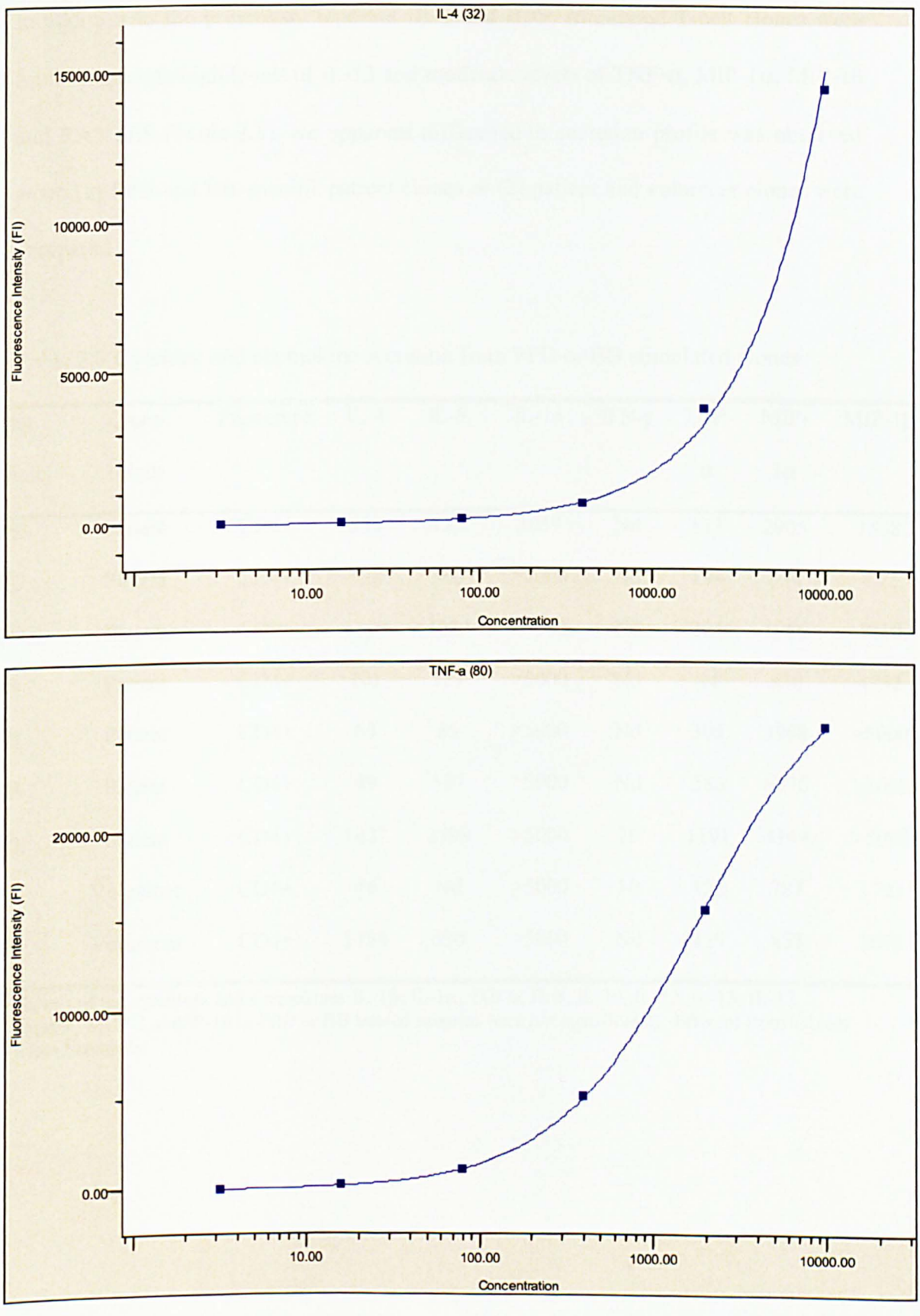


Figure 2.17. Standard curve of IL-4 (top) and TNFα (bottom)

In addition to the previously reported IL-4 and IL-5, stimulated T-cell clones were found to secrete high levels of IL-13 and moderate levels of TNF-α, MIP-1α, MIP-1β and RANTES (Table 2.3). No apparent difference in secretion profile was observed when (1) PPD and BB-specific patient clones or (2) patient and volunteer clones were compared.

Table 2.3 Cytokine and chemokine secretion from PPD or BB stimulated clones

Clone specificity	Clone origin	Phenotype	IL-4	IL-5	IL-13	IFN-γ	TNF-α	MIP-1α	MIP-1β	RANTES
PPD	Patient	CD4+	535	125	3059	Nd	113	2905	1538	316
PPD	Patient	CD4+	528	360	>5000	Nd	194	634	452	221
PPD	Patient	CD4+	1129	4023	>5000	Nd	224	1986	3210	3288
BB	Patient	CD4+	Nd	189	>5000	Nd	44	838	4752	213
BB	Patient	CD4+	63	85	>5000	Nd	305	3968	>5000	1526
BB	Patient	CD4+	49	597	>5000	Nd	586	6376	>5000	2574
BB	Patient	CD4+	1427	2598	>5000	20	1191	4149	>5000	1561
BB	Volunteer	CD4+	46	Nd	>5000	10	122	787	1792	2697
BB	Volunteer	CD4+	1754	630	>5000	Nd	129	853	1078	740

*levels of the cytokines and chemokines IL-1β, IL-1α, TGFα, IL-8, IL-10, IL-12, IL-15, IL-17, eotaxin, MCP-1 and IP-10 in PPD or BB treated samples were not significantly different from solvent treated controls.

2.4 Discussion

Topical application of low concentrations of the PPD oxidation product BB to mice has been associated with increased proliferation of draining lymph node cells (White *et al.*, 2006; Farrell *et al.*, 2008). Similarly in PPD-allergic patients BB has also been shown to stimulate *ex vivo* lymphocyte proliferation (Krasteva *et al.*, 1993; Sieben *et al.*, 2002). Furthermore, in the present *in vitro* investigation T-cells from allergic patients have been found to be BB-specific. However, in contrast with previous investigations of T-cell responses to PPD and its oxidation products (Krasteva *et al.*, 1993; Sieben *et al.*, 2002), it was shown that BB can stimulate a proliferative response to lymphocytes from volunteers (14/16), with or without known hair dye exposure (figure 2.6). 2/16 volunteers did not respond to BB, despite repeated testing, even though these cells were responsive to the protein antigen (tetanus toxoid 5μg/ml; Figure 2.7). This suggests that there is an immunogenic threshold of BB exposure that must be crossed to stimulate a proliferative response in healthy volunteers

It is possible that the reason why volunteer lymphocytes respond to non-toxic concentrations of BB could be attributed to the fact that PPD is found in dark blue and black clothing dyes, so virtually everyone is exposed to PPD and thus acquire sub-clinical sensitisation over time. Importantly, a second independent research group in Southampton have also detected BB-specific stimulation of volunteer lymphocytes, using the same cell culture methodologies. Work performed in their laboratory with cord blood samples has also revealed that incubation with BB failed to stimulate a response in these cells (Figure 2.10). These findings have not only strengthened the results of this investigation by validating the reproducibility of the data, but also added weight to the hypothesis that BB sensitisation could be acquired due to

environmental exposure and indicate that BB-specific lymphocyte stimulation is dependent on prior exposure.

These data clearly indicate that BB is can stimulate T-cells from both PPD-allergic patients and volunteers. Ex-vivo stimulation with BB is seen even when an individual had never dyed their hair (Figure 2.9). However, the relationship between T-cell stimulation with BB and development of an allergic reaction is ill defined. It is possible that BB stimulates a T-cell response in allergic patients, while volunteer cells might express a more regulatory T-cell phenotype. To explore whether the observed lymphocyte response to BB represents a true antigen specific response, T-cell clones were generated from both responsive allergic patients and volunteer lymphocyte cultures. Low numbers of CD4+ BB-specific clones were generated (Figure 2.16a), supporting the theory of an antigen-specific pathway for lymphocyte stimulation. These data are comparable with the findings of Cavani *et al.*, (1998;2003) showing firstly the presence of nickel specific CD8+ T-cell clones in allergic patients alone and secondly nickel-specific CD4+ T-cell clones in non-allergic individuals displaying regulatory T cell markers and secreting low levels of the inflammatory cytokine IFN-γ and/or higher levels of IL-10 production compared with T-cell clones from allergic patients. However recent evidence has implied that the ability of certain individuals to evade a cytotoxic proliferative response to nickel is attributed to oral tolerance that can block the development of an inflammatory response mediated by CD8+ T-cells (Schwarz *et al.*, 2004; Cavani *et al.*, 2005). Similarly in mouse models single intra-gastric administration of hapten was able to block *in vitro* induction of 2,4-ditroflurobenzene (DNFB)-specific CD8+ CTL cells responsible for tissue inflammation, and that a subset of regulatory CD4+ T-cells (IL-10 producing

CD25+CD4+ T-cells) mediate oral tolerance by inhibiting expansion of specific CD8+ effector T-cells in lymph nodes (Desvignes *et al.*, 2000). Therefore further investigations are required to identify whether this phenomenon occurs with non-allergic PPD individuals.

Recently White *et al.*, (2006) have shown that only 16% of PPD patch test positive patients produce a weak positive patch test response when exposed to gradually increasing topical concentrations of BB, suggesting that BB-specific T-cells may not actually play a pathogenic role in PPD allergy following exposure to hair dye. In support of this hypothesis, lymphocytes from allergic patients but not volunteers were found to be additionally stimulated with PPD (Figures 2.5,2.6). However, due to the instability of PPD it was important to develop a system to assess whether these data represent a true PPD response or are the cells stimulated by one of its oxidation products i.e.: BB. Initial experiments were designed to monitor the stability, *in vitro* distribution and irreversible binding of PPD and BB to cells and serum (Figure 2.14) PPD was found to degrade rapidly in culture (Figure 2.11) where as BB was more stable over the 130h time period. Degradation of PPD was associated with detection of unbound BB from 24h onwards. PPD-derived material and BB both associated irreversibly with cellular and serum protein, however binding of BB occurred at a much lower rate. This could explain the reason why a weaker patch test is seen to BB as the threshold needed for it to bind to protein and become immunogenic would occur much more slowly. In further experiments glutathione, which has previously been shown to prevent BB formation (Coulter et *al.*, 2007), was included in a subset of experiments. This study confirmed previous data that the presence of GSH in PPD-containing incubations prevented the formation of BB (Figure 2.12). Interestingly

GSH increased the rate of degradation of BB. The only radiolabelled product produced was highly polar and could not be associated confidently with a putative parent ion with available mass spectrometric data (Figure 2.13). In both cases presence of glutathione decreased but did not prevent irreversible binding of PPD and BB to protein. A comparison of binding of PPD to cells and serum from allergic patient and volunteer lymphocytes was also carried out to see if PPD is more readily oxidised in the patients. No significant difference was observed in the quantity of PPD irreversibly bound to protein when patients and volunteers were compared, therefore differences in cellular distribution of PPD does not account for the diverse T-cell response in patients and volunteers. To confirm that patient lymphocytes are specifically stimulated with PPD, the cells were pulsed with PPD and glutathione for 4 and 16h. A proliferative response was seen at 16 hours when compared to vehicle control treated cells (Figure 2.15) and was of similar strength to that seen with PPD alone. This data provides evidence of a discrimination between the patient and volunteer groups, showing that only T-cells from allergic patients are specifically stimulated with PPD. This was further confirmed by the generation of PPD-specific T-cell clones from patients, but not volunteers (Figure 2.16b,c). The mechanism by which PPD can stimulate T-cell proliferation has not been fully elucidated. Sieben *et al* (2002) suggested that PPD can be recognised by T-cells through a processing independent pathway, as antigen presenting cells pulsed with PPD for 4h failed to stimulate proliferation in clones. The authors hypothesise that PPD could bind directly to MHC and/or the T-cell receptor. This study has also shown that incubation of PPD with lymphocytes for 4h fails to stimulate proliferation in lymphocytes, however a longer incubation of 16h was associated with a PPD-specific proliferative response. Importantly 16h is the time need for 1) maximal irreversible binding of PPD to

cellular and serum proteins and 2) antigen processing which indicates that PPD associated covalently with protein may be an antigenic determinant. This possibility is discussed in greater detail in chapters 3 and 4.

Contact dermatitis elicited by exposure to chemicals such as PPD has always been thought to be instigated by IFN-γ producing T-cells. However, it has recently been postulated that IL-4 producing T-cells might play an important role in the development of tissue pathology (Salerno *et al.,* 1995) in certain types of cutaneous allergic reactions. In support of this hypothesis, high levels of IL-4 and related cytokine IL-13 are detected in skin biopsy samples from allergic patients (Asherson *et al.*, 1996; Neis *et al.*, 2006). A murine model of PPD-induced contact sensitisation found Th2 cytokines, IgE antibodies and mast cells are crucial in the development of tissue pathology (Yokozeki *et* al., 2003), while Sieben *et al.,* (2002) reported high levels of IL-4 and IL-5 secretion by PPD and BB stimulated clones from hair dye allergic patients. Herein, similar data is presented with allergic patient clones stimulated with PPD and BB. Furthermore a similar pattern of cytokine secretion was detected from BB stimulated T-cell clones from volunteers (Table 2.3.). In line with the histological observations described above, patient and volunteer clones were also found to secrete high levels of IL-13. As Luminex technology permitted a more global analysis of cytokine / chemokine secretion, stimulated T-cell clones were shown to secrete the cytokine TNFα, which might relate to the inflammatory symptoms associated with contact dermatitis, and the CCR5 ligands MIP-1α, MIP-1β and RANTES. Expression of these chemokines has been shown to be increased in the presence of TNF-α and IL-13, while increased expression is also associated with various immunological diseases including rheumatoid arthritis and systemic lupus

erythematous (Vila et al., 2006; Torikai et al., 2007); however the role of MIP-1α, MIP-1β and RANTES in contact dermatitis warrants further investigation.

In conclusion, data presented clearly show that patient and most volunteer lymphocytes are stimulated with BB. However, PPD provides an additional chemical signal to specifically stimulate T-cells from allergic patients. These data represent an important laboratory-based discrimination between allergic and non-allergic groups.

CHAPTER 3

A mechanistic investigation into the antigenicity and irreversible binding of *p*-phenylenediamine and structurally related compounds

3.1 Introduction

p-Phenylenediamine (PPD), is part of a large group of chemicals used in textile products such as printing ink, photo development, plastics and rubber (Chung *et al.*, 2004). More commonly PPD is used as an intermediate in permanent hair dye formulations (Corbett *et al.*, 1973) and recently in henna dyes (Le Coz *et al.*, 2000; Mautlich & Sullivan 2005). PPD is classed as a strong sensitizer, based on murine local lymph node assay data (Warbrick *et al.*, 1999; White *et al.*, 2006), and on human exposure causes T-cell-mediated severe allergic contact dermatitis (ACD; White *et al.*, 2006; Sieben *et al.*, 2002; Blomeke *et al.*, 2008). Reports of PPD-associated ACD have increased over recent years (McFadden *et al.*, 2007) and due to the broad-spectrum use of PPD-containing products; exposure in everyday life is high.

In solution and on contact with skin it is susceptible to sequential oxidation and self-conjugation reactions resulting in the formation of an unstable primary quinonediimine intermediate, products of dimerization and trimerization, and a re-arrangement product of the trimer known as Bandrowski's base (Picardo *et* al., 1990; Coulter *et* al., 2006). However it has never been confirmed whether PPD or an oxidation product is responsible for PPD-associated ACD, and previous results have given conflicting evidence (Krasteva *et al.*, 1993; Sieben *et al.*, 2002). In chapter 2 we have demonstrated that cells from both patients and volunteers will proliferate in the presence of BB but only patients cells respond to PPD. As these data suggest that PPD may trigger T cell activation upon exposure our aim was to investigate the mechanism of PPD-specific adduct formation.

As we know a variety of factors including skin penetration, cell stress, cytotoxicity, dendritic and T-cell activation determine the sensitizing potential of low molecular weight chemicals such as PPD. Despite this the formation of a protein adduct is the

primary chemical factor required to initiate the response (Landsteiner & Jacobs 1935; Sanderson *et al.*, 2007). Subsequent uptake and processing of modified protein by antigen presenting cells, such as Langerhans cells, results in immune priming and programming. The electrophilicity and therefore protein reactivity of a chemical is thought to closely relate to its ability to promote an immune reaction (Ashby *et al.*, 1995; Basketter *et al.*, 1988) and indeed, protein reactivity models based on cysteine and lysine binding interactions are currently considered useful screening approaches for skin sensitization testing (Aleksic *et al.*, 2009; Gerberick *et al.*, 2004; 2007; Natsch *et al.*, 2007:2008). Despite this, little is known about the nature of compound-specific protein modifications and how this relates to the initiation of an immune response. The scarcity of data in this respect is largely due to the number of potential target proteins for reactive chemicals (2000 have been separated from skin, Divkovic *et al.*, 2005; Celis *et al.*, 1998), the abundance of amino acid binding sites on any given protein candidate and the accessibility of binding sites for reactive chemicals. It is possible that sensitising potential of a compound relates to its chemical reactivity towards a few critical amino acids. In this respect p-benzoquinonediimines (first oxidation derivatives of allergic p-amino aromatic compounds) have been shown to react with nucleophilic amino acid residues through a set of complex mechanisms (Eilstein *et al.*, 2006; Eilstein *et al.*, 2007), indicating a potential route to protein haptenation. Furthermore, Eilstein et al (2008), have shown the selectivity of 2,5-dimethyl-p-benzoquinonediimine binding to lysine in a model peptide containing potentially reactive amino acids (except cysteine). 2,5-dimethyl-p-benzoquinonediimine reacts with lysine via Schiff base formation and an oxido-reduction process giving rise to an aldehyde intermediate and peptide cyclization.

Data presented in chapter 2 demonstrates that protein irreversibly modified with oxidation products of PPD provides an antigenic signal to stimulate T-cells from allergic patients however, the fact the nature of the PPD-protein binding interaction has not been characterized. Therefore the objective of this chapter was to utilize state-of-the-art mass spectrometric methods to define the amino acid binding site(s) and nature of binding interaction when PPD and structurally-related compounds (Figure 3.1) were incubated with model peptides and the cellular protein glutathione-*S*-transferase Pi (GSTP), which is particularly suitable as a target protein as it is an abundant cytosolic protein highly expressed in both liver and skin (Jenkins *et al.*, 2008).

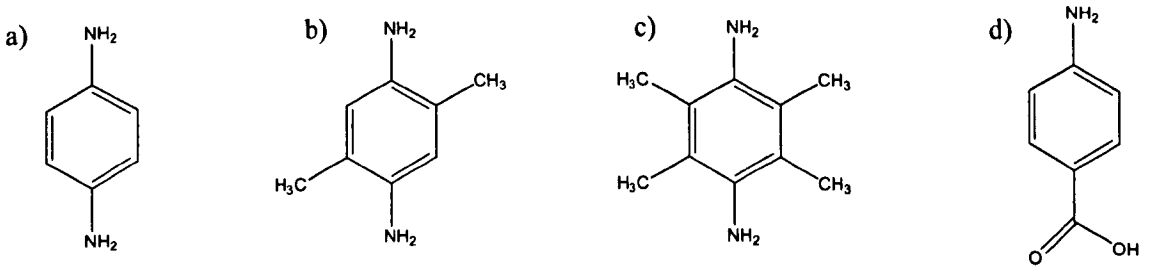


Figure 3.1 Structure of a) p-phenylenediamine b) 2,5-dimethyl-p-phenylenediamine c) 2,3,5,6-tetramethyl-p-phenylenediamine d) 4-aminobenzoic acid; added as a negative control which should not associate covalently with protein

Further experiments were conducted using lymphocytes from PPD allergic patients to explore the relationship between protein binding and T-cell reactivity.

3.2 Methods

3.2.1 Materials

PPD, 2,5-Dimethyl-1,4-phenylenediamine, 2,3,5,6-Tetramethyl-*p*-phenylenediamine, 4-Aminobenzoic acid were obtained from Sigma Chemical Co (Poole, Dorest, UK). [^{14}C]PPD (specific activity 60 mCi/mmol) was synthesized by GE Healthcare (Bucks, UK).

Peptides (AcFAACAA, AcFAAHAA, FAAAAA & AcFAAAAA, AcFAAKAA AcFAARAA, AcFAAYAA & AcFAGAGA) and DS3 peptide (VLSPADKTNWGHEY RMFCQIG) were kindly donated by Unilever.

3.2.2 Generation of His-tagged Glutathione-S-Transferase pi

Glutathione-*S*-transferase pi constitutes an ideal protein for studies of adduct formation by low molecular weight chemicals for the following reasons: it is relatively small (23.5 kDa) and therefore its analysis is straightforward; it contains only four cysteine residues so that modification at sulphydryl groups results in few changes to the mass spectral profile; and, mass spectrometric methods for the analysis are available and have been described recently (Jenkins *et al.*, 2008).

The cDNA for human GSTP was cloned into the vector pET-15b (Novagen) and expressed as previously described (Chang *et al.*, 1999). Briefly, transformed BL21 cells were grown under carbenicillin selection, induced by the addition of 2 mM IPTG, and the proteins were released by sonication using a Soniprep 150 (MSE, London, UK). The supernatant was recovered following centrifugation (13000g) at 4°C for 10 mins. His-Select nickel affinity gel (Sigma) was used to isolate His-GSTP from bacterial lysate. The gel was washed with 5 changes of phosphate buffer, and

100 µL beads were incubated with 2 mg lysate for 10 mins at room temperature with agitation. The beads were washed with 5 x 1 mL phosphate buffer. On-bead BCA assays (Pierce, Rockford, IL, USA) to determine protein concentration were performed as described (Stich 1990).

3.2.3 Detection of nucleophilic peptides following incubation with p-phenylenediamine and related compounds (peptide depletion assay)

Peptide stock solutions (AcFAACAA, AcFAAHAA, FAAAAA & AcFAAAAA; pH 10- AcFAAKAA AcFAARAA & AcFAAYAA) were prepared to a final concentration of 2.5mM in degassed ultra pure water. Test chemicals were prepared in acetonitrile (ACN) at a concentration of 125 mM. Triplicate incubations in 96 well tissue culture plates contained peptide (2.5 mM, 50 µl; final concentration 0.5 mM), chemical (125 mM, 100 µl; final concentration 50 mM), phosphate buffer solution (90 µl; pH 7.4) and an internal standard (AcFAGAGA; (2.5 mM, 10 µl; final concentration 0.5 mM). Samples without chemical were used as a control. Calibration standards were prepared manually from peptide stock solution at final well concentrations of 0.025, 0.05, 0.08, 0.1, 0.2, 0.3, 0.4 & 0.5mM. Plates were capped and incubated at room temperature in darkness for 24 h.

Samples and calibration standards were analysed using an Agilent (Cheshire, UK) 1100 series HPLC and Waters (Milford, MA) Quattro Micro API tandem quadrupole MS using multiple reaction monitoring in electrospray positive mode. Samples were enclosed in the autosampler at 4°C and 2µL aliquots were injected onto a Gemini C18, 150 x 2mm, 3µl column (Phenomenex, Cheshire, UK) at a 0.2mL/min flowrate. For peptides AcFAAKAA, AcFAAHAA and AcFAARAA 93% mobile phase A (0.05% formic acid in ultrapure water) and 7% mobile phase B (0.05% formic acid in ACN)

was initially held for 0.5 minutes before the percentage of B was increased to 90% over 7 minutes before being decreased back to 7% B for a run time of 12 minutes. For peptides AcFAACAA, and AcFAAYAA 90% mobile phase A and 7% mobile phase B was initially held for 1 minutes before the percentage of B was increased to 90% over 7.5 minutes before being decreased back to 10% B for a run time of 13 minutes. Peptide reactivity with the test chemicals is reported as percent peptide depletion, which was determined as the reduction of the peptide concentration in the samples relative to the average concentration of the controls.

3.2.4 Characterization of specific amino acid modifications of the peptide DS3 by p-phenylenediamine and related compounds

DS3 peptide solutions were prepared to a final concentration of 80 µM in ammonium acetate (50mM). PPD, 2,5-Dimethyl-1,4-phenylenediamine, 2,3,5,6-Tetramethyl-*p*-phenylenediamine or 4-Aminobenzoic acid (800 µM) were prepared in a solution of ACN / ammonium acetate (50 mM; 50:50 v/v), and mixed with the DS3 peptide (peptide : chemical ratio 1:10). Control of DS3 peptide was prepared without chemical in a mixture of ACN and ammonium acetate (50 mM; 25:75 (v/v). Samples were incubated the dark at room temperature for 24 hours. Prior to analysis unbound material was removed using SepPak C18 cartridges (Millipore; Watford, Hertforshire). Samples were reconstituted in 50mM ammonium acetate containing trypsin (20:1 ratio trypsin to protein) and digested overnight before being reconstituted in 5µl ACN (5 % v/v) / trifluoroacetic acid (0.1 % v/v)

3.2.5 Characterization of the time-dependent irreversible binding of p-phenylenediamine to the peptide DS3

DS3 peptide (40 μM) was incubated with PPD (400 μM; 1% DMSO) for 72 h in phosphate buffer (pH 7.4; 100 μL). After 2, 16, 48 and 72 h, a 10 μL aliquot was taken, unbound material removed using C18 ZipTip columns according to the manufacturer's instructions (Millipore; Watford, Hertfordshire), and reconstituted in an aqueous solution containing ACN (5 % v/v) / trifluoroacetic acid (0.1 % v/v); 10μL final volume.

Aliquots of sample (0.5 μL) were spotted onto a target plate together with an equal volume of α-cyano-4-hydroxycinnamic acid (10 mg/mL; LaserBiolabs, CITY, France) in ACN (50 %) / trifluoroacetic acid (0.1%) for matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS). Peptide mass fingerprints were acquired on a Voyager DE Pro MALDI instrument (Applied Biosystems, California, USA) in positive ion reflector mode.

To test reversibility of PPD binding, glutathione (GSH) was included in certain experiments. The peptide DS3 (40μM) was incubated for 16 hours alone, in the presence of PPD (400μM) only, GSH (1mM) only, and PPD (400μM) and GSH (1mM) simultaneously. Peptide samples were analysed on a Voyager De Pro MALDI Instrument as described previously.

3.2.6 Characterization of specific amino acid modifications of the protein glutathione-S-transferase Pi by p-phenylenediamine and related compounds

His-GSTP captured on nickel beads was eluted with an excess of histidine (200mM). To evaluate whether PPD binds irreversibly to the protein, [^{14}C] PPD (400 μM [1 μCi]; ± GSH [1mM]) was incubated with His-GSTP (40μM; peptide: chemical ratio

1:10) for 16 hours. On completion of the incubation period, unbound PPD was removed by exhaustive solvent extraction and protein was precipitated by the addition of ACN (100 µL). An aliquot containing 20 µg of total protein was taken and added to an equal volume of reducing sample buffer. Samples were then heated at 95 °C for 10 min and the proteins were separated by electrophoresis on SDS-polyacrylamide gels using the discontinuous buffer system described by Laemmli (1970). Bands were then excised and digested. Briefly the gel was cut into $0.5cm^3$ sections and digested with soluene at 50°C for 1 hour before the addition of 30µL glacial acetic acid. The level of [^{14}C] PPD incorporated into each band was then determined by liquid scintillation counting.

Specific amino acid modification of His-GSTP by PPD, 2,5-Dimethyl-1,4-phenylenediamine, 2,3,5,6-Tetramethyl-*p*-phenylenediamine or 4-Aminobenzoic acid was analyzed following incubation of nickel bead-bound His-GSTP (40µM) suspended in phosphate buffer (13.08 mM KH_2PO_4, 62.27 mM Na_2HPO_4, pH7.4) with the compounds (400µM; DMSO [1 %]; peptide: chemical ratio 1:10), or DMSO alone. Samples were incubated for 16 h at room temperature before washing (5 x 1 mL changes of phosphate buffer and 2 x 500µL of 50mM ammonium bicarbonate buffer). Modified protein was then digested on-bead by suspension in 30 µl ammonium bicarbonate buffer with 20 ng trypsin and incubation for 16 h at 37 °C. The samples were desalted using C18 ZipTip cartridges (Millipore; Watford, Hertfordshire), and reconstituted in 5µL (acetonitrile [5 % / trifluoroacetic acid [0.1%]) for mass spectrometric analysis.

3.2.7 Mass Spectrometry

For LC-MS/MS analysis of DS3 and His-GSTP samples, aliquots of 1μL were delivered into a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems) by automated in-line liquid chromatography (integrated LCPackings System, 5mm C18 nano-precolumn and 75μm x 15cm C18 PepMap column (Dionex, California, USA)) via a nano-electrospray source head and 10μm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 5% ACN/0.05% TFA (v/v) to 48% ACN/0.05% TFA (v/v) in 60mins was applied at a flow rate of 300nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA) (Analyst, Applied Biosystems). Database searching was performed using ProteinPilot version 2 (Applied Biosystems) against the latest version of the SwissProt database, with the confidence level set at 90%, and with p-phenylenediamine included as a high probability user-defined modification on cysteine.

3.2.8 Donor Characteristics

Peripheral blood mononuclear cells were isolated from venous blood (as described in Chapter 2) obtained from 10 PPD allergic, patch test positive patients and 10 healthy volunteers. Approval for the study was obtained from Liverpool local research ethics committee; informed written consent was obtained from all donors. Clinical characteristics of the allergic patients and volunteers are shown in Table 3.1.

Table 3.1. Clinical details from PPD allergic patients

	Age	Sex	Dye use	PPD allergy	Other allergies	Months since reaction	Patch test
1	37	F	Yes	Yes	Nickel, cobalt , nail polish, pollen, latex, benzoic acid, benzyl peroxide	252	+
2	43	F	Yes	Yes	-	17	+
3	77	F	Yes	Yes	-	18	+
4	51	F	Yes	Yes	Hay fever	24	+
5	33	F	Yes	Yes	nickel, colourings & preservatives	22	+
6	68	F	Yes	Yes	Nickel, copper, latex	44	+
7	52	F	Yes	Yes	E45 cream, orange colouring, biological soap	14	+
8	70	F	Yes	Yes	Penicillin, make up, creams, nickel, leather, tea tree oil, fragrances	11	+
9	56	F	Yes	Yes	Nickel, perfumes	22	+
10	43	F	Yes	Yes	Local anaesthetics, Nickel, sunglasses & clothes dye.	96	+

3.2.9 Determination of lymphocyte proliferation

Proliferation of lymphocytes from PPD allergic (n = 10) patients was measured using the lymphocyte transformation test, as described previously (See section 2.2.3 & 2.2.5). Lymphocytes were cultured with PPD, 2,5-dimethyl-1,4-phenylenediamine, 2,3,5,6-tetramethyl-*p*-phenylenediamine or 4-aminobenzoic acid (final concentrations ranged from 0.1-20 μM); absence of the chemicals was taken as a negative control.

3.2.10 Statistical analysis

The Mann-Whitney test was used for comparison of control and test values, accepting $P < 0.05$ as significant.

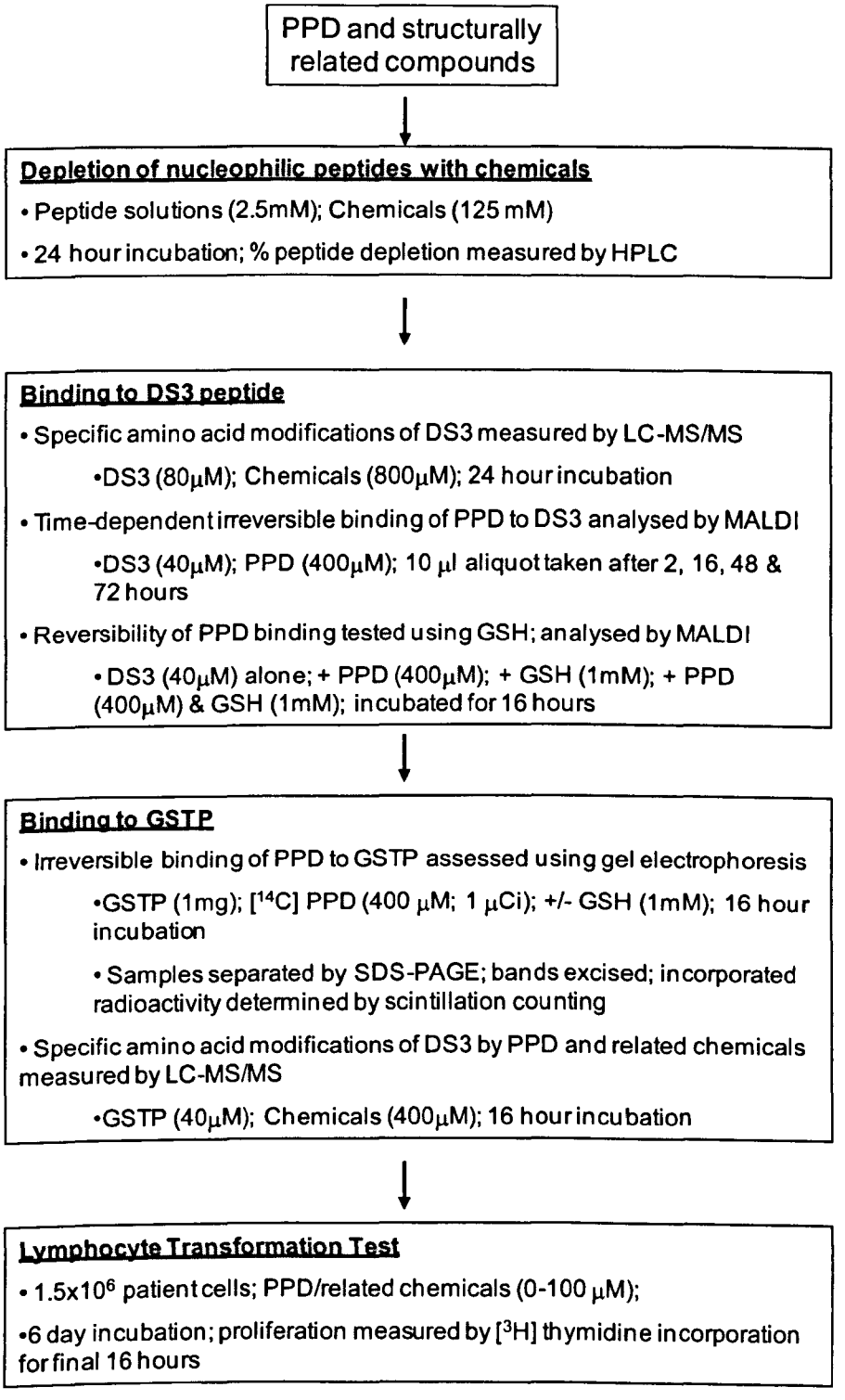


Figure3.2. Overview of methods used to assess irreversible binding

3.3 Results

3.3.1 Reactivity of p-phenylenediamine and related compounds with synthetic peptides

To explore selectivity of the amino acid binding, reactivity of PPD, 2,5-dimethyl-1,4-phenylenediamine, 2,3,5,6-tetramethyl-*p*-phenylenediamine and 4-aminobenzoic acid towards synthetic nucleophilic peptides was assessed using previously described optimal conditions for time (24 h) and chemical:peptide ratio (Gerberick *et al.*, 2004). Three of the four compounds tested, namely PPD, 2,5-dimethyl-1,4-phenylenediamine and 2,3,5,6-tetramethyl-*p*-phenylenediamine caused greater than 90% depletion of both cysteine- and lysine-containing peptides. None of the chemicals depleted peptides containing histidine, arginine and tyrosine to below 85 % of control levels. 4-aminobenzoic acid peptide treatment was not associated with peptide depletion (Table 3.2).

Table 3.2 % depletion of cysteine, lysine, histidine, arginine and tyrosine peptides after incubation with p-phenylenediamine, 2,5-dimethyl-p-phenylenediamine, 2,3,5,6-tetramethyl-p- phenylenediamine and 4-amino benzoic acid

	Peptide				
	AcFAACAA	AcFAAKAA	AcFAARAA	AcFAAYAA	AcFAAHAA
p-phenylenediamine	96.69	90.13	6.47	9.66	10.01
2,5-dimethyl-1,4-phenylenediamine	96.84	98.91	3.9	0	0
2,3,5,6-tetramethyl-p-phenylenediamine	96.84	30.72	0	4.33	15.61
4-aminobenzoic acid	3	2.1	0	0.28	10.08

Figure 3.3 shows the examples of MRM chromatograms for the depletion reactions.

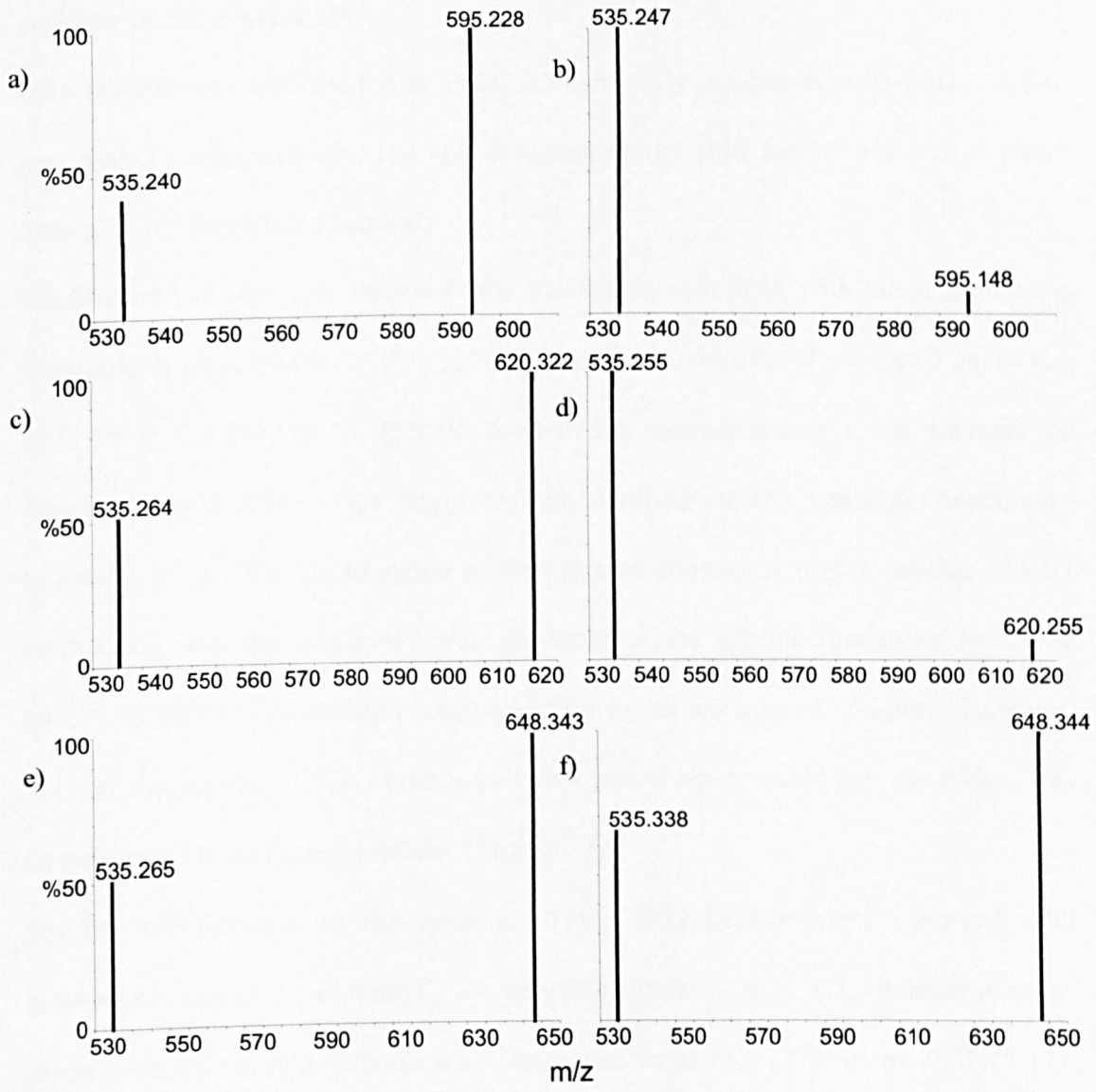


Figure 3.3.MRM chromatograms for depletion reactions m/z 535 represents standard control; a) relative amount of control cysteine peptide (m/z 595.228) b) relative amount of cysteine peptide after 24hr incubation with PPD (m/z 595.148) c) relative amount of control lysine peptide (m/z 620.322) d) relative amount of lysine peptide after 24hr incubation with PPD (m/z 620.255) e) relative amount of control arginine peptide (m/z 648.343) f) relative amount of arginine peptide after 24hr incubation with PPD (m/z 648.344).

3.3.2 p-Phenylenediamine and related compounds specifically modify cysteine residues in the peptide DS3

DS3 peptide was incubated with PPD, 2,5-dimethyl-1,4-phenylenediamine, 2,3,5,6-tetramethyl-*p*-phenylenediamine and 4-aminobenzoic acid for 16 hours at a molar ratio of 1:10 (peptide to chemical).

Modification of DS3 was observed after incubation with PPD, with the peptide mass increasing from *m/z* 2451.15 (2+, 1226.58; 3+ 818.05; 4+ 613.79 – figure 3.3a) to *m/z* 2557.15 (2+, 1279.58; 3+ 853.38; 4+ 640.29), corresponding to an increase of 106amu (figure 3.4b). This suggested the addition of the benzoquinonediimine intermediate of PPD. Modification of the cysteine-containing tryptic peptide of DS3 (MFCQIG) was also observed, with the mass of the peptide increasing from *m/z* 697.29 to 803.29 (2+ 402.64), corresponding to an increase of 106amu. Collision-induced dissociation (CID) of the *m/z* 402.64 parent ion revealed that the adduct was associated with the cysteine residue (Figure 3.5b).

Similar modifications to the cysteine of the DS3 peptide were observed after incubation with 2,5-dimethyl-1,4-phenylenediamine and 2,3,5,6-tetramethyl-*p*-phenylenediamine. The peptide mass increased from *m/z* 2451.15 to 2601.15 (2+, 1301.58; 3+ 868.05; 4+ 651.29) with the former corresponding to an increase of 152amu (Figure 3.4c). Modification of the cysteine containing tryptic peptide of DS3 (MFCQIG) was again observed, with the mass of the peptide increasing from *m/z* 697.29 to 849.29 (2+ 425.65). Collision-induced dissociation (CID) of the *m/z* 425.65 ion revealed that a 134amu adduct was associated with the cysteine residue plus there was oxidation of the methionine (Figure 3.5c). Incubation with 2,3,5,6-tetramethyl-*p*-

phenylenediamine lead to a peptide mass increase from *m/z* 2451.15 to 2663.15 (3+ 888.71) corresponding to an increase of 212amu (Figure 3.4d).

Modification of DS3 was not observed in incubations containing 4-aminobenzoic acid.

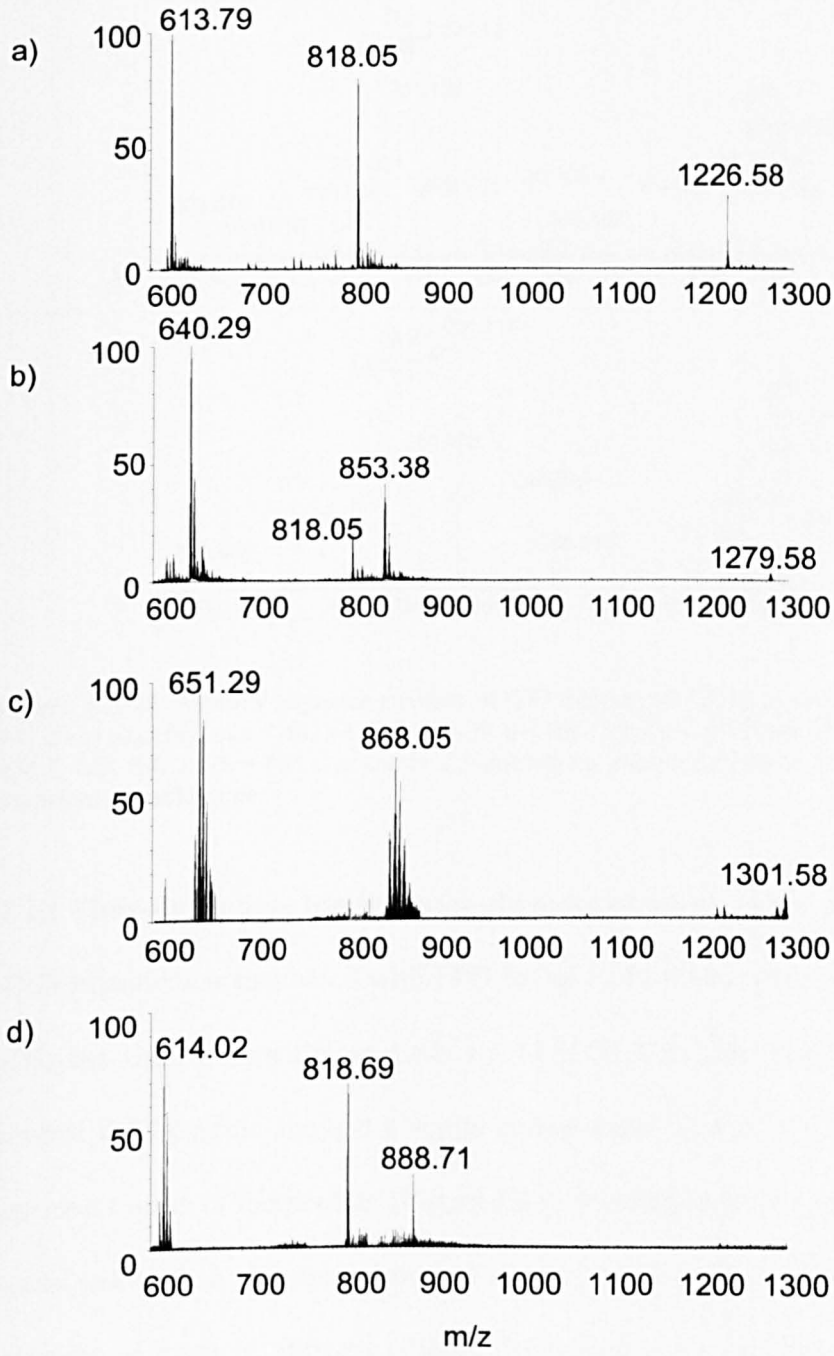


Figure 3.4 Mass spectrum of. a) control peptide MW 2451.15; *m/z* 2+, 1226.58; 3+ 818.05; 4+ 613.79. b) PPD modified peptide MW 2557.15; *m/z* 2+, 1279.58; 3+ 853.38; 4+ 640.29 c) 2,5-dimethyl-1,4-phenylenediamine modified peptide MW; 2601.15; *m/z* 2+, 1301.58; 3+ 868.05; 4+ 651.29 d) 2,3,5,6-tetramethyl-*p*-phenylenediamine MW 2663.15; *m/z* 3+ 888.71.

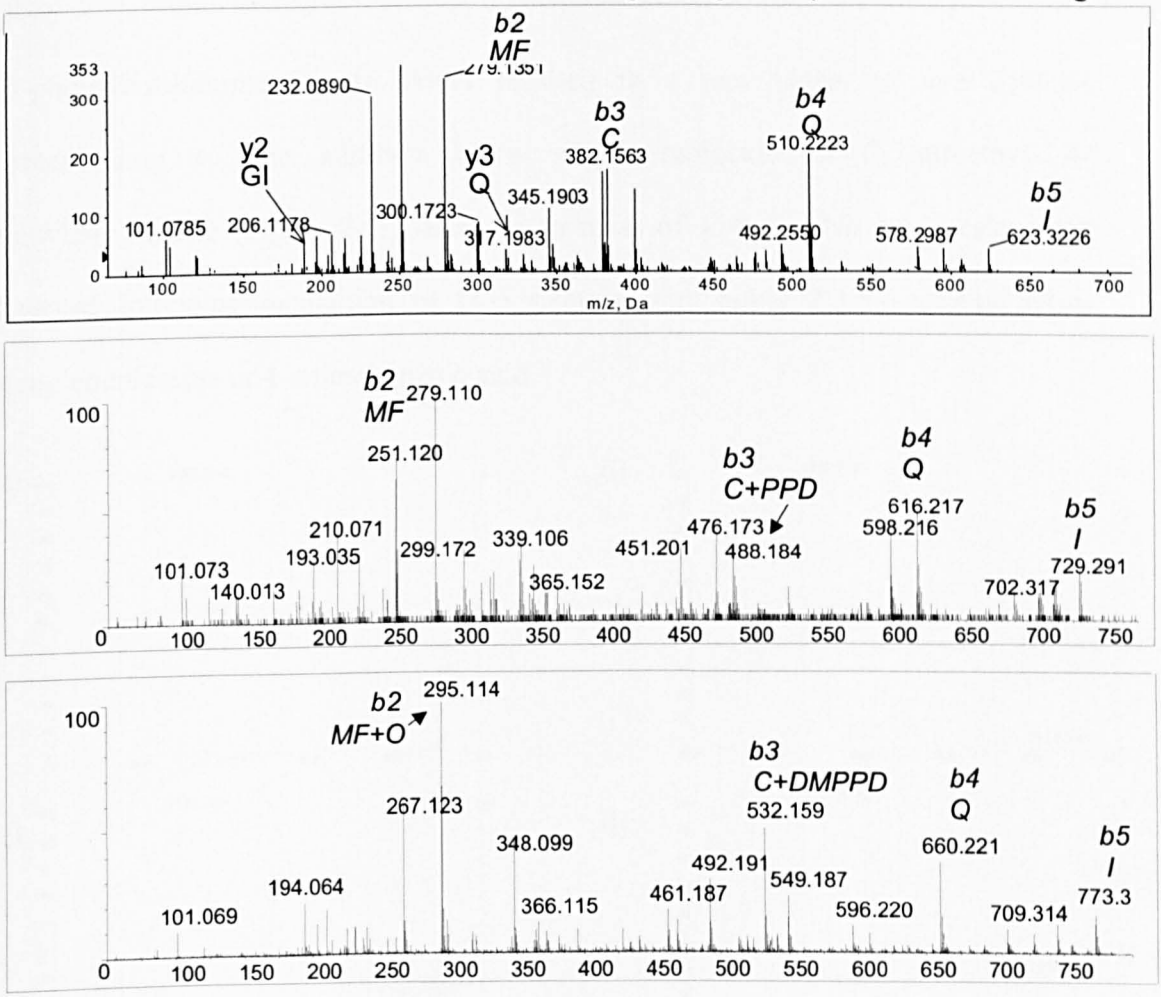


Figure 3.5. LC-MS/MS sequence analysis of DS3 peptide MFCQIG a) unmodified m/z= 349.5; MR 697.29 b) modified m/z = 402.64; MR 803.29 **Δ = 106** Cys shows PPD modification. c) modified m/z = 424.5; MR 847.29 **Δ = 134** Cys shows 2,5-dimethyl-p-phenylenediamine modification; + **Δ = 16** for oxidation of methionine

3.3.3 Time-dependent binding of p-phenylenediamine to the peptide DS3

DS3 peptide was incubated with PPD for up to 72 hours, with aliquots being taken at different time points for analysis by MALDI-MS. The MALDI spectrum of the control DS3 peptide showed a single strong signal at *m/z* 2452.45, corresponding to the intact mass of the peptide (Figure 3.6a). Incubation of the peptide with PPD for 2 hours resulted in the appearance of a new signal at *m/z* 2558.87, representing an increase in mass of 106amu (Figure 3.6b), and corresponding to the addition of a single molecule of PPD. The relative intensity of this new peak increased over the 72 hour incubation period (Figure 3.6c-e). Incubation of the peptide with 2,5-dimethyl-

1,4-phenylenediamine for 16 hours resulted in a new signal at *m/z* 2586.84 corresponding to the addition of a single molecule of 2,5-dimethyl-1,4-phenylenediamine (Figure 3.6f); increase in mass of 134amu. No new peaks were observed following incubation of DS3 peptide with either 2,3,5,6-tetramethyl-*p*-phenylenediamine or 4-aminobenzoic acid.

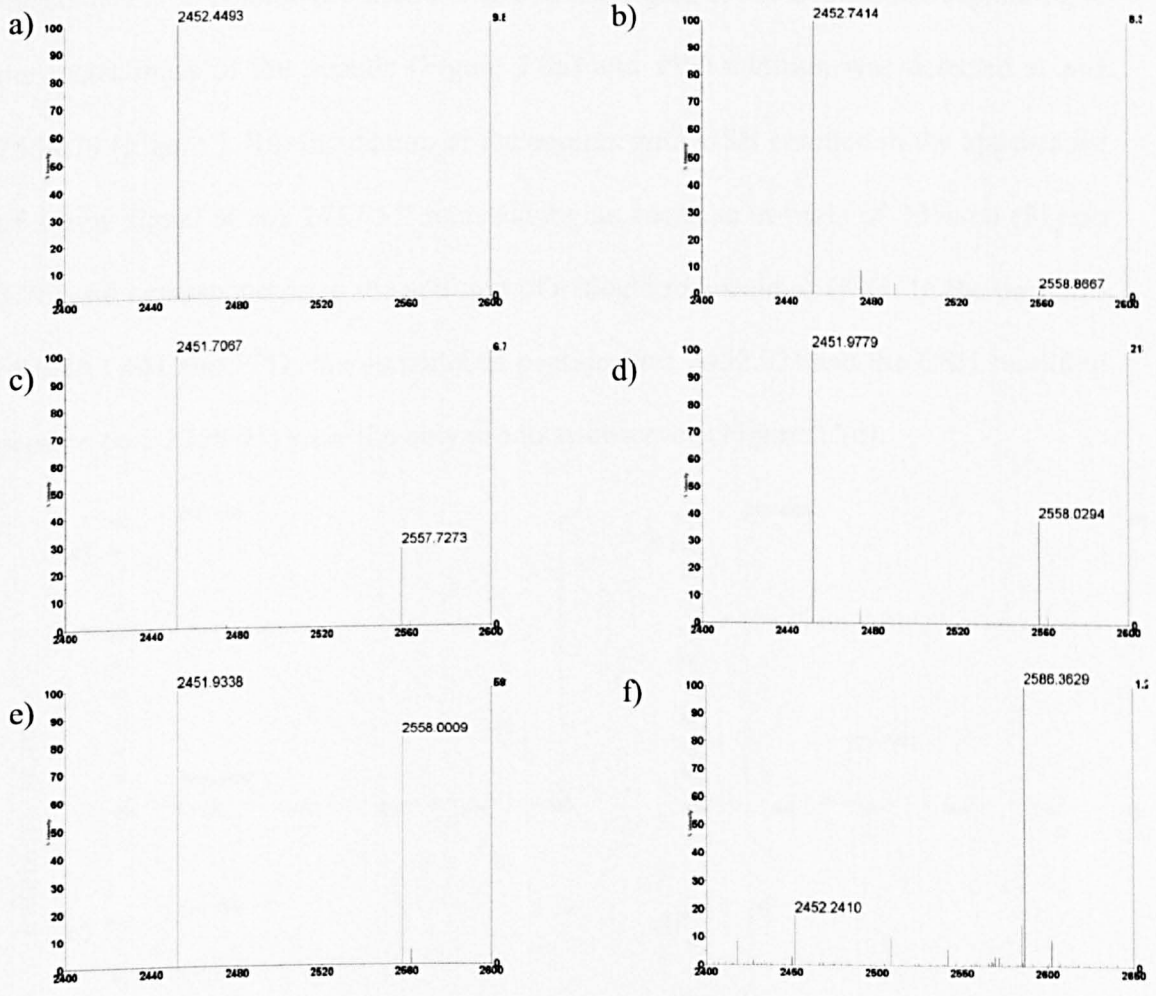


Figure 3.6 DS3 peptide (H-Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Try-Gly-His-Glu-Tyr-Arg-Met-Phe-Cys-Gln-Ile-Gly-OH) time course
a) Control; b) PPD+DS3 2 hours; c) PPD+DS3 24 hours; d) PPD+DS3 48 hours; e) PPD+DS3 72 hours Unmodified peptide MR = 2452.45; Modified peptide MR = 2558.86; Δ = 106
f) Representative spectra for 2,5-dimethyl-p-phenylenediamine +DS3 at 16 hours; Unmodified peptide MR = 2452.61; Modified peptide MR = 2586.36; Δ = 134 (+16 for oxidation of met)

3.3.4 Effect of glutathione on irreversibility of PPD-protein adduct formation

GSH was added to a subset of experiments to assess the reversibility of PPD-protein adduct formation. DS3 peptide was incubated in the absence of drug, with PPD alone, with GSH alone, and with PPD plus GSH for 16 hours. As before, MALDI analysis of the control DS3 peptide revealed a single strong signal at *m/z* 2452.45 corresponding to the intact mass of the peptide (Figure 3.7a) and PPD addition was detected at *m/z* 2558.74 (Figure 3.7b). Incubation of the peptide with GSH resulted in the appearance of a new signal at *m/z* 2757.55, representing an increase in mass of 305amu (Figure 3.7c) and corresponding to the addition of a single molecule of GSH. In the presence of both GSH and PPD, the unmodified peptide (*m/z* 2452.83) and the GSH modified peptide (*m/z* 2758.01) were the only products observed (Figure 3.7d).

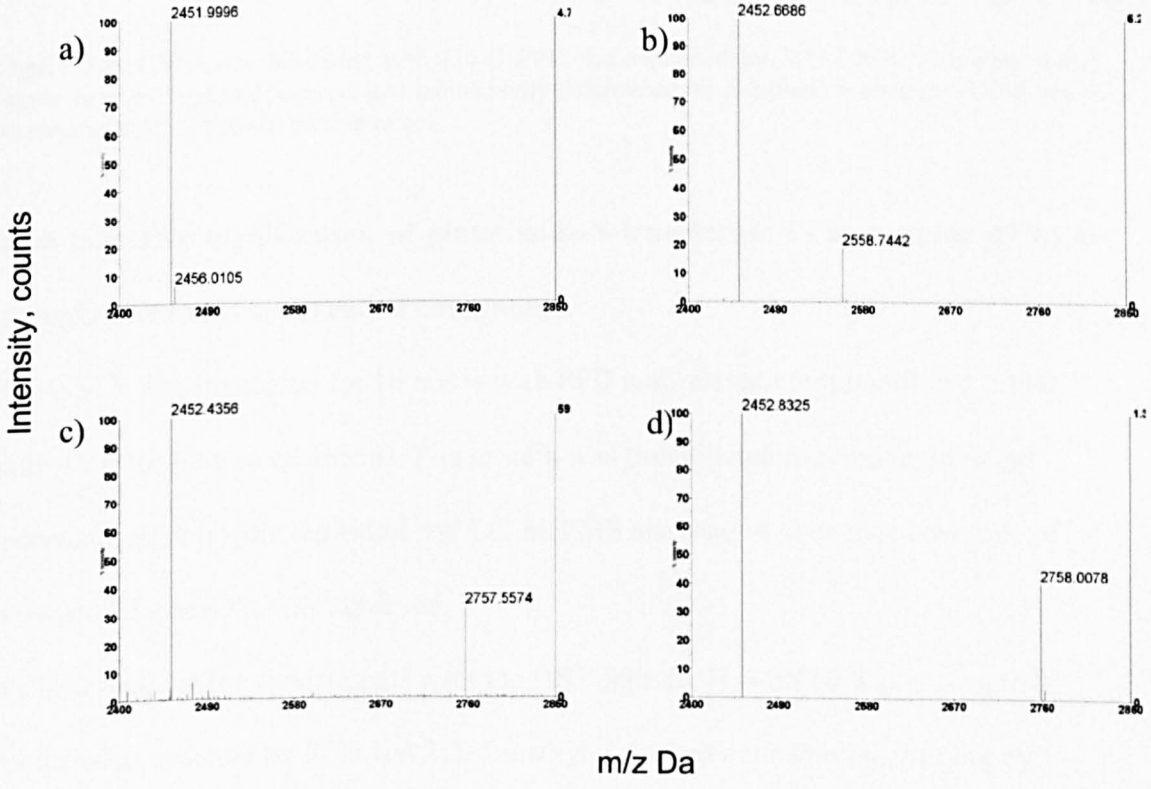


Figure 3.7. DS3 peptide PPD +/- GSH 16 hours a) Control; b) DS3+PPD; c) DS3+GSH; d) DS3+PPD+GSH ; Unmodified peptide MR = 2452; PPD modified peptide MR = 2558; Δ = 106; GSH modified peptide MR= 2757, Δ = 305

3.3.5 p-Phenylenediamine binds irreversibly to the model protein glutathione-S-transferase Pi

Denaturing gel electrophoresis of His-GSTP exposed to radiolabelled PPD followed by band excision and scintillation counting revealed that PPD bound irreversibly to His-GSTP (Figure 3.8).

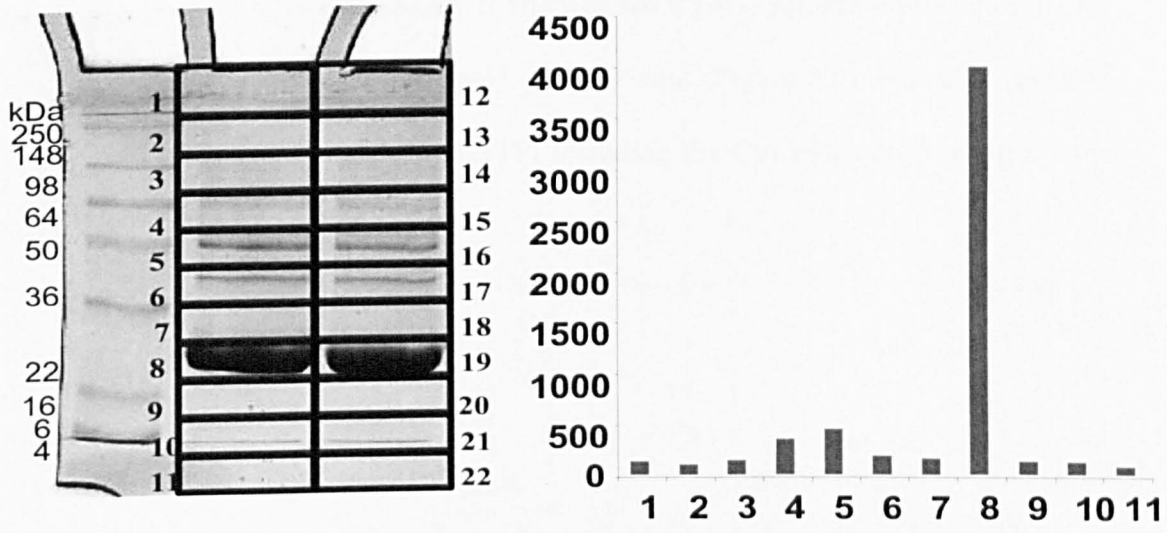


Figure 3.8. GSTP was incubated with [14]C PPD and separated by SDS-PAGE (10μg/per track). Bands were excised and incorporated radioactivity determined by scintillation counting. DPM values correspond to each excised section of gel.

3.3.6 Selective modification of glutathione-S-transferase Pi at cysteine 47 by p-phenylenediamine and related compounds

His-GSTP was incubated for 16 hours with PPD and related compounds at a molar ratio 1:10 (protein to chemical). The protein was precipitated to remove unbound material before tryptic digestion and LC-MS/MS analysis. A sequence coverage of between 82 and 89% was achieved.

As observed for the experiments with the DS3 peptide, His-GSTP was shown to be modified at cysteine by PPD and 2,5-dimethyl-1,4-phenylenediamine, but not by 2,3,5,6-tetramethyl-*p*-phenylenediamine and 4-aminobenzoic acid.

Modification of the Cys47-containing peptide 45-54 (ASCLYGQLPK) was observed after incubation with PPD for 16 hours. The peptide mass increased from *m/z* 1078.6 (2+, 540.3) to *m/z* 1184.6 (2+, 593.3), corresponding to an increase of 106amu. These data suggest that the primary PPD oxidation intermediate benzoquinonediimine binds covalently to His-GSTP. Fragmentation of the *m/z* 593.3 ion revealed the absence of the y8 ion at m/z 921 corresponding to unmodified Cys47, and the presence of the b3 ion at *m/z* 368 corresponding to Cys47 plus 106amu (Figure 3.9). No other residues were found to be modified in His-GSTP, including the Cys residues at positions 14, 101 and 169.

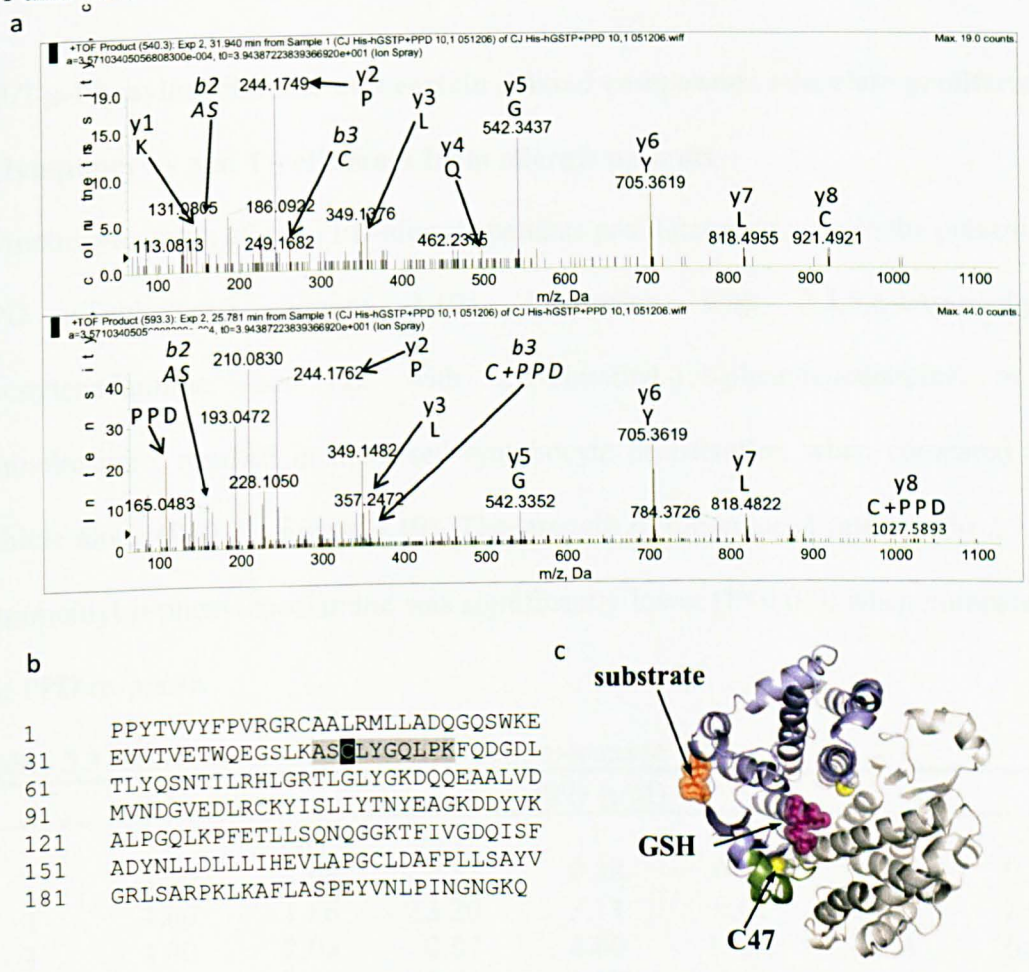


Figure 3.9. Exposure of GSTP to PPD leads to adduct formation through modification of cysteine residues. a) LC-MS/MS sequence analysis of human GSTP peptide ASCLYGQLPK a top) unmodified m/z=540.3; MW 1078.6 a bottom) modified m/z = 593.3; MW 1184.6 Δ = 106 Cys34 shows PPD modification. b) Amino acid sequence of human GSTP1-1. Tryptic peptide 45-54 and Cys47 are indicated. c) Molecular model of the dimer of human GSTP1, with the sites of substrate and GSH binding indicated. Cys47 is located close to the GSH binding site. The model is based on structure 6GSS contained in the Protein Data Bank (Oakley *et al.*, 1997) and has been modified using PyMol (DeLano, 2002).

Incubation of His-GSTP with 2,5-dimethyl-1,4-phenylenediamine also resulted in modification of the Cys47-containing peptide 45-54. The peptide mass increased from *m/z* 1078.6 (2+, 540.3) to 1212.6 (2+, 607.3), consistent with modification by the chemical, and the partial sequence of the peptide could be interpreted. However, no adduct was detected in the MS/MS spectrum, possibly due to lability of the adduct in the collision chamber of the MS (data not shown). Modification of His-GSTP was not observed in incubations containing 2,3,5,6-tetramethyl-*p*-phenylenediamine and 4-aminobenzoic acid.

3.3.7 p-Phenylenediamine and certain related compounds stimulate proliferation of lymphocytes and T-cell clones from allergic patients

Lymphocytes from all ten PPD-allergic patients proliferated *in vitro* in the presence of PPD (Table 3.3, Figure 3.10). Incubation with 2,3,5,6-tetramethyl-*p*-phenylenediamine, but not with 2,5-dimethyl-1,4-phenylenediamine or 4-aminobenzoic, resulted in increased lymphocyte proliferation when compared with vehicle alone ($P<0.01$; Figure 3.10). The strength of the induced response to 2,3,5,6-tetramethyl-*p*-phenylenediamine was significantly lower ($P<0.01$), when compared to the PPD response.

Table 3.3. Stimulation Index for patients responses to PPD

	PPD (μM)						
	0	0.1	0.5	1	5	10	20
1	1.00	1.68	2.33	0.38	0.43	0.25	0.12
2	1.00	1.86	3.20	3.11	9.01	10.70	2.03
3	1.00	2.03	2.87	4.00	11.02	8.44	0.22
4	1.00	3.32	2.08	0.97	1.00	1.11	0.79
5	1.00	2.42	12.18	4.58	2.68	1.81	0.20
6	1.00	2.46	4.91	4.60	4.24	5.50	2.85
7	1.00	2.40	3.23	3.66	12.32	3.23	0.13
8	1.00	0.66	1.07	0.85	17.76	1.39	0.37
9	1.00	0.97	1.32	1.55	8.65	15.33	4.99
10	1.00	4.47	13.78	26.74	33.06	34.89	28.21

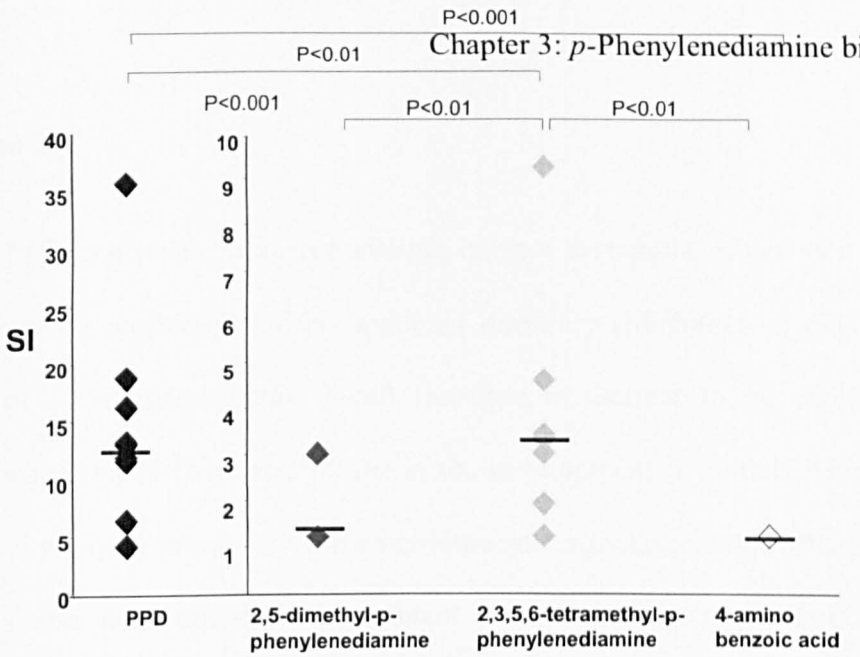


Figure 3.10. Antigen specific stimulation of lymphocytes from allergic patients. Proliferative response of lymphocytes form 10 allergic patients stimulated with PPD, 2,5-dimethyl-p-phenylenediamine, 2,3,5,6-tetramethyl-p-phenylenediamine and 4-aminobenzoic acid. Lymphocytes from all allergic patients proliferated in a concentration dependent manner to PPD. 2,3,5,6-tetramethyl-*p*-phenylenediamine and 2,5-dimethyl-1,4-phenylenediamine, stimulated low levels of proliferation in 8/9 and 1/9 patients, respectively. A proliferative response to 4-aminobenzoic acid was not observed. Proliferation was measured by [3H] thymidine incorporation for the last 16 hours of the experiment. Statistical analysis was performed by comparing incubations in the presence and absence of drug ($P<0.05$ was accepted as significant).

Figure 3.11 shows concentration dependent effects of PPD and 2,3,5,6-tetramethyl-*p*-phenylenediamine from 2 representative patients expressed as cpm to demonstrate the inter-variability between individuals.

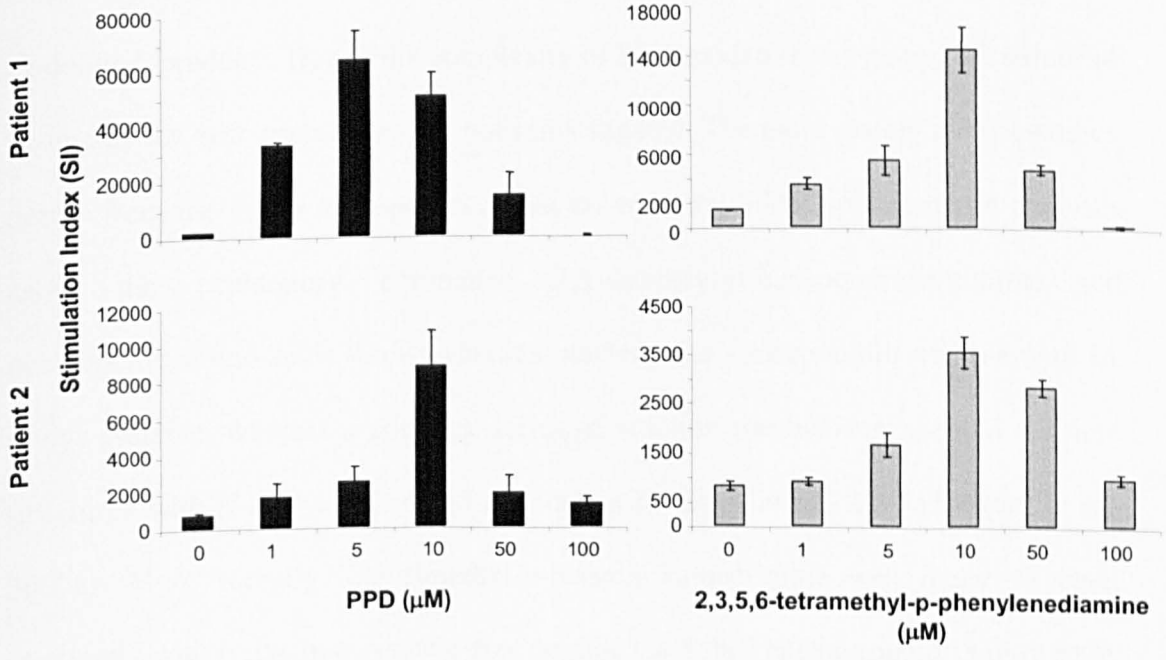


Figure 3.11. Concentration dependent effects of p-phenylenediamine and 2,3,5,6-tetramethyl-p phenylenediamine on lymphocyte proliferation from 2 representative patients. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16 h of the experiment. Results are expressed as mean cpm of triplicate culture ± SD.

3.4 Discussion

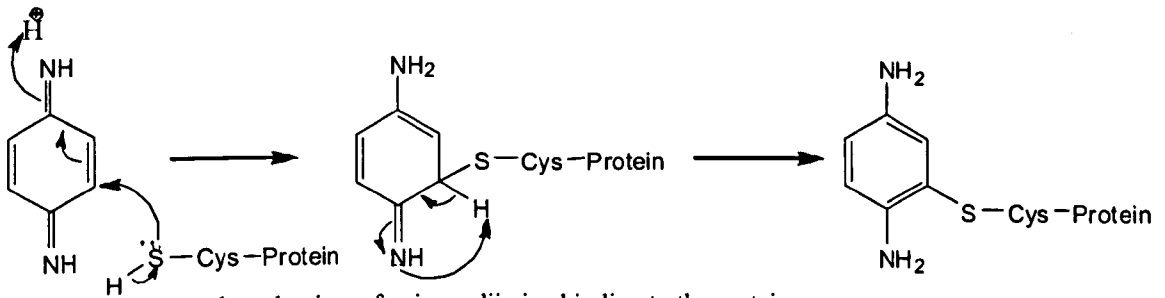
Exposure to PPD can result in severe allergic contact dermatitis, which is a major cause of increasing morbidity and occupational disability (Mcfadden *et al.*, 2007). Stimulation of an antigen-specific T-cell response is thought to be decisive in determining whether PPD exposure results in the development of contact dermatitis. In this respect, we have previously shown lymphocyte stimulation with PPD but not Bandrowski's base, is an important determinant of individual susceptibility (Chapter 2). This finding corroborates Landsteiner and Jacobs' theory of hapten recognition of low molecular weight compounds by the immune system, and underlines the importance of delineating the site and nature of PPD-specific protein interaction(s), and whether particular modifications stimulate a T-cell response.

PPD is readily converted into *p*-benzoquinonediimine in solution, which is susceptible to hydrolysis yielding *p*-benzoquinone, and self-conjugation generating several, as yet ill-defined, products. Due to the complexity of PPD oxidative chemistry, the nature of its interaction with protein has not been investigated. The most closely related studies derive from the group of Lepoittevin and co-workers; NMR spectrometric analyses using the exploratory compound 2,5-dimethyl-*p*-benzoquinonediimine and nucleophilic amino acids show a classical nucleophile – electrophile reaction with *N*-acetyl cysteine, whereas a complex series of reaction mechanisms yielded multiple structures with *N*-acetyl lysine and tryptophan (Eilstein *et al.*, 2006, Eilstein *et al.*, 2007a). More recently, 2,5-dimethyl-*p*-benzoquinonediimine was found to react covalently with lysine in a cysteine-free peptide via Schiff base formation followed by an oxido-reduction process giving rise to an aldehyde intermediate and peptide cyclization (Eilstein *et al.*, 2007b). The authors conclude that lysine should be

considered an important amino acid for conjugate formation for allergenic *p*-benzoquinonediimines; however, the ratio of binding to specific amino acids in a cysteine-containing peptide and the nature of the binding interaction with protein were not evaluated. Thus, using a PPD and 3 structurally related compounds, we have adopted a mass spectrometric approach to address these issues.

We used specifically designed peptides containing single nucleophilic amino acid residues to examine intrinsic chemical reactivity of PPD and 3 structurally related chemicals. PPD, 2,5-dimethyl-1,4-phenylenediamine and 2,3,5,6-tetramethyl-*p*-phenylenediamine were found to modify only cysteine and lysine residues in the peptide depletion assay, whereas no modification with 4-aminobenzoic acid was observed. This simple assay dependent on the disappearance of a parent molecular ion does not discriminate between adduct formation and peptide degradation, thus the synthetic peptide DS3 was used to explore selectivity to amino acids in a more complex environment.

PPD was found to bind exclusively to cysteine in DS3, despite each of the 20 common amino acids being represented in the peptide sequence. LC-MS/MS analysis of the DS3 peptide following incubation with PPD revealed a mass shift of 106amu in modified samples compared to control, suggesting a direct nucleophilic/electrophilic reaction between PPD benzoquinonediimine and sulfur of the cysteine residue (Scheme 1).



Scheme 1. Proposed mechanism of quinonediimine binding to the cysteine.

2,5-dimethyl-1,4-phenylenediamine and 2,3,5,6-tetramethyl-*p*-phenylenediamine were also found to exclusively modify the cysteine residue of DS3 peptide with mass additions of 134 and 194 amu detected, respectively. These experiments, conducted at physiological pH 7.4, suggest that cysteine modification could be a key determinant in the induction of allergic contact dermatitis. Although reactions with amino acids other than cysteine are chemically feasible and occur naturally in a cysteine-free environment, the context of the amino acid residue has an important effect on the binding profile of a chemical.

GSTP was used as a model protein to further explore the covalent interaction of PPD with biological macromolecules. The Cys47-containing tryptic peptide (ASCLYGQLPK) of His-GSTP, but not the other cysteine residues, was found to be modified by PPD and 2,5-dimethyl-1,4-phenylenediamine, consistent with the relative reactivity of the Cys residues in this protein. Cys47 has a lower pKa value than usual (3.5-4) making it highly reactive and subject to modification by Michael addition (LoBello *et al.*, 1993; Orton & Lieber, 2007). The unusual nucleophilicity of the residue is thought to be due to its deprotonation by the nearby lysine 54 residue. The selective binding of PPD to a single binding site in an isolated protein such as GSTP suggests that PPD protein binding *in vivo* might also display some degree of

selectivity. However the lysine residues of His-GSTP do not exhibit low pKa values (range 9.52-10.50, PropKa (http://propka.ki.ku.dk/~drogers/) (Jensen *et al.*, 2005; Li *et al.*, 2005) therefore selectivity of the binding interaction will further be examined in Chapter 4, using a different protein with known reactivity on lysine residues. In on-going experiments, structurally-defined PPD-protein conjugates are being used to probe the nature of the antigen that interacts with MHC and specific T-cell receptors.

Interestingly no 2,3,5,6-Tetramethyl-*p*-phenylenediamine modification was detected on GSTP even though cysteine modification with this compound had been detected in the model peptides. This suggests again that the context of the amino acid residue in protein profoundly influences its chemical reactivity.

To explore the relationship between protein binding *per se* and immune reactivity, PPD, 2,5-dimethyl-1,4-phenylenediamine, 2,3,5,6-tetramethyl-*p*-phenylenediamine and 4-aminobenzoic acid were tested for their ability to stimulate lymphocytes from PPD allergic patients. A vigorous PPD-specific proliferative response was observed with all patients. 2,3,5,6-tetramethyl-*p*-phenylenediamine and 2,5-dimethyl-1,4-phenylenediamine stimulated low levels of proliferation in 8/9 and 1/9 patients, respectively. No mechanism for binding of the 2,3,5,6-tetramethyl-*p*-phenylenediamine to protein could be identified and no real conclusions could be drawn as to why this compound and not the 2,5-dimethyl-1,4-phenylenediamine would cause a proliferative response. A proliferative response to 4-aminobenzoic acid, which does not bind irreversibly to protein, was not observed. These data suggest that there is a relationship between protein binding and stimulation of lymphocytes from PPD allergic patients for this panel of compounds. However, the

site of specific protein modification, the nature of the irreversible bond, the effect of the binding interaction on protein structure and the chemical itself affects the level of cross reactivity and the strength of the induced response.

Finally reversibility of the PPD-peptide adduct was tested using the nucleophilic tripeptide GSH as it has been shown that GSH can prevent the formation of oxidation products of PPD (Coulter *et al.*, 2006). Inclusion of GSH reduced the binding of PPD to cysteine residue of the DS3 peptide. Originally it was presumed PPD would favour a reaction with the sulphydryl group of GSH. However no evidence of a PPD-GSH conjugate was found (data not shown). The most feasible explanation is that GSH is simply driving the reformation of PPD from the quinonediimine intermediate, resulting in an equilibrium reaction between the two. This would explain why the binding of PPD decreases in the presence of GSH but is never fully inhibited and how it prevents Bandrowski's base being formed. Direct interaction between the GSH and the cysteine 47 residue of peptide of GSTP was also observed by MS/MS. This suggests GSH may out compete PPD for binding to the cysteine residue, thereby decreasing its availability of binding sites for PPD.

In conclusion, we have identified the amino acid cysteine as the primary target of PPD modification in a model protein and verified the nature of this interaction by accurate mass shift determination and sequencing. Further work is needed to relate selective adduct formation to the induction of a hapten-specific T-cell response; linking this chemical knowledge through to biological function is vital.

CHAPTER 4

Characterization of *p*-phenylenediamine-albumin binding sites and T-cell responses to hapten-modified protein

4.1 Introduction

Chemicals associated with skin sensitisation are electrophilic, low molecular weight molecules that form strong irreversible bonds with self protein to generate immunogenic hapten-protein complexes (Divkovic *et al.*, 2005). PPD is one of the most frequently encountered sensitisers due to its wide use as an intermediate in permanent hair dye formulations (Corbett *et al.*,1973), in printing ink & photo development (Chung *et al.*,1993) and more recently in henna tattoos (Mautlich & Sullivan, 2005). The incidence of allergic reactions to PPD is on the increase, because of the increasing use of consumer products (McFadden *et al.*, 2007).

PPD itself is not directly protein reactive. However, auto-oxidization on skin and in aqueous solution generates an electrophilic quinonediimine intermediate, which is susceptible to subsequent sequential oxido-conjugation reactions. The product of these reactions is Bandrowski's base, a derivative of three conjugated PPD molecules (Picardo *et* al., 1990; Coulter *et* al., 2007; Aeby *et* al., 2008). Oxidation processes generate haptenic determinants that may bind to different proteins, to different amino acids or the same amino acid at different positions in the protein structure (Lepoittevin *et al.*, 2006).

PPD and BB are classed as potent skin sensitizers in the mouse (Warbrick *et al.*, 1999; White *et al.*, 2006). In these model systems, the immuno-stimulatory effects of PPD are thought to be dependent on cutaneous oxidation and the generation of BB (Farrell et al., 2008; Aeby *et* al., 2008). Patch testing studies on humans and *in vitro* analysis of lymphocyte proliferation suggest that the nature of the antigenic determinant is different in allergic patients. Patch testing using PPD, which has a high positive

predictive value (Ho *et al.*, 2005), is time-dependent (Basketter *et al.*,2006) indicating that oxidation is important for immune reactivity. Only 16% of PPD patch test positive patients are responsive to BB (White *et al.*, 2006); therefore, primary PPD oxidation products are thought to be major antigenic determinants in human subjects. On examination of the data generated in chapter 2, BB was found to stimulate a T cell response in allergic patients and tolerant individuals, which is apparently not converted into an allergic reaction. T-cell responses to PPD, which are highly diagnostic, are restricted to allergic patients, adding further weight to the theory that PPD or one of it's primary oxidation products (ie; benzoquinonediimine intermediate) is responsible for sensitisation. It is generally thought that for a small compound, such as PPD, to cause ACD it must first bind to a protein to form a hapten complex large enough to be recognised by the immune system (Gober & Gaspari, 2008; Lepoittevin, 2006; Watanabe *et al.*, 2002). Thus, understanding how PPD binds is vital in our understanding of mechanisms of ACD. The reaction profile of the PPD related compound 2,5-dimethyl-*p*-benzoquinonediimine with single nucleophilic amino acids has been studied in detail (Eilstein *et al.*, 2006;2007). 2,5-dimethyl-*p*-benzoquinonediimine binds directly to cysteine, and other amino acids, through a series of complex chemical pathways. The selectivity of 2,5-dimethyl-*p*-benzoquinonediimine towards lysine residues in a model peptide containing potentially reactive amino acids (except cysteine) suggests that lysine might be an important amino acid for hapten complex formation (Eilstein *et al.*, 2007). However data obtained in Chapter 3 suggests otherwise. We found that PPD bound specifically and selectively to cysteine residues of small designer peptides and the protein glutathione-*S*-transferase (GSTP). Antigen-specific T-cells are thought to be stimulated by MHC-associated peptides derived from the hapten-protein complex

following internal processing by antigen presenting cells therefore it is important to relate this selective adduct formation to the induction of a hapten-specific T-cell response.

The primary aim of this project was to use human serum albumin to further characterize PPD-protein complex formation and the T-cell stimulatory capacity of this PPD-modified protein. HSA was chosen as a model protein for several reasons: first, HSA is the most abundant plasma protein; second, it contains a single cysteine in a reduced form and therefore available for hapten complex formation; and finally, the T-cell stimulatory capacity of hapten-HSA complexes has been shown previously (Brander *et al.*, 1995) using penicillin.

Also presented in this chapter are data exploring fundamental cytokine differences between allergic patients and volunteers' lymphocyte responses, when stimulated with PPD, BB and PPD-modified HSA. It is well documented that ACD is a common eczematous disease mediated by T-cells that occurs in the skin at the sites of contact with small chemicals, such PPD, however our understanding of the cellular basis of ACD and reasons for individual susceptibility are ill-defined, Therefore functional studies assessing cytokine secretion following hapten stimulation are important as cytokines are known to directly related to the induction of specific chemical manifestations of the disease. After encountering antigen-bearing dendritic cells, T-lymphocytes differentiate into distinct subtypes representing polarised forms of the highly heterogeneous CD4+ Th cells; Th1 & Th2, or CD8+ Tc cells; Tc1 & Tc2, that differ in their expression of cytokines and chemokine receptors (Romagnani 2004). Briefly, type 1 responses are characterised by the prevalent production of IL-2, IL-12,

IFN-γ, and TNF-β, whereas by contrast, type 2 response pattern is characterised by IL-4, IL-5, IL-10 and IL-13.

Known contact sensitisers DNCB and nickel have been shown cause secretion of predominantly Type 1 cytokines (Pickard *et al.*, 2007; Lecart *et al.*, 2001; Cavani *et al.*, 2000). In contrast a murine model of PPD-induced contact sensitisation found Type 2 cytokines, IgE antibodies and mast cells are crucial in the development of tissue pathology (Yokozeki *et al.*, 2003). Coulter *et al.*, (unpublished data) showed that stimulation of patient lymphocytes with PPD and BB was associated with significantly higher levels of Type 2 cytokine secretion and increased gene expression when compared with volunteers, suggesting the ability to produce cytokines such as IL-4, IL-5 and IL-13 may contribute to the development of contact dermatitis. Data presented within this chapter utilise an independent and expanded allergic patients and tolerant volunteer cohort to confirm that PPD and BB induced T-cell activation is associated with type 2 cytokine secretion and whether PPD-modified HSA T-cell stimulation promotes a similar response.

4.2 Materials and Methods

4.2.1 Culture medium for lymphocyte culture and T-cell cloning

Culture medium consisted of RPMI-1640 supplemented with pooled heat-inactivated human AB serum (10%, v/v), HEPES (25 mM), L-glutamine (2 mM), transferrin (25 $\mu g\ ml^{-1}$), streptomycin (100 $\mu g\ ml^{-1}$) and penicillin (100 U ml^{-1}). T-cell lines and clones were maintained in culture medium supplemented with IL-2 (25U/ml).

4.2.2 Cell lines

EBV-transformed B-cell lines (referred to as antigen presenting cells) were generated from peripheral blood lymphocytes of allergic patients using supernatant from the EBV producing cell line B9-58 according to established methods (Wu *et al.*, 2007). Cell lines were maintained in medium consisting of RPMI-1640 supplemented with 10% FCS, HEPES (25 mM), L-glutamine (2 mM), streptomycin (100 $\mu g\ ml^{-1}$) and penicillin (100 U ml^{-1}).

PPD was obtained from Sigma Chemical Co (Poole, Dorest, UK). [^{14}C]PPD (specific activity 60mCi/mmol) was synthesized by GE Healthcare (Bucks, UK). Stock solutions (10 mg ml^{-1}) were prepared in culture media and dimethyl sulphoxide (4:1 v/v) and diluted as required.

4.2.3 Donor Characteristics

Peripheral blood mononuclear cells were isolated from venous blood (as described in Chapter 2) obtained from 10 PPD allergic, patch test positive patients and 10 healthy volunteers Approval for the study was obtained from Liverpool local research ethics committee; informed written consent was obtained from all donors. Clinical characteristics of the allergic patients and volunteers are shown in Table I.

Table 1. Clinical details from PPD allergic patients and tolerant individuals

	Age	Sex[a]	Dye use	PPD allergy	Other allergies	Months since reaction	Patch test	LTT (SI)	
								PPD	PPD-HSA
Allergic Patients									
1	37	F	Yes	Yes	Nickel, cobalt , nail polish, pollen, latex, benzoic acid, benzyl peroxide	252	+	2.3	2.5
2	43	F	Yes	Yes	-	17	+	10.7	76.2
3	77	F	Yes	Yes	-	18	+	11.02	20.2
4	51	F	Yes	Yes	Hay fever	24	+	3.3	2.4
5	33	F	Yes	Yes	nickel, colourings & preservatives	22	+	12.2	18.9
6	68	F	Yes	Yes	Nickel, copper, latex	44	+	5.5	51.2
7	52	F	Yes	Yes	E45 cream, orange colouring, biological soap	14	+	12.3	54.9
8	70	F	Yes	Yes	Penicillin, make up, creams, nickel, leather, tea tree oil, fragrances	11	+	17.8	2.2
9	56	F	Yes	Yes	Nickel, perfumes	22	+	15.3	38.2
10	43	F	Yes	Yes	Local anaesthetics, Nickel, sunglasses & clothes dye.	96	+	34.9	33.5
Volunteers									
11	26	F	Yes	No		na[c]	np[b]	-[d]	-
12	28	M	No	No		Na	Np	-	-
13	24	F	Yes	No		Na	Np	-	-
14	24	F	Yes	No		Na	Np	-	-
15	24	F	Yes	No		Na	Np	-	-
16	28	F	Yes	No		Na	Np	-	-
17	37	M	No	No		Na	Np	-	-
18	23	F	Yes	No		Na	Np	-	-
19	27	M	No	No		Na	Np	-	-
20	27	M	No	No		Na	Np	-	-
21	33	F	Yes	No		Na	Np	3.20	2.11
22	34	F	No	Unknown		Na	Np	10.76	6.29

[a]M, male; F, female; [b]np, not performed; [c]na, not applicable; [d]-, SI less than 2

4.2.4 p-Phenylenediamine-human serum albumin complex formation

[^{14}C]PPD (150 μM [0.2 μCi]) was incubated with HSA (1 mg/ml; in HBSS, pH 7.4; total volume 6 ml) for 0–130hours (h) to permit hapten-protein complex formation (molar ratio 10:1; PPD: protein). After 0, 4, 24, 48, 72, and 130h, the reaction was terminated and protein extracted by exhaustive solvent extraction with acetonitrile; ACN (4 x 1ml). HSA conjugates were dissolved in NaOH (0.2 M; 1ml). Aliquots (100μl) were taken from each precipitation step for quantification of hapten-protein complex formation and protein estimation. The time-dependency of [^{14}C]PPD-HSA complex formation was explored directly by scintillation counting.

To show that PPD binds irreversibly to HSA, an aliquot of protein was taken after 24h and unbound PPD was removed by exhaustive solvent extraction and protein was precipitated by the addition of ACN (100 μL). An aliquot containing 20 μg of total protein was taken and added to an equal volume of reducing sample buffer. Samples were then heated at 95 °C for 10 min and the proteins were by electrophoresis on SDS-polyacrylamide gels under reducing conditions using the discontinuous buffer system described by Laemmli (1970). Bands were then excised and digested. Briefly the gel was cut into 0.5cm^3 sections and digested with soluene at 50°C for 1h before the addition of 30μL glacial acetic acid. The level of [^{14}C] PPD incorporated into each band was then determined by scintillation counting.

Glutathione (GSH) was added to a subset of experiments to assess the reversibility of PPD binding, as it has previously been shown to prevent formation of BB (Coulter *et al.*, 2007) through the establishment of a redox cycling pathway (Chapter 3). [^{14}C]PPD (150 μM [0.2 μCi]) was incubated with HSA (1 mg/ml; in HBSS, pH 7.4;

total volume 6 ml) with 1mM GSH for 0–130h. Experiments were carried out as described above.

Specific amino acid modification of HSA by PPD was determined by incubating HSA (15 μM) with PPD (150 μM; 100μl) for 16h in phosphate buffer at 37°C. Samples were reduced with dithiothreitrol (DTT; 100 mM), alkylated with iodoacetamide (55 mM) and solvent washed with methanol (10:1), to remove unbound material, and suspended in 50 mM ammonium bicarbonate. Samples (100 μg) were digested with modified trypsin (5 ng; in 50mM ammonium bicarbonate) for 16h at 37°C and desalted using C18 ZipTip columns according to the manufacturer's instructions (Millipore, Watford, Hertfordshire). The resultant peptide mixture was reconstituted in ACN/trifluoroacetic acid (5 μl; 5:0.1%). For LC-MS/MS analysis, aliquots of sample (1μL) were delivered into a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems; Warrington, UK) by automated in-line liquid chromatography (integrated LCPackings System, 5mm C18 nano-precolumn and 75μm x 15cm C18 PepMap column (Dionex, California, USA)) via a nano-electrospray source head and 10μm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 5% ACN/0.05% TFA (v/v) to 48% ACN/0.05% TFA (v/v) in 60mins was applied at a flow rate of 300nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA) (Analyst, Applied Biosystems). Database searching was performed using ProteinPilot version 1 (Applied Biosystems) against the latest version of the SwissProt database, with the confidence level set at 90%. PPD and carboxamidomethyl were set as a high and medium probability user-defined modifications on specific amino acids ie: cysteine.

4.2.4.1 Determination of PPD-modified HSA stability

To assess the stability of the PPD-modified HSA complex [^{14}C]PPD (150 μM [0.2 μCi]) was incubated with HSA (1 mg/ml; in HBSS, pH 7.4; total volume 6 ml) for 16h. After which the reaction was terminated and protein extracted by exhaustive solvent extraction with acetonitrile (4 x 1ml) and reconstituted in 1ml media. The PPD-modified HSA solution was incubated at 37 °C for 130h; duration of LTT. After 0, 4, 24, 48, 72, and 130h an aliquot of the supernatant was collected and analysed by radiometric HPLC for presence of dissociated PPD. Samples were eluted from a Zorbax SB-C18 column (250 mm × 46 mm id; Phenomenex, Macclesfield, Cheshire, UK) at room temperature with a gradient of methanol (5 % for 5 min; 5 – 60 % over 15 min) in 10mM ammonium acetate (pH 6.9); the flow rate was 0.9 ml/min. Mobile phase was delivered by a Kontron 325 pump (Watford, Herts., U.K.). Analytes in the eluate were monitored with a Spectra Physics UV1000 spectraphotometer (254nm; Hemel Hempstead, Herts.,U.K.) and radiolabelled analytes were quantified using a Canberra-Packard Radiometric Flo-One/β radioactivity detector. The eluate was mixed with Ultima-Flo AP scintillant at a rate of 1 ml/min.

4.2.5 Determination of lymphocyte proliferation

Proliferation of allergic patient and tolerant individuals lymphocytes with PPD and PPD-modified HSA (0.1-1000μg/ml) was quantified by measurement of [^{3}H] thymidine, as described previously in Chapter 2 (2.2.5) (Pichler and Tilch, 2004). Proliferative responses were calculated as a stimulation index (SI; c.p.m. in drug-treated cultures/c.p.m. in cultures with DMSO alone). An SI of greater than or equal to 2 was considered significant.

4.2.6 Measurement of lymphocyte cytokine secretion

Supernatants (100µl) were collected from the lymphocyte transformation test of patients and volunteers prior to the addition of [^{3}H] thymidine. Samples from individual patients and volunteers were pooled for each condition (0, PPD, BB or PPD-modified HSA). Concentrations of IL-1α, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IFNγ and TNFα were measured in the supernatants using the human cytokine LINCOplex multiplex assay kit (Milipore) Cytokine content was measured using a Liquichip 100 workstation (Qiagen Ltd, West Sussex, UK) with LiquiChip IS 2.3 software as described in Chapter 2 (section 2.2.9.1)

4.2.7 Determination of purified CD4+ and CD8+ T-cell proliferation

To measure proliferation of purified CD4+ and CD8+ T-cells from allergic patients and tolerant volunteers, PBMC were isolated on a density gradient of Lymphoprep and separated into two aliquots. To generate monocyte-derived dendritic cells, as a source of antigen presenting cell in the proliferation assay, the first aliquot of PBMC was cultured in 24-well plates for 4 h followed by repeated washing to remove non-adherent cells. Adherent cells were then cultured in medium, supplemented with IL-4 (800 U/ml), and GM-CSF (800 U/ml). Half of the medium was replaced with fresh complete medium on days 1, 3, and 5, and immature dendritic cells were ready for use on day 6. The second PBMC aliquot was frozen, then thawed on day 6 and CD4+ and CD8+ T cells were positively selected by incubating PBMC with immunomagnetic microbeads coated with anti-CD4$^+$ or anti-CD8$^+$ antibodies and magnetic cell sorting (Miltenyi Biotec, Bisley, UK). The purity of the sorted T-cells was determined by flow cytometry (Coulter EPICS XL-MCL flow cytometer; Beckman Coulter Inc,

Fullerton, Calif) with conjugated CD3+, CD4+, and CD8+ antibodies (BD Biosciences, Oxford, UK).

To test the antigen-specificity of the purified CD4+ and CD8+ T-cells, T-cells (1×10^5; total volume, 0.2 mL) were incubated with autologous irradiated (60 Gy) dendritic cells (0.2×10^5) and PPD or BB (0.5 - 2 μg/ml). After 5 days, [^{3}H] thymidine was added and proliferation measured by scintillation counting.

Supernatant was collected and stored from antigen-stimulated PBMC and purified T-cell cultures prior to the addition of [^{3}H] thymidine, for the analysis of IL-5, IL-13 and IFN-γ secretion using methods outlined above (Section 4.2.6).

4.2.8 Statistical analysis

The Mann-Whitney test was used for comparison of control and test values, accepting $P < 0.05$ as significant.

4.3 Results

4.3.1 *p*-Phenylenediamine binds irreversibly to the model protein human serum albumin

[^{14}C]PPD binding was detectable after 2 h and increased steadily over a 72 h period (Figure 4.1a). Gel electrophoresis followed by band excision and scintillation counting for incorporated radioactivity established that [^{14}C]PPD binds irreversibly to HSA (Figure 4.1c). Radioactivity was not associated with non-HSA containing bands (Figure 4.1d). Presence of GSH significantly reduced binding of PPD to HSA (Figure 4.1b&e)

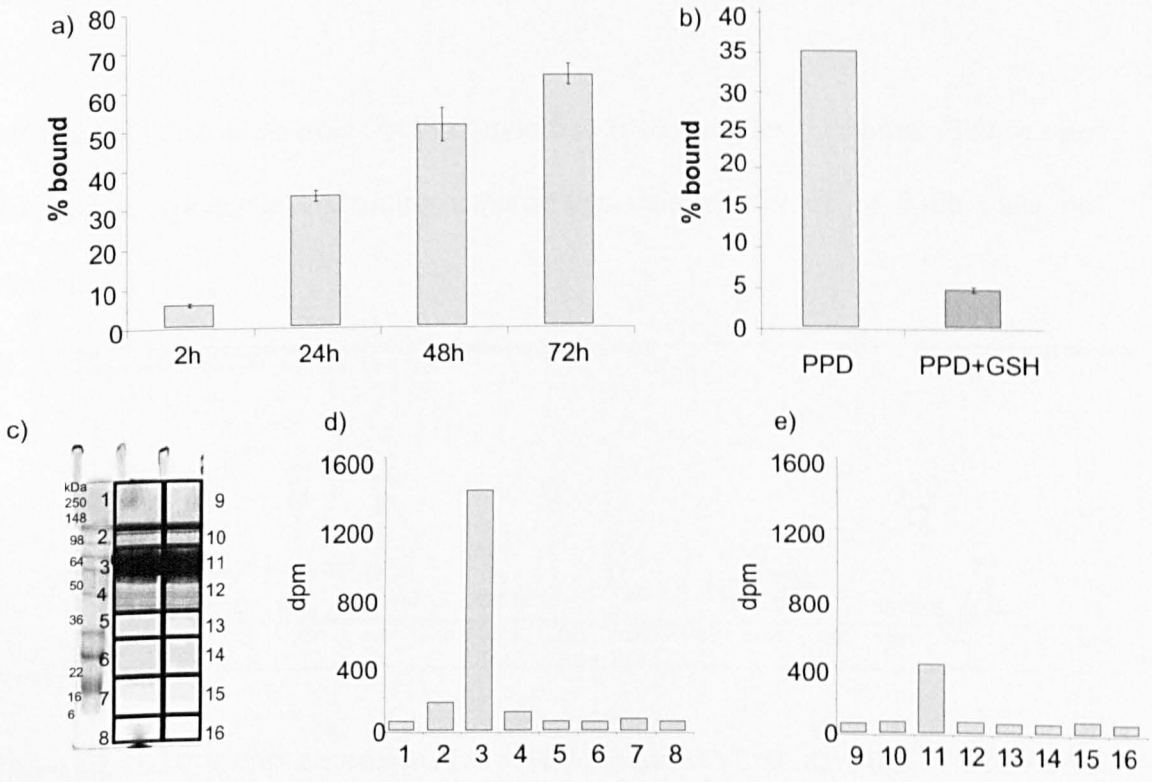


Figure 4.1. Irreversible binding of PPD to HSA. [^{14}C]PPD (150 μM [0.2 μCi]) was incubated with HSA (1 mg/ml; in HBSS, pH 7.4; total volume 6 ml) for 0–72h (a) Time-dependent binding of [^{14}C]PPD to HSA measured by scintillation counting. (c)[^{14}C]PPD-treated HSA separated by electrophoresis on SDS-polyacrylamide gels under reducing conditions. (d) Bands excised and incorporated [^{14}C]PPD determined by scintillation counting. (b&e) GSH reduced binding of PPD to HSA

4.3.2 Selective *p*-phenylenediamine modification of human serum albumin at cysteine 34

PPD was incubated for 16h with HSA at a 10:1 ratio (chemical to protein). Samples were reduced and alkylated and methanol precipitated to remove unbound material before tryptic digestion and LC-MS/MS analysis. PPD modification of the cys-34 containing peptide 21-41 (ALVIAFAQYLQQC PFEDHVK) was observed; the peptide mass increased from 2432.6 (3+, m/z 811.8) to 2538.6 (3+, m/z 847.2) corresponding to an increase of 106amu. Fragmentation of *m/z* 847.2 ion revealed the y8 fragment ion at *m/z* 1080, corresponding to Cys34 plus 106 amu, confirming a direct cysteine modification (Figure 4.2). No modification of lysine residues as detected by Eilstein *et al.*, (13) was detected.

PPD did not dissociate from the PPD-modified HSA complex. Unbound PPD was not detected in conjugate containing supernatants cultured for up to 130h (data not shown).

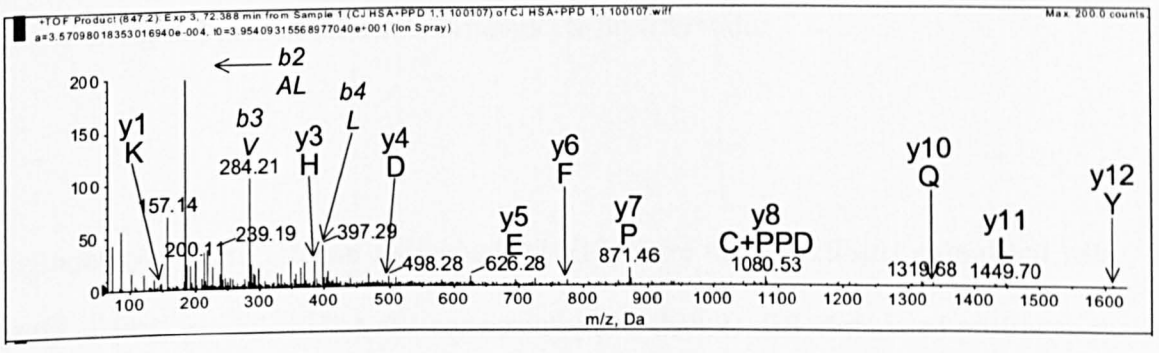
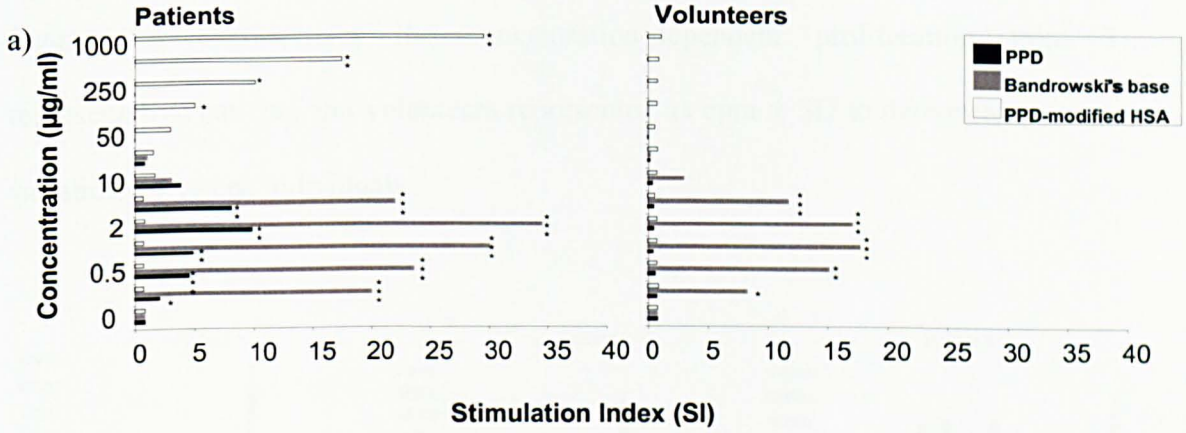


Figure 4.2. (a) LC-MS/MS sequence analysis of HSA peptide ALVLIAFAQYLQQCPFE DHVK MR 2538.6; 3+,m/z 847.2. (unmodified peptide 2432.6; 3+,m/z = 811.8 D 106) Cys34 shows PPD modification

4.3.3 *p*-phenylenediamine and *p*-phenylenediamine-modified human serum albumin stimulate lymphocytes from allergic patients

Lymphocytes from allergic patients (n=10) were found to proliferate following *in vitro* stimulation with PPD and PPD-modified HSA (Table 4.1). Low PPD concentrations (0.1-10μg/ml; SI 10.02 at 2μg/ml) stimulated a T-cell response, while higher concentrations inhibited lymphocyte proliferation; a phenomenon associated with the previously described *in vitro* toxicity of PPD (Coulter *et al.*, 2007; Figure 4.3a). Significantly higher concentrations of PPD-modified HSA (250-2000μg/ml) were required to stimulate a proliferative response (SI 29.68 at 1000μg/ml; Figure 4.3a), however, direct comparison of the strength of the induced proliferative response with equivalent PPD concentrations associated with HSA and soluble PPD (figure 4.3b), revealed that irreversibly bound PPD represents a more potent antigenic signal. Importantly, unbound PPD was not detected by radiometric HPLC, mass spectrometry or scintillation counting in experiments containing PPD-modified HSA; indicating that the observed response of lymphocytes was not due to PPD itself. Untreated HSA (up to 2 mg/ml) did not stimulate lymphocyte proliferation.

Lymphocytes from tolerant individuals (10/12) were not specifically stimulated with PPD or PPD-modified HSA (SI consistently less than 2). BB stimulated proliferation in patient and volunteer cells (SI 34.70 & 17.87 at 2μg/ml respectively).



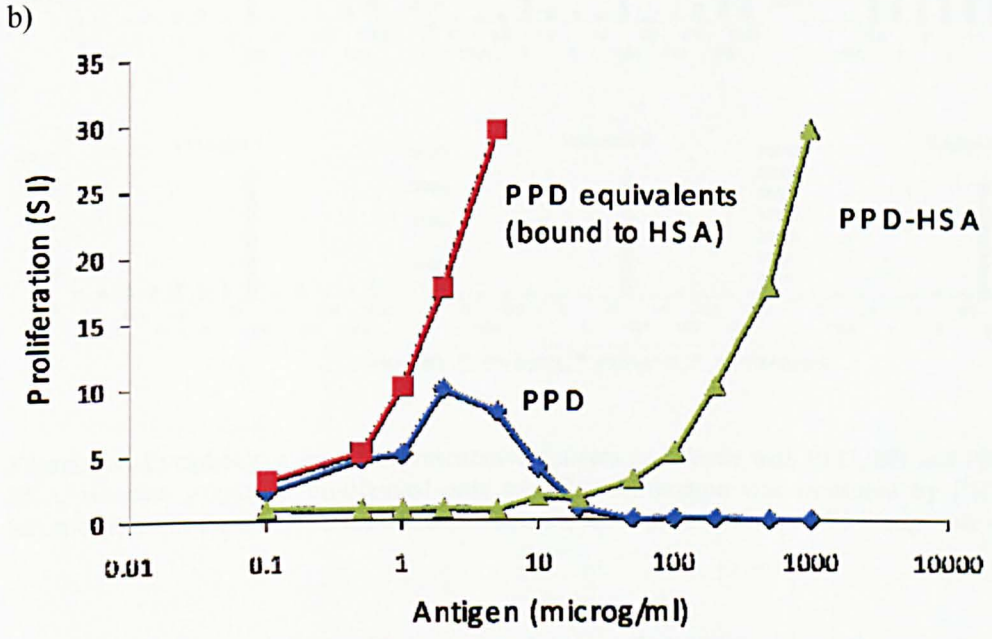


Figure 4.3 Antigen specific stimulation of lymphocytes from allergic patients and volunteers. Mean proliferative responses of lymphocytes from 10/10 allergic patients and 10/12 volunteers. PPD and PPD-modified HSA stimulate proliferation of lymphocytes from allergic patients, but not volunteers. BB stimulates proliferation in both sets. Proliferation was determined by incorporation of [^{3}H] thymidine. Statistical analysis compares incubations in the presence and absence of antigen ($^*P < 0.05$; $^{**}P<0.01$; $^{***}P<0.001$). (b) Lymphocyte proliferation with PPD and PPD-modified HSA with a curve superimposed to represent the estimated concentration of PPD associated with HSA at each concentration tested

Figure 4.4 characterises the concentration-dependent proliferation from 3 representative patients and volunteers represented as cpm ± SD to demonstrate inter-variability between individuals.

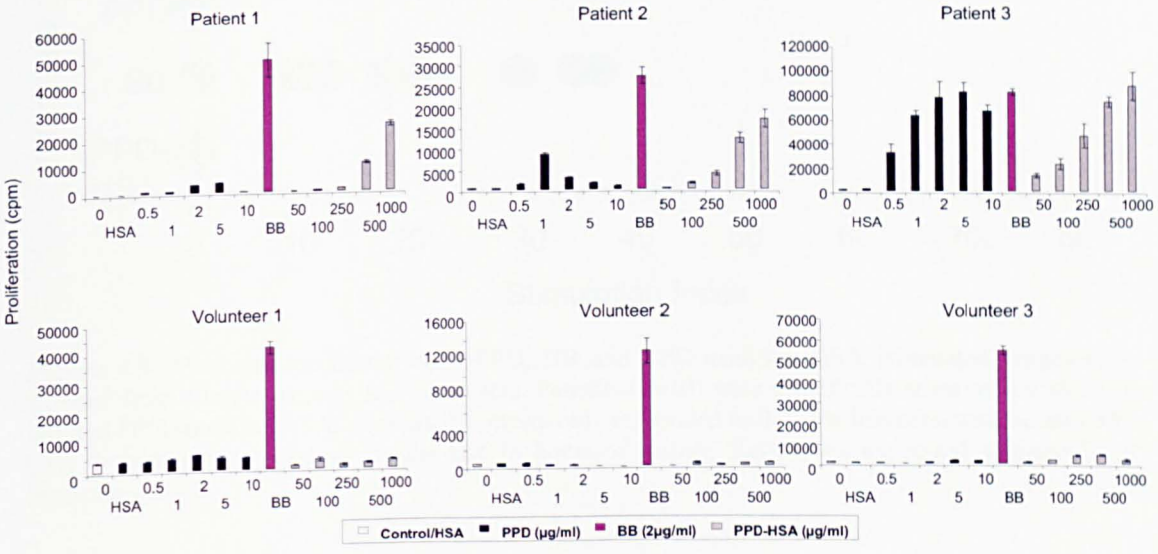


Figure 4.4. Lymphocytes from 3 representative patients proliferate with PPD, BB and PPD-modified HSA, whereas volunteers proliferated only to BB. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16 hours of culture. Results are expressed as mean SI of triplicate culture.

Figure 4.5 shows the maximum stimulation of patient and volunteer lymphoctyes when exposed to PPD, BB and PPD-modified HSA. Although large inter-individual variations were seen with both experimental groups, consistent stimulation of patient cells to PPD, BB and PPD-modified HSA was observed. In contrast stimulation of volunteer cells was only seen with BB.

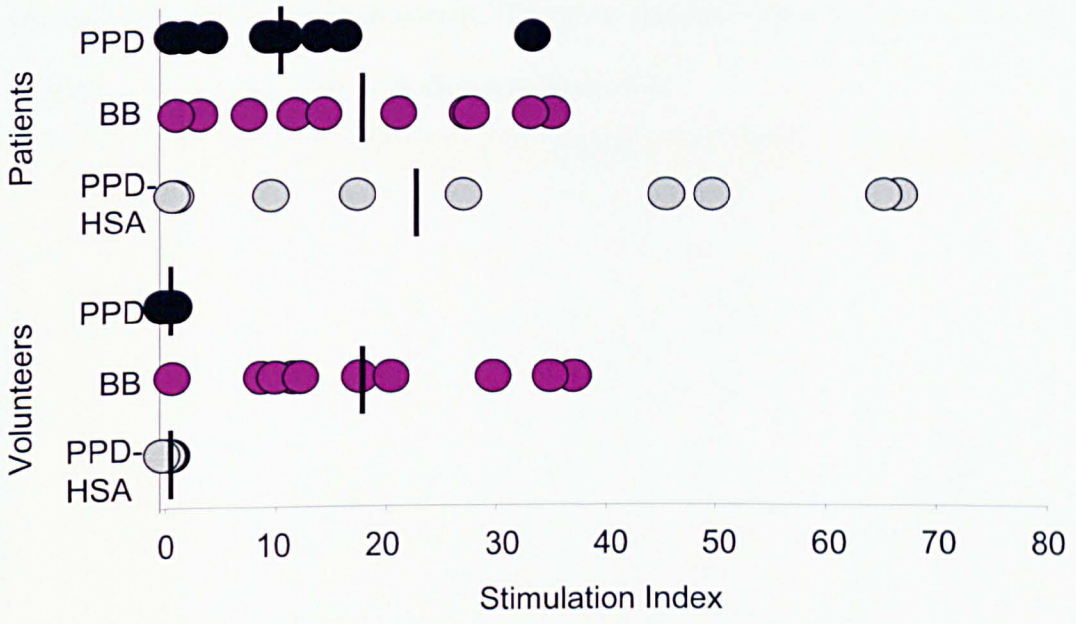


Figure 4.5. Maximum proliferation of PPD, BB and PPD-modified HSA stimulated lymphocytes isolated from allergic patients and volunteers. Patients (n=10) were specifically stimulated with PPD, BB and PPD-modified HSA, whereas volunteers only responded to BB. Proliferation was measured by [^{3}H] thymidine incorporation for the last 16 hours of culture. Results are expressed as mean SI of triplicate culture

4.3.4 Cytokine secretion from lymphocytes stimulated with *p*-phenylenediamine, bandrowski's base and *p*-phenylenediamine-modified human serum albumin

Supernatants were collected from PPD, BB and PPD-modified HSA exposed allergic patient and volunteer lymphocytes cultures and analysed for cytokine secretion using Luminex technology.

Samples were analysed per well using a minimum of 50 beads per region. The raw data (mean fluorescence intensity, MFI) were captured using LiquiChip IS 2.3 software; for data analysis, a 5-parameter logistic (5-PL) or curve-fitting method for calculating cytokine/chemokine concentrations in samples was applied to each standard curve and sample concentrations were interpolated from the standard curve. The gating specifications / settings ranged from 8,060 to 13,000 with a limit of detection (LOD), defined as the lowest concentration of analyte that can be detected, of 3.2 pg/ml. Standard curves for culture supernatant for all 10 cytokines were

generated in eight individual assays, the mean standard curve for two representative cytokines, IL-13 and TNF-α, is shown in figure 4.6.

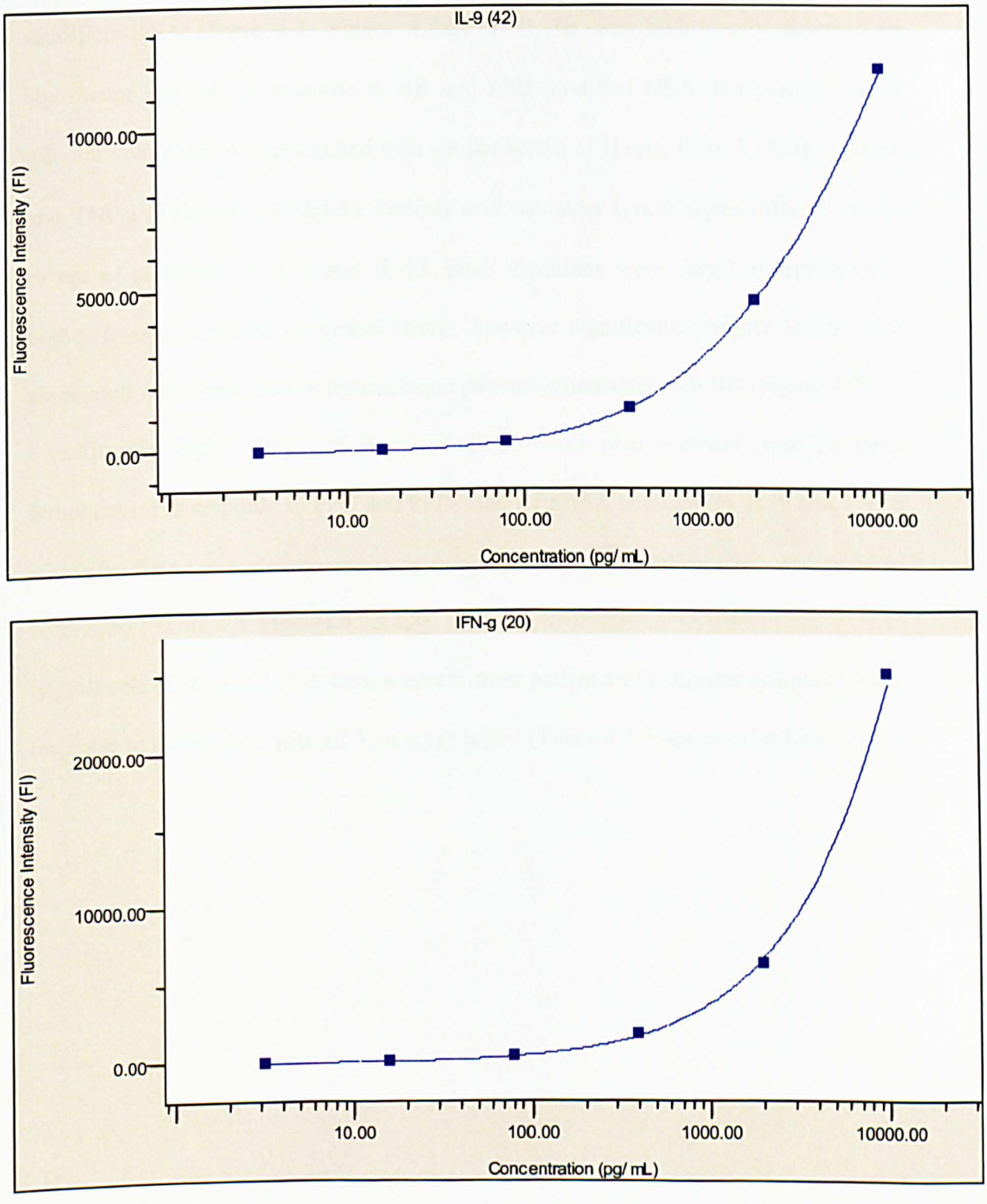


Figure 4.6. Standard curve of IL-9 (top) and IFNγ (bottom)

Significant quantities (>10pg/ml) of IL-5, IL-6, IL-8, IL-9, IL-13, IFNγ and TNFα were detected from allergic patient lymphocytes stimulated with PPD, BB and PPD-modified HSA (Table 4.2, Figure 4.7&4.8). IL-1α was additionally detected in significant amounts in response to BB and PPD-modified HSA. BB stimulation of tolerant volunteers was associated with similar levels of IL-1α, IL-6, IL-8, IL-9, IFNγ and TNFα (Table 4.2, 4.7&4.8). Patients and volunteer lymphocytes differed in the extent of secretion of IL-5 and IL-13. Both cytokines were found at significantly higher levels compared to control levels, however significantly higher levels were associated with lymphocytes from allergic patients stimulated with BB (Figure 4.7). Significantly higher levels of IL-5 and IL-13 were also secreted from patients' lymphocytes in response to PPD and PPD-modified HSA stimulation. IL-9 and TNFα were also found in higher quantities in response to this stimulation when compared to volunteers (Table 4.2, Figures 4.7&4.8).

High levels of IL-6 and IL-8 were secreted from patient and volunteer lymphocytes in response to stimulation with all 3 antigens tested (Table 4.2, Figures 4.7&4.8).

Table 4.2. Cytokine secretion after exposure of patient and volunteer lymphocytes to PPD, BB and PPD-modified HSA*

		Cytokines pg/ml									
	Antigen (SI)	IL-1	IL-4	IL-5	IL-6	IL-8	IL-9	IL-10	IL-13	IFNγ	TNFα
Patients n=10	PPD (10.1±3.2)	2.8 ±0.9	4.3 ±1.4	524.8 ±166.1	1329.8 ±420.8	730.9 ±231.3	48.1 ±15.2	2.7 ±0.8	1684.3 ±533	70.2 ±22.2	230.7 ±73
	BB (34.7±10.9)	29.2 ±9.2	1.7 ±0.5	248.3 ±78.6	2157.5 ±682.7	695.6 ±22.1	90.7 ±28.7	6.6 ±2.1	1389.7 ±439.8	291.2 ±92.2	164.3 ±52
	PPD-HSA (29.7±9.4)	52.2 ±16.5	1.1 ±0.3	76.5 ±24.2	4261.8 ±1348.7	1024.63 ±324.3	91.2 ±28.9	16.3 ±5.1	899.4 ±284.6	270.4 ±85.6	410.1 ±129.8
Volunteers n=10	PPD (1.8±0.6)	0±0	0±0	0.1±0	777.7 ±246.1	345.9 ±109.5	0±0	0±0	0±0	2.5 ±0.8	0±0
	BB (17±5.4)	72.1 ±22.8	0.3 ±0.1	26.9± 8.5	1962.9 ±621.2	1302.1 ±412.1	15.2 ±4.8	9.9 ±3.2	158.9± 50.3	34.1 ±10.8	77.01 ±24.4
	PPD-HSA (1.82±0.6)	27.9 ±8.8	1.8 ±0.6	0.1±0	2100.1 ±664.6	621.8 ±196.8	1.1 ±0.4	8.1 ±2.6	12.8 ±4	4.02 ±1.3	65.7 ±20.8

* Lymphocytes from allergic patients (n=10) and volunteers (n=10) were exposed to PPD, BB, PPD-modified HSA and cell culture medium (negative control) for the duration of the LTT. The concentration of cytokine secretion was measured using Luminex technology. Data are represented as mean ± SD.

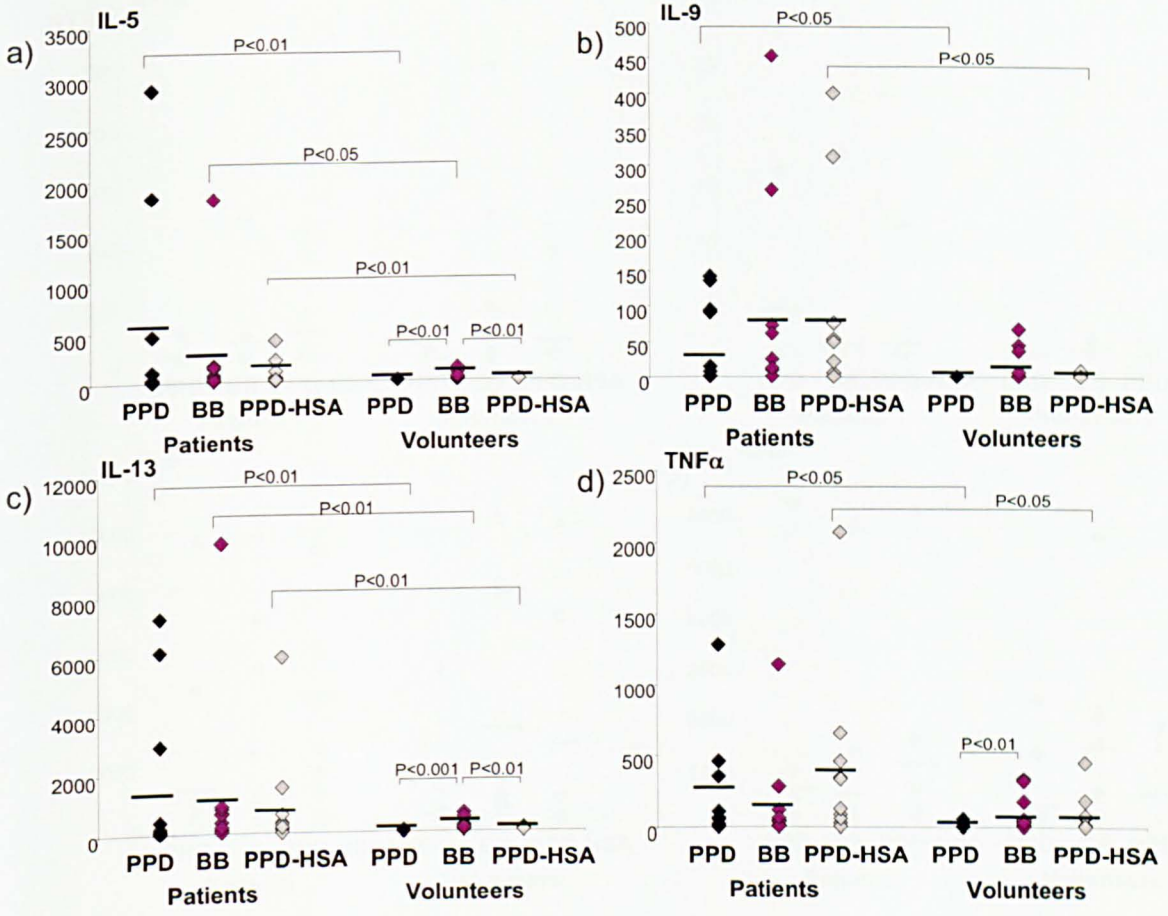


Figure 4.7. IL-5(a), IL-9 (b), IL-13 (c) and TNFα(d) secretion from patient (n=10) and volunteer (n=10) lymphocytes in response to PPD, BB and PPD-modified HSA exposure. Lymphocytes were incubated with PPD and BB for the duration of the LTT, prior to addition [3H] thymidine incorporation, aliquots of supernatant were taken and the sample concentration which provided the maximal proliferative response in the LTT was analysed using the human cytokine / chemokine LINCOplex multiplex assay kit. Statistical analysis was performed by comparing incubations of patients and volunteers. Co-efficient of variation was consistently less than 20%.

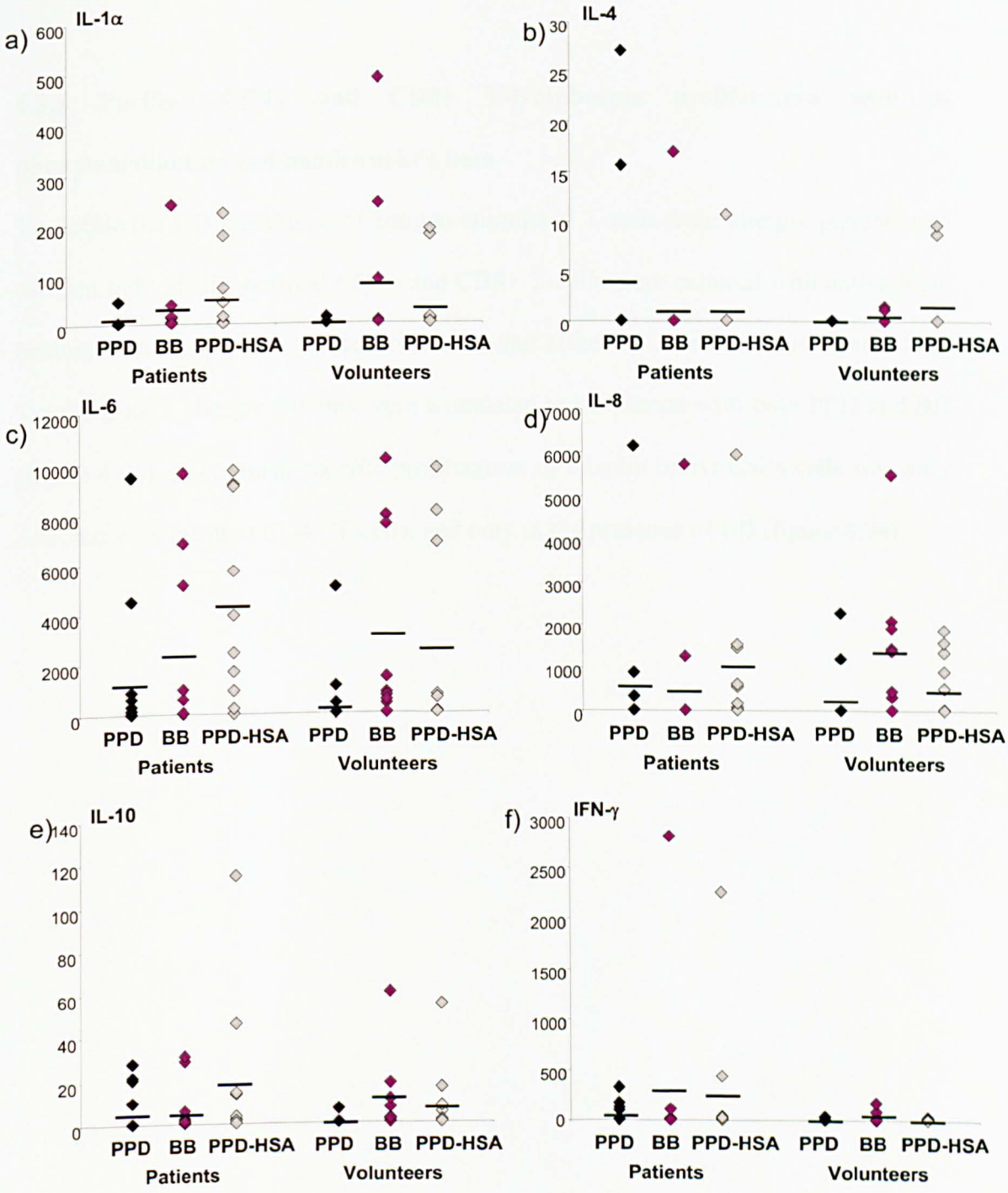
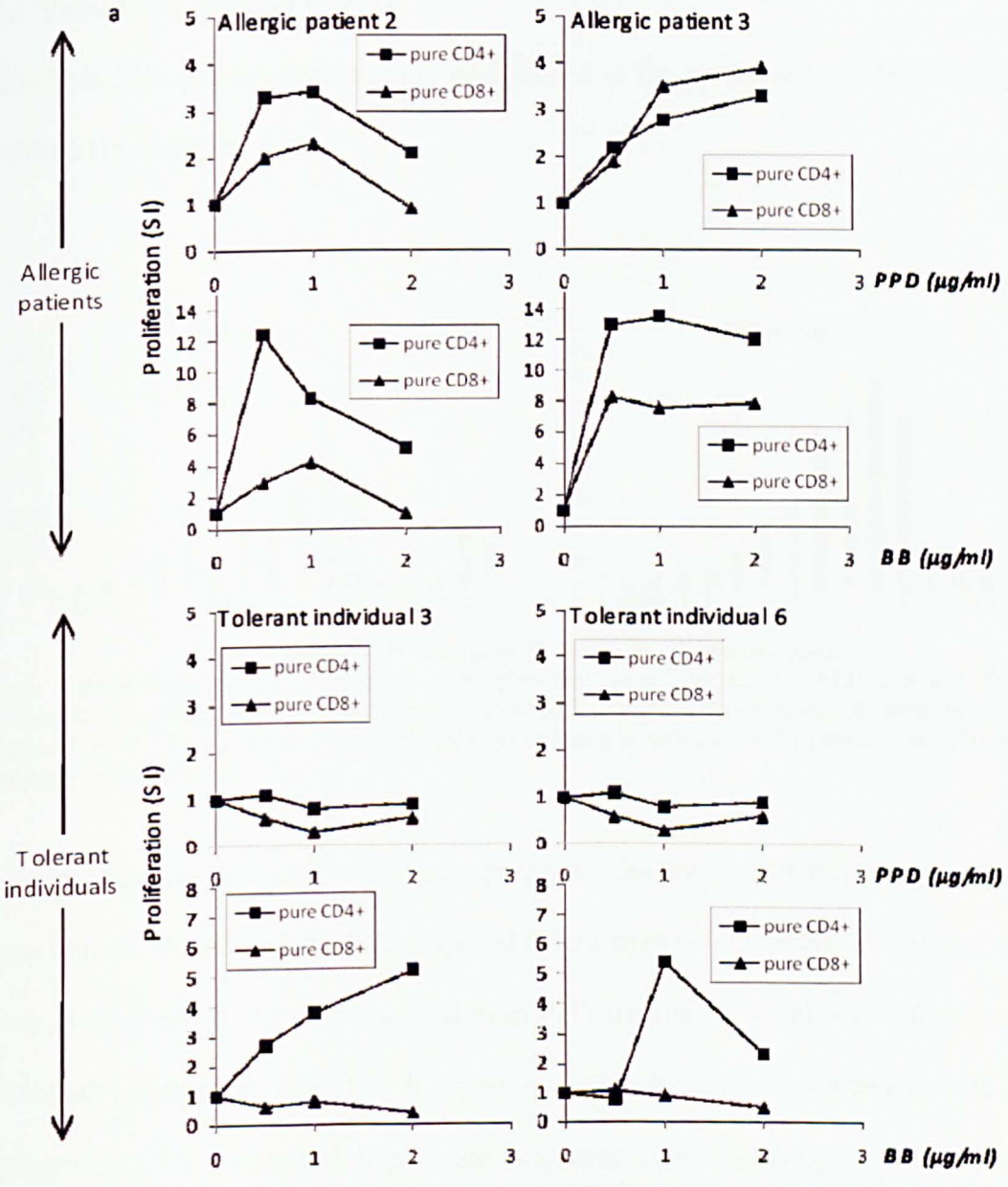


Figure 4.8. IL-1-α (a), IL-4 (b), IL-6 (c), IL-8 (d), IL-10 (e) and IFNγ (f) secretion from patient (n=10) and volunteer (n=10) lymphocytes in response to PPD, BB and PPD-HSA exposure. Lymphocytes were incubated with PPD, BB & PPD-modified HSA for the duration of the LTT, prior to addition [3H] thymidine incorporation, aliquots of supernatant were taken and the sample concentration which provided the maximal proliferative response in the LTT was analysed using the human cytokine LINCOplex multiplex assay kit. No significant difference was found in secretion of these cytokines.

4.3.5 Purified CD4+ and CD8+ T-lymphocyte proliferation with *p*-phenylenediamine and bandrowski's base

To define the CD phenotype of antigen-stimulated T-cells from allergic patients and tolerant individuals, purified CD4+ and CD8+ T-cells were cultured with autologous, irradiated monocyte-derived dendritic cells and either PPD or BB. CD4+ and CD8+ T-cells from 2 allergic patients were stimulated to proliferate with both PPD and BB (figure 4.9a). In contrast, specific proliferation of tolerant individual's cells was only detected with purified CD4+ T-cells, and only in the presence of BB (figure 4.9a).



	Antigen	IL5 (pg/ml)		IL-13 (pg/ml)		IFNγ (pg/ml)	
		CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
Allergic patients	PPD	914	768	1583	849	<10	<10
	BB	1757	757	3444	1137	<10	<10
Tolerant volunteers	PPD	62	87	<10	42	<10	<10
	BB	393	203	993	127	<10	<10

Figure 4.9. Stimulation of CD4+ and CD8+ T-cells from allergic patients and tolerant individuals with PPD and BB. (a) Antigen-specific proliferation of CD4+ and CD8+ T-cells in the presence of autologous dendritic cells as antigen presenting cells. Proliferation was determined by incorporation of [^{3}H]-thymidine. Coefficient of variation was consistently less than 20%. (b) IL-5, IL-13 and IFN-γ secretion from allergic patient and tolerant individual CD4+ and CD8+ T-cells in response to PPD and BB.

4.3.6 Volunteers respond to PPD

Cells from 2/12 patients significantly proliferated in the presence of PPD and PPD-modified HSA (Figure 4.10).

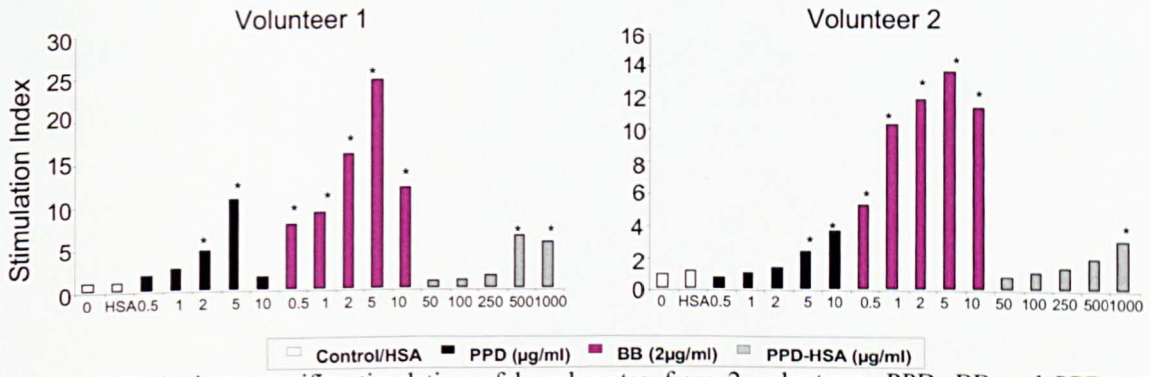


Figure 4.10 Antigen specific stimulation of lymphocytes from 2 volunteers. PPD, BB and PPD-modified HSA stimulate proliferation of lymphocytes the 2 volunteers Proliferation was determined by incorporation of [^{3}H] thymidine. Statistical analysis compares incubations in the presence and absence of antigen ($^*P < 0.05$).

Volunteer one has no known previous exposure to hair dye, but multiple exposure to henna tattoos. Volunteer 2 has been exposed to hair dyes on numerous occasions. Low levels of IL-5 and IL-13 were secreted from PPD stimulated lymphocytes from both individuals (Volunteer 1 IL-5 – 0.19pg/ml; IL-13- 0.52pg/ml; Volunteer 2 IL-5- 1.23pg/ml; IL-13- 1.21pg/ml). Significant quantities of the regulatory cytokine IL-10 were not detected (Volunteer 1 – 2.76pg/ml; Volunteer 2 – 0.09pg/ml).

4.4 Discussion

Allergic contact dermatitis, a delayed-type, cell mediated immune reaction, occurs in response to several electrophilic low molecular weight molecules. PPD, a chemical found in many consumer products and associated with a high incidence of allergic reactions (McFadden *et al.*, 2007) is able to induce dendritic cell co-stimulatory signalling (Coulter *et al.*, 2007; Aeby *et al.*, 2008; Hulette *et al.*, 2005) and provide the antigenic signals (Sieben *et al.*, 2002; Krasteva *et al.*, 1993) required to stimulate T-cell responses.

As shown in chapter two, T-cell responses to the PPD oxidation product BB have been detected with lymphocytes from allergic patients and tolerant individuals; however, T-cell stimulation with PPD itself is discernable with lymphocytes from allergic patients alone. Thus, detection of PPD-specific T-cell responses represents an important discrimination between allergic and tolerant patient groups and is the reason this chapter focuses on characterization of T-cell responses to PPD and primary products of auto-oxidation, but not synthetic BB. In chapter 3 we have shown that PPD binds selectively to cysteines residues of model peptides and the protein GSTP. In this chapter we chose HSA as a model protein to investigate the T-cell stimulatory capacity of a conjugated form to PPD.

Since the landmark studies of Landsteiner and Jacobs (1935), reactivity of chemical allergens towards nucleophilic amino acid residues on protein has been coupled with sensitization potential (Basketter *et al.*, 1997). This resulted in the hypothesis that binding of chemical allergens to specific loci on protein might be associated activation of immune cells. The strong sensitizer 2,4-dinitrochlorobenzene binds to a large number of cellular proteins (Pickard *et al.*, 2007); however, studies using

isolated proteins have identified a highly restricted modification of nucleophilic amino acids residing in specific microenvironments of the protein structure (Aleksic *et al.*, 2007; 2008). In this chapter, PPD bound to the single cysteine residue on HSA existing in a reduced form (cys-34) (Figure 4.2). Cys-34 is attached to the hydroxyl oxygen of Tyr84 by a hydrogen bond in unmodified HSA (Stewart *et al.*, 2005). PPD ligation will disrupt this hydrogen bonding interaction inducing a conformational change that alters functional activity (Kawakami *et al.*, 2006) and possibly generating new antigenic determinants.

PPD modification of HSA at both cys-34 was characterized by the addition of 106 amu on the relevant tryptic peptide, corresponding to the addition of a single molecule of PPD and not secondary oxidation products, including BB. As illustrated by Eilstein *et al.*, (2006) the reaction likely proceeds via a classical nucleophilic mechanism (Chapter 3; Scheme 1). Unlike previous studies exploring reaction profiles with isolated nucleophilic amino acids and small peptides, excluding cysteine, hapten-protein complex formation with other amino acid residues (e.g., lysine and tryptophan) was not detected. These findings imply that covalent modification of cysteine residues predominate over alternative conjugating pathways in protein; hence, the reason for investigating the antigenic potential of PPD and PPD-modified HSA with lymphocytes and T-cell clones from allergic patients.

In initial *in vitro* experiments, the lymphocyte transformation test, which is a sensitive and specific assay to diagnose drug allergy and contact sensitisation (Wu *et al.*, 2006; Sieben *et al.*, 2002; Pickard *et al.*, 2007; Pichler and Tilch 2004; Naisbitt *et al.*, 2005), was used to explore lymphocyte stimulation with PPD and PPD-modified HSA. In

agreement with previous work (Coulter *et al.*, 2007; Sieben *et al.*, 2002) lymphocytes from allergic patients (10/10), but not the majority of tolerant volunteers (2/12), were stimulated to proliferate in response to low concentrations of PPD; higher concentrations inhibited proliferation due to the direct toxicity of PPD in culture (Figure 4.3). The strength of the proliferative response observed (SI greater than 10 in 7/10 patients) compares favourably with most drug and chemical antigens (Pichler and Tilch 2004; Nyfeler and Pichler 1997), which indicates that exposure of lymphocytes to PPD directly represents an important antigenic determinant in many patients. Patient lymphocytes were also stimulated to proliferate vigorously with PPD-modified HSA. Although much higher concentrations of PPD-modified HSA were required to trigger a proliferative response, relative levels of PPD in culture are estimated, based on differences in molecular weight and the number of PPD molecules bound, to be 2-3 orders of magnitude lower. Of particular importance to our discussion was an absence of detectable free PPD in PPD-modified HSA cultures that might be immuno-stimulatory; analyses performed included radiometric HPLC, LC-MS and scintillation counting.

Interestingly, the response of lymphocytes from two patients (patients 6 and 8) differed in terms of their preferred antigenic determinant (i.e., PPD or PPD-modified HSA; table 1); possibly highlighting alternative pathways of antigen presentation. Lymphocytes from 10/12 tolerant volunteers did not proliferate in the presence of PPD or PDD-modified HSA. Interestingly 2/12 volunteers lymphocytes proliferated significantly in the presence of PPD and PPD-modified HSA. One volunteer has previously been exposed to hairdye on a number of occasions, whereas the other hadn't. The cytokine secretion from the PPD samples was not significantly different to the volunteer samples, possibly indicating the presence of tolerant/ regulatory cells.

However no increase in IL-10 was detected. Due to limited ethical approval no patch test could be performed on these volunteers to determine if this PPD associated proliferation is translated into a physical response. More work needs to be done of characterising T cells present in order to determine if this is a true response.

To explore fundamental functional differences between patient and volunteer responses to PPD and PPD-modified HSA we used cytokines as a measurement. It is well documented that ACD is a common eczematous disease mediated by T-cells that occurs in the skin at the sites of contact with small chemicals, such PPD, therefore functional studies assessing cytokine secretion following hapten stimulation are important as cytokines are known to directly related to the induction of specific chemical manifestations of the disease. Results from Coulter et al (unpublished data) showed that PPD-specific proliferation was associated with secretion of high levels of Type 2 cytokines in allergic patients but not volunteers. Therefore, using a new patient and volunteer cohort we aimed to see if IL-5 and IL-13 secretion represents a true functional difference between patient groups that could be used as a functional biomarker to diagnose PPD-mediated contact dermatitis.

Previously, understanding of the cellular basis of contact dermatitis has been derived from of murine models. Yokozeki et al (2003), using PPD-challenged mice found Th2 cytokines, IgE antibodies and mast cells are crucial in the development of tissue pathology. Coulter et al (unpublished data) examined the nature of secreted cytokine profile between patients and volunteers lymphocytes to characterise the functionality of the hypersensitivity response. Significantly higher levels of Type 2 cytokines IL-4, IL-5 and IL-13 secretion were associated with patient lymphocytes exposed to PPD

and BB, suggesting that measurement of these cytokines could act as a potential biomarker to discriminate between allergic and non-allergic individuals.

Using a new patient and volunteer cohort we have examined the functionality of the antigen-specific cells by measuring cytokine secretion after exposure to PPD, BB and PPD-modified HSA.

Lymphocytes from allergic patients and tolerant volunteers secreted IL-1α, IL-5, IL-6, IL-8, IL-9, IL-13, IFNγ and TNFα in response to BB stimulation (Table 4.2). Significantly higher levels of IL-5 (90%) and IL-13 (90%) were secreted from patient lymphocytes stimulated with BB compared to volunteer levels.

Secretion of the Type 2 cytokines IL-5 and IL-13 supports previous studies indicating that secretion of Type 2 cytokines after antigen stimulation may relate to an individuals susceptibility towards contact dermatitis. IL-5 and IL-13 secretion have previously been shown drive IgE production by activation of antigen-specific T-cells (Dearman *et al.*, 2002). However, it is also possible that decreased cytokine secretion in BB-treated volunteer PBMC might be a reflection that only one T-cell population is responding and that perhaps certain Th2 producing T-cells are missing. To address this issue, purified CD4+ and CD8+ T-cells from allergic patients and tolerant individuals were stimulated with PPD and BB and proliferation and cytokine secretion assessed. CD4+ and CD8+ T-cells from allergic patients proliferated and secreted high levels of the Th2 cytokines IL-5 and IL-13 following PPD and BB stimulation. In contrast, in tolerant individuals, proliferation and low levels of cytokine secretion was only detected in the CD4+ T-cell population. Detection of antigen-specific, cytokine secreting CD8+ T-cells from allergic patients alone provides an explanation our observed results with PBMC and supports to hypothesis that CD8+ T-cells are important effectors in contact allergic dermatitis (Vocanson *et al.*, 2006;2008).

Patients lymphocytes also secreted significantly higher levels of IL-9 and TNFα (as well as IL-5 and IL-13) in response to PPD and PPD-modified HSA stimulation, when compared to volunteers. Increased levels of IL-9 secretion from allergic patients lymphocytes has not been previously described or associated with contact dermatitis. IL-9 is a type 2 cytokine produced by T-cells, eosinophils and mast cell (Kajiyama *et al.*, 2007). It promotes IgE production by B-cells and is thought to be important in the development of certain allergic diseases. It has also been shown to synergise with IL-13 to increase eotaxin release and IL-5 to drive eosinophil maturation (Fawaz *et al.*, 2007). The increase in TNFα secretion from patient cells may be associated may relate to the recently described genetic association between TNFA-308 G/A polymorphism and susceptibility toward contact allergy to chemicals such as PPD (Blomeke *et al.*, 2008).

As regards to the high secretion levels of IL-6 and IL-8, both are associated with inflammatory conditions (IL-6 is produced by a variety of cells such as keratinocytes, dendritic cells and T-cells and is known as the principal endogenous circulating pyrogen; IL-8 is a chemotactic protein produced by a variety of cells including macrophages), perhaps their presence suggests that there is some sub clinical response to PPD (and PPD-modified HSA) in volunteers that manifests differently to that seen in allergic patients.

In conclusion, we have shown that cysteine is the primary target of PPD modification on HSA and identified PPD hapten protein complex formation as an important mechanistic step in the stimulation of T-cells from allergic patients. Individual susceptibility is likely dependent on several factors including those relating to

differential levels of PPD exposure, different thresholds for co-stimulatory signalling, expression of as yet undefined HLA haplotypes and cytokine polymorphisms, similar to those recently described for TNFα (Blomeke et al., 2008).

CHAPTER 5

Characterisation of the chemical basis of PPD-specific T-cell activation

5.1 Introduction

Chemicals associated with skin sensitisation are electrophilic, low molecular weight molecules that form strong irreversible bonds with self-protein to generate immunogenic hapten-protein complexes (Divkovic *et al.*, 2005). Antigen-specific T-cells are thought to be stimulated by MHC-associated peptides derived from the hapten-protein complex following processing by antigen presenting cells. However debates on the nature of specific hapten protein binding interactions and the way in which hapten-protein complexes stimulate immune cells are still ongoing. It remains a possibility that haptens bind directly to MHC molecules or MHC embedded peptides to stimulate a specific T-cell response (Pichler, 2003). In fact it is possible that T cells can be activated via 3 different mechanisms – (1) classical hapten mechanism in which a covalently modified protein conjugate is taken up by antigen presenting cells (APC), processed into peptide fragments, and translocates to the cell surface of the MHC for presentation to T cells, ie; as shown for the model chemical allergen DNCB. Generation of specific clones demonstrated that chemical bound to a protein requires metabolic processing by APC to form hapten-peptide MHC complexes in order to initiate a T cell response (Pickard *et al.*, 2007); (2) through direct binding to the MHC molecules expressed on the cell surface of APC, avoiding the requirement of APC processing machinery as shown for nickel; (3) Non-reactive or chemically inert compounds have been shown to undergo process independent and non-covalent binding mechanisms which allows the chemical to bind directly to the MHC and activate T-cells, as shown for nickel (Sieben *et al.*, 2002; Naisbitt *et al.*, 2003; Zanni *et al.*, 1998).

Brander *et al.*, (1995) characterised mechanisms of antigen presentation using free penicillin and penicillin bound to human serum albumin using T-cell clones specific

for each compound. They found that with free penicillin processing was not required and the penicillin specific clones still proliferated in the presence of fixed antigen presenting cells. However the penicillin-bound HSA had to undergo processing in order to stimulate the specific clones. It is possible therefore that more than one mechanism of T-cell activation is involved in the induction of allergic contact dermatitis in patients.

Even though PPD is one of the most frequently encountered sensitisers, little is known about how this small compound elicits an allergic response. Data obtained from chapter 2,3 & 4 have shown that; first, only PPD and not its oxidation product BB will elicit a proliferative response in lymphocytes from allergic patients (section 2.1, data also supported by the generation of PPD-specific T-cells from patient cells but not from volunteers), secondly that PPD binds selectively to cysteine residues of small designer peptides, and the proteins GSTP and HSA (sections 3.1 & 4.1) and thirdly that allergic patients lymphocytes will specifically proliferate in the presence of PPD-modified HSA (section 4.2). Despite this the mechanism by which PPD stimulates T-cell proliferation hasn't been fully elucidated. Sieben *et al.*, (2002) suggested that PPD might be recognised by T-cells via a processing independent pathway ie: - via direct binding to MHC and/or the T-cell receptor. Data shown in chapter 2 show that a longer incubation time of 16h, which is the time required for optimal hapten-protein complex formation, resulted in proliferative response. This suggests that PPD protein complexes may be recognised via a processing dependent pathway.

Therefore, the aim of this chapter was to use PPD and PPD-modified human serum albumin as antigens for T-cell clones from allergic patients in order to characterize mechanisms of PPD-specific T-cell activation.

5.2 Materials and Methods

5.2.1 Culture medium for lymphocyte culture and T-cell cloning

Culture medium consisted of RPMI-1640 supplemented with pooled heat-inactivated human AB serum (10%, v/v), HEPES (25 mM), L-glutamine (2 mM), transferrin (25 $\mu g\ ml^{-1}$), streptomycin (100 $\mu g\ ml^{-1}$) and penicillin (100 U ml^{-1}). T-cell lines and clones were maintained in culture medium supplemented with IL-2 (25U/ml).

5.2.2 Cell lines

EBV-transformed B-cell lines (referred to as antigen presenting cells) were generated from peripheral blood lymphocytes of allergic patients using supernatant from the EBV producing cell line B9-58 as described previously in Chapter 2 (Section 2.2.4). Cell lines were maintained in medium consisting of RPMI-1640 supplemented with 10% FCS, HEPES (25 mM), L-glutamine (2 mM), streptomycin (100 $\mu g\ ml^{-1}$) and penicillin (100 U ml^{-1}).

PPD was obtained from Sigma Chemical Co (Poole, Dorest, UK). [^{14}C]PPD (specific activity 60mCi/mmol) was synthesized by GE Healthcare (Bucks, UK). Stock solutions (10 mg ml^{-1}) were prepared in culture media and dimethyl sulphoxide (4:1 v/v) and diluted as required.

5.2.3 Donor Characteristics

Peripheral blood mononuclear cells were isolated from venous blood obtained from 3 PPD allergic, patch test positive patients. Approval for the study was obtained from Liverpool local research ethics committee; informed written consent was obtained from all donors. Clinical characteristics of the allergic patients are shown in Table I.

. **Table 5.1** Clinical details from PPD allergic patients used to generate clones

	Age	Sex	PPD allergy	Clinical symptoms	Other allergies	Months since reaction	Patch test	LTT (SI)	
								PPD	PPD-HSA
Allergic Patients									
1	56	F	Yes	Angiodema/ erythema multiforme	Nickel, perfumes	22	+	15.3	38.2
2	52	F	Yes	Exfoliative dermatitis	E45 cream, orange colouring, biological soap	14	+	12.3	54.9
3	43	F	Yes	Angiodema	Local anaesthetics, Nickel, sunglasses & clothes dye.	96	+	34.9	33.5

5.2.4 p-Phenylenediamine-human serum albumin complex formation

PPD (150 μM) was incubated with HSA (1 mg/ml; in HBSS, pH 7.4; total volume 6 ml) for 16h to permit hapten-protein complex formation for use in cloning assays.

5.2.4.1 Specific amino acid modification of human serum albumin by PPD

Specific amino acid modification of HSA by PPD was determined by incubating HSA (15 μM) with PPD (150 μM; 100μl) for 16h in phosphate buffer at 37°C. Samples were reduced with dithiothreitrol (DTT; 100 mM), alkylated with iodoacetamide (55 mM) and solvent washed with methanol (10:1), to remove unbound material, and suspended in 50 mM ammonium bicarbonate. Samples (100 μg) were digested with modified trypsin (5 ng; in 50mM ammonium bicarbonate) for 16h at 37°C and desalted using C18 ZipTip columns according to the manufacturer's instructions (Millipore, Watford, Hertfordshire). The resultant peptide mixture was reconstituted in acetonitrile/trifluoroacetic acid (5 μl; 5:0.1%). For LC-MS/MS analysis, aliquots of

sample (1μL) were delivered into a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems; Warrington, UK) by automated in-line liquid chromatography (integrated LCPackings System, 5mm C18 nano-precolumn and 75μm x 15cm C18 PepMap column (Dionex, California, USA)) via a nano-electrospray source head and 10μm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 5% acetonitrile/0.05% TFA (v/v) to 48% acetonitrile/0.05% TFA (v/v) in 60mins was applied at a flow rate of 300nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA) (Analyst, Applied Biosystems). Database searching was performed using ProteinPilot version 1 (Applied Biosystems) against the latest version of the SwissProt database, with the confidence level set at 90%. PPD and carboxamidomethyl were set as a high and medium probability user-defined modifications on specific amino acids ie: cysteine.

5.2.4.2 Determination of PPD-modified HSA stability

To assess the stability of the PPD-modified HSA complex [^{14}C]PPD (150 μM [0.2 μCi]) was incubated with HSA (1 mg/ml; in HBSS, pH 7.4; total volume 6 ml) for 16h. After which the reaction was terminated and protein extracted by exhaustive solvent extraction with acetonitrile (4 x 1ml) and reconstituted in 1ml media. The PPD-modified HSA solution was incubated at 37°C for 130h; duration of LTT. After 0, 4, 24, 48, 72, and 130h an aliquot of the supernatant was collected and analysed by radiometric HPLC for presence of dissociated PPD. Samples were eluted from a Zorbax SB-C18 column (250 mm × 46 mm id; Phenomenex, Macclesfield, Cheshire, UK) at room temperature with a gradient of methanol (5% for 5 min; 5 – 60% over 15 min) in 10mM ammonium acetate (pH 6.9); the flow rate was 0.9 ml/min. Mobile

phase was delivered by a Kontron 325 pump (Watford, Herts., U.K.). Analytes in the eluate were monitored with a Spectra Physics UV1000 spectraphotometer (254nm; Hemel Hempstead, Herts.,U.K.) and radiolabelled analytes were quantified using a Canberra-Packard Radiometric Flo-One/β radioactivity detector. The eluate was mixed with Ultima-Flo AP scintillant at a rate of 1 ml/min.

5.2.5 Generation and characterisation of p-phenylenediamine and p-phenylenediamine-protein complex-specific T-cell clones

For generation of T-cell lines, lymphocytes ($1x10^6$; total volume 1ml) from 3 allergic patients were incubated with PPD (2μg/ml) or PPD-modified HSA (1mg/ml). IL-2 was added on day 6 and 9 to maintain antigen specific proliferation. After 14 days T-cell lines were cloned by serial dilution using established methodology (Wu *et al.*, 2006; Naisbitt *et al.*, 2003; Schnyder *et al.*, 1997) and described in Chapter 2.2.8.

To test the specificity of the clones, T-cells (0.5×10^5; total volume 0.2 ml) were incubated with irradiated (60 Gy) autologous Epstein-Barr transformed B-cells (B-LCL; 0.1×10^5) as antigen-presenting cells and the compound that the lymphocytes were initially stimulated with; PPD 1 and 2 μg/ml or PPD-modified HSA 0.1-1mg/ml. After 48h, [^{3}H] thymidine was added and proliferation determined by scintillation counting. Cell cultures with an SI of greater than or equal to 2 were expanded further.

Antigen-specific T-cell clones were characterised in terms of CD phenotype by flow cytometry using fluorescent labelled anti-CD4+ and anti-CD8+ antibodies (Serotec Ltd, Oxford UK). Cells were suspended in 100 μl of HBSS and stained with fluorescent PE-conjugated mAbs to CD4 and FITC-conjugated mAbs to CD8 for 30

min at 4°C. Cells were washed twice (with 1ml HBSS) and analyzed using flow cytometry (Coulter Epics XL software; Beckman Coulter, Inc).

5.2.6 Determination of the functionality of p-phenylenediamine and p-phenylenediamine -protein complex-specific T-cell clones

The concentration of PPD and PPD-modified HSA required to stimulate proliferation of individual clones was determined by incubating T-cells (0.5×10^5; total volume 0.2ml) with B-LCL (0.1×10^5) and titrated antigen (PPD, 0.1-5 μg/ml; PPD-modified HSA, 0.1-1 μg/ml). Antigen-specific proliferative responses were measured by [^{3}H] thymidine incorporation. Cytokine secretion (IL-1α, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, TNFα and IFNγ) secretion was measured using a Liquichip 100 workstation (Qiagen Ltd, West Sussex, UK) with LiquiChip IS 2.3 software as described previously (Chapter 2, section 2.2.9.1). Cytotoxic potential was determined by measuring cell surface expression of CD107a, a molecule transported to the cell surface by T-cell receptors during degranulation (Mittendorf *et al.*, 2005; Betts *et al.*, 2003). T-cell clones ($0.5x10^5$; total volume 0.2ml) were incubated with B-LCL ($0.25x 10^5$) in the presence and absence of PPD (2μg/ml). After 2 and 16h cells were harvested, washed and stained for 30 mins at 4°C with a FITC-labelled CD107a mAB (Serotec Ltd., Oxford, United Kingdom) and analyzed by flow cytometry (Coulter Epics, XL software; Beckman Coulter, Luton, UK). A minimum of 10000 events per sample were analyzed. The fluorescence intensity of stimulated T-cells was compared directly with T-cells treated with solvent alone. Data was analyzed by generating a gate around the T-cell population based on the side scatter / forward scatter profile as follows. Forward and side scatter were measured simultaneously and the forward

threshold was raised so as to exclude cellular debris; cells were then gated for analysis and fluorescence was measured on the FL1 channel. At least 10000 cells were analysed for each sample. All data on forward and side scatter, and the two way fluorescence signals for each of the 10000 cells was stored and analysed with WinMDI 2.8 software.

5.2.7 Determination of mechanism of antigen presentation to p-phenylenediamine and p-phenylenediamine-protein complex-specific T-cell clones

The involvement of irreversible protein binding and processing in the presentation of PPD and PPD-modified HSA to specific T-cells was studied by modifying the standard assay for assessment of T-cell proliferation assay as follows:

5.2.7.1 Pulse assay

B-LCL were pulsed with PPD (2 μg/mL) or PPD-modified HSA (1mg/ml) for 16h; the optimal time for PPD oxidation and protein complex formation (Coulter *et al.*, 2007; Aeby *et al.*, 2008). Pulsed B-LCL were washed repeatedly, suspended in PPD-free medium, and added to the proliferation assay (T-cells - 0.5×10^5; total volume 0.2ml) in the absence of soluble antigen.

5.2.7.2 Fixation assay

B-LCL ($2x10^6$; 0.5 mL) were fixed chemically with glutaraldehyde (2%; 12.5 μL) a procedure that prevents processing but not the presentation of pre-processed antigens to T-cells (19, 21, 22), for 30 seconds before the reaction was stopped by the addition of 1ml of 15μM L-glycine solution. Cells were then washed with 4mls HBSS twice,

resuspended in medium and added to the proliferation assay. Irradiated B-LCLs were used as a positive control in certain wells.

5.2.7.3 T cell receptor down regulation

The kinetics of T-cell receptor internalization, an early marker of antigen T-cell receptor engagement, was measured after 1, 4 and 16 h using a FITC-labelled anti-CD3 mAb (Serotec Ltd., Oxford, United Kingdom) and flow cytometry following incubation of T-cell clones ($0.5x10^5$; total volume, 0.2 mL) with B-LCLs ($0.25x10^5$) and either PPD (1-2 μg mL) or PPD-modified HSA.

5.2.8 Statistical analysis

The Mann-Whitney test was used for comparison of control and test values, accepting $P < 0.05$ as significant.

5.3 Results

5.3.1 Selective p-phenylenediamine modification of human serum albumin at cysteine 34

PPD was incubated for 16h with HSA at a 10:1 ratio (chemical to protein). Samples were reduced and alkylated and methanol precipitated to remove unbound material before tryptic digestion and LC-MS/MS analysis. PPD modification of the cys-34 containing peptide 21-41 (ALVIAFAQYLQQC PFEDHVK) was observed; the peptide mass increased from 2432.6 (3+, m/z 811.8) to 2538.6 (3+, m/z 847.2) corresponding to an increase of 106amu. Fragmentation of *m/z* 847.2 ion revealed the y8 fragment ion at *m/z* 1080, corresponding to Cys34 plus 106 amu, confirming a direct cysteine modification (Figure 4.2). No modification of lysine residues as detected by Eilstein et al. (13) was detected.

PPD did not dissociate from the PPD-modified HSA complex. Unbound PPD was not detected in conjugate-containing supernatants cultured for up to 130h (data not shown).

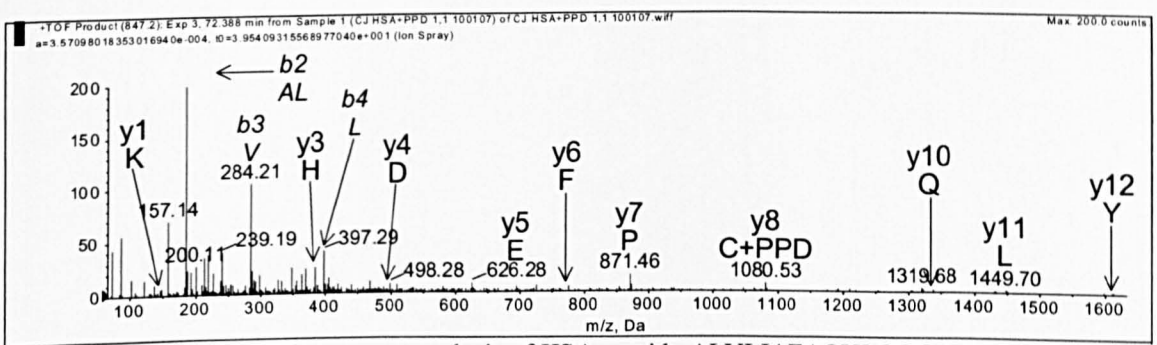


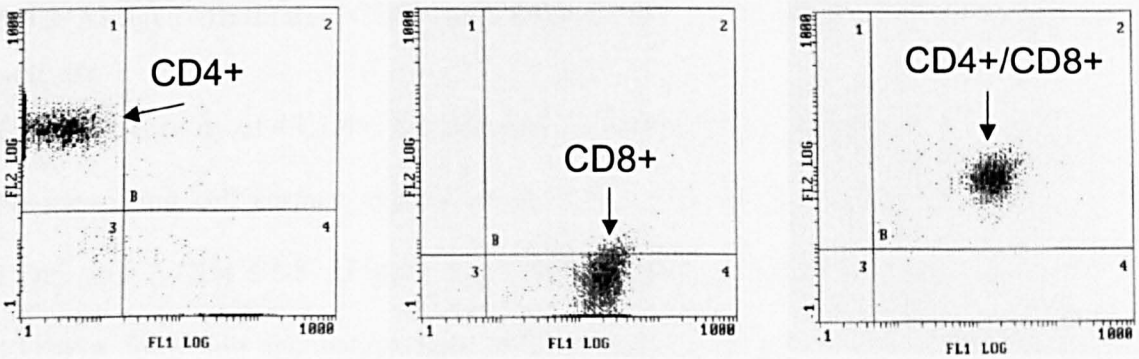
Figure 5.1. (a) LC-MS/MS sequence analysis of HSA peptide ALVLIAFAQYLQQCPFE DHVK MR 2538.6; 3+,m/z 847.2. (unmodified peptide 2432.6; 3+,m/z = 811.8 D 106) Cys34 shows PPD modification

5.3.2 Characterisation antigen-specific CD4+, CD8+ and CD4+CD8+ T-cell clones from p-phenylenediamine allergic patients

Following serial dilution of PPD-stimulated lymphocytes from 3 allergic patients, 70 antigen-specific T-cell clones were identified by measurement of proliferation. Ten further T-cell clones were detected from lymphocytes stimulated with PPD-modified HSA (Table 5.2). Clones displayed a remarkable heterogeneity in CD marker expression; CD4+ and CD8+ clones were characterized from blood of all three allergic patients, while clones expressing relatively high levels of CD4+ and CD8+ were found in 2 patients (Table 5.2; Figure 5.2a&b).

Table 5.2. Proliferation and CD phenotype of T-cell clones generated from 3 allergic patients

	CD phenotype	PPD-specific (n)	Proliferation (cpm) 0	Proliferation (cpm) PPD	Activity with PPD-HSA (n tested)	PPD-HSA-specific (n)	Proliferation (cpm) 0	Proliferation (cpm) PPD-HSA	Activity with PPD (n tested)
1	CD4+	21	599.3	2438.6	19% (16)	0	-	-	-
	CD8+	9	1344.8	8365.7	50% (8)	0	-	-	-
	CD4+CD8+	0	-	-	-	0	-	-	-
2	CD4+	2	239.3	6609.5	50% (2)	2	251.3	643	100% (2)
	CD8+	4	207	6711.4	0% (3)	3	269.8	1529.7	100% (3)
	CD4+CD8+	11	2371.1	16047.4	64% (11)	0	-	-	-
3	CD4+	20	427.3	6585.3	70% (20)	5	550.7	2826.3	100% (5)
	CD8+	2	1134.8	19118.3	50% (2)	0	-	-	-
	CD4+CD8+	1	240.5	4286.5	0% (1)	0	-	-	-



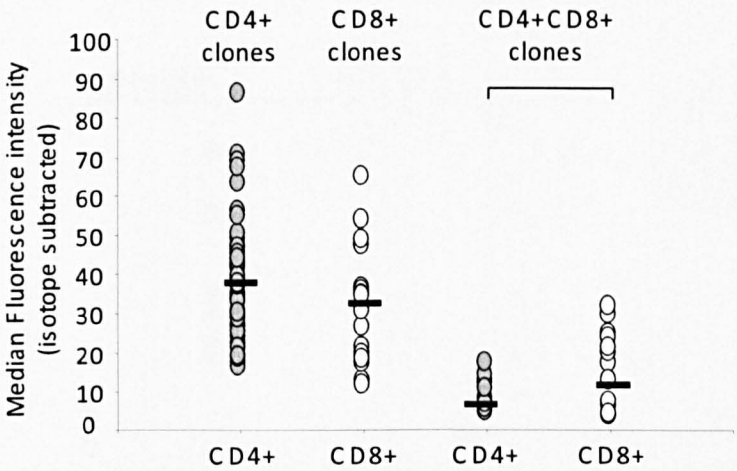


Figure 5.2 (a) Traces showing the phenotype of representative CD4+, CD8+ and dual positive T-cell clones. Analysis was performed using dual colour flow cytometry with cells stained with fluorescent labelled anti-CD4+ and anti-CD8+ antibodies. (b) Expression of CD4+ and CD8+ receptors on T-cell clones generated from allergic patients. Receptor expression measured by flow cytometry.

5.3.3 Antigen-stimulated CD8+ and CD4+CD8+ T-cell clones display cytotoxic activity

Cytotoxic activity of 4 CD4+, 8 CD8+ and 11 CD4+CD8+ T-cell clones was assessed by measuring cell surface expression of CD107a following antigen stimulation. 4 $CD8^+$ and 7 $CD4^+CD8^+$ (Figure 5.3) clones exhibited increased expression of the CD107a following incubation with PPD. Increased CD107a expression was not observed on PPD-stimulated CD4+ T-cell clones.

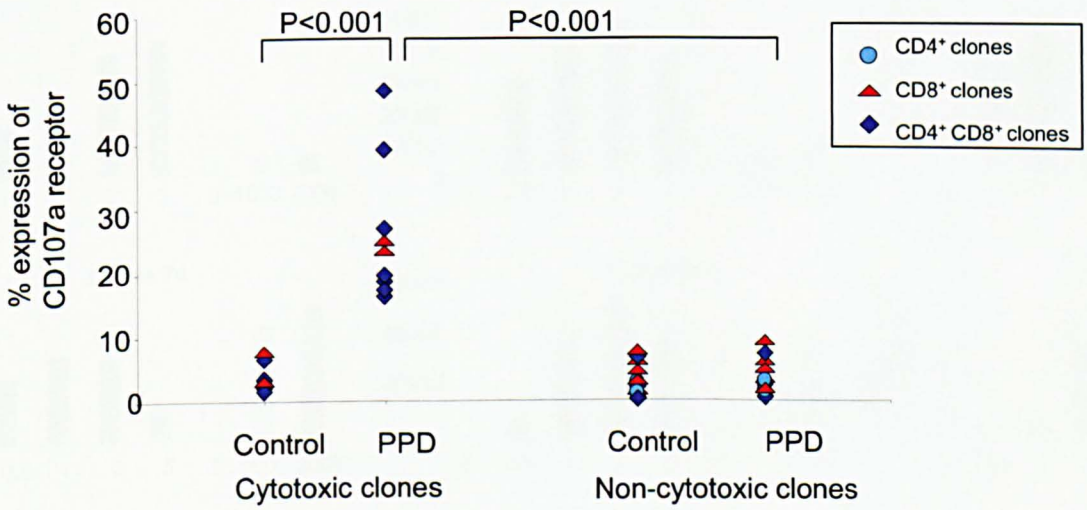


Figure 5.3. Expression of CD107a receptors on T-cell clones generated from allergic patients. Receptor expression measured by flow cytometry.

5.3.4 Stimulation of T-cell clones with p-phenylenediamine and p-phenylenediamine-modified human serum albumin

Proliferation of T-cell clones was antigen concentration-dependent; maximal responses were observed with 0.5-2μg/ml PPD and 500-2000μg/ml PPD-modified HSA. The majority of PPD-specific T-cell clones (40 out of 73) generated from three allergic patients were additionally stimulated with PPD-modified HSA (Figure 5.4; Table 5.3).

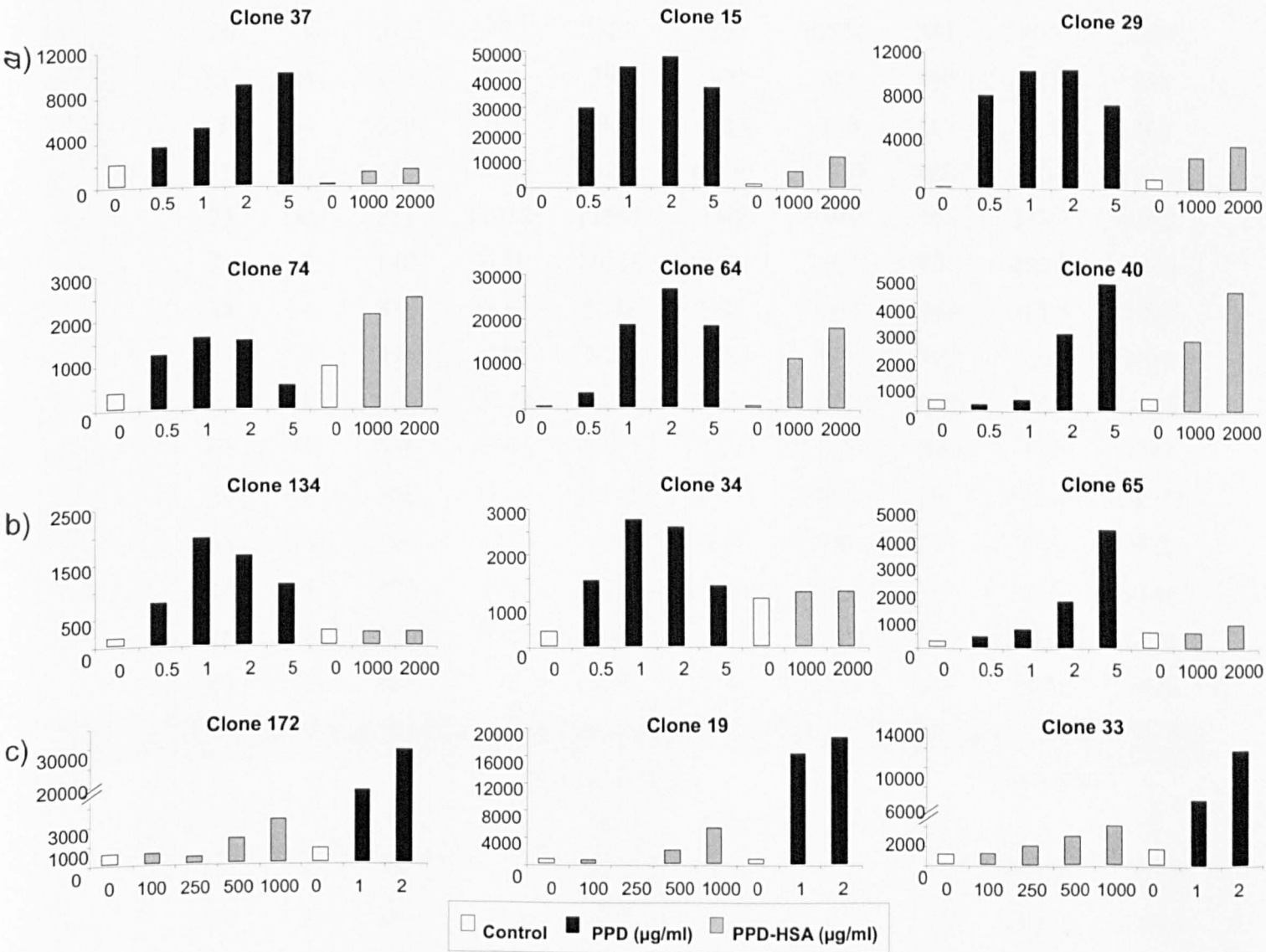


Figure 5.4. Concentration dependent proliferation of PPD & PPD-modified HSA clones (a) 6 representative cross- reactive PPD specific clones (b) 3 representative non-cross reactive PPD specific clones (c) 3 representative PPD-modified HSA specific cross reactive clones.

Table 5.3. Stimulation of T-cell clones with *p*-phenylenediamine and *p*-phenylenediamine-modified human serum albumin

Patient	Clone	CD	Proliferation (cpm)							
			PPD (μg/ml)					PPD-HSA (μg/ml)		
			0	0.5	1	2	5	0	1000	2000
Clones generated from PPD-stimulated lymphocytes										
1	46	8	1385	2267	5918	16159	14857	275	493	707
	37	8	2069	3547	5267	9040	10054	228	1304	1494
	34	8	541	10208	19667	16643	9241	-	-	-
	26	8	2602	1477	1829	3841	12711	341	603	1304
	107	4	102	114	598	1107	1336	440	397	348
	134	4	159	789	1968	1624	1110	287	252	268
2	15	Dual	167	29267	44159	47560	36769	1450	6161	11478
	23	Dual	251	11252	11591	11460	9409	705	3637	4032
	29	4	140	8231	10358	10471	7362	929	2822	3939
	34	4	338	1439	2748	2584	1307	1048	1185	1206
	74	8	391	1220	1621	1550	529	951	2109	2488
	111	8	129	15979	17289	18081	15230	2079	2111	2798
3	63	4	631	699	1005	7134	12579	1163	980	2361
	64	8	568	3311	18560	26413	18459	691	11110	18172
	65	Dual	240	407	689	1690	4286	577	594	902
	18	4	479	362	610	1244	3309	724	621	1541
	26	4	210	692	1821	9201	15766	831	5032	5591
	40	4	386	227	416	2881	4699	469	2641	4429
Clones generated from PPD-modified HSA-stimulated lymphocytes										
			PPD-HSA (μg/ml)					PPD (μg/ml)		
			0	100	250	500	1000	0	1	2
2	171	8	56	142	314	368	488	422	87149	107191
	172	8	541	598	499	1503	2447	769	18552	29304
3	19	4	798	738	136	2093	5326	801	16269	18538
	33	4	592	682	1082	1597	2188	915	6824	11809

5.3.5 Antigen-stimulated T-cell clones secrete high levels of Type 2 cytokines

When culture supernatants from incubations containing T-cells, B-LCL and PPD or PPD-modified HSA were analysed for cytokine secretion, high levels of the Type 2 cytokines IL-4, IL-5 and IL-13 detected (Table 5.4, Figure 5.5) Antigen stimulation was also associated with secretion of the cytokines IFNγ (at low levels) and TNFα.. No difference was seen when comparing secretion from CD4+ clones, CD8+ clones and dual positive clones directly.

Table 5.4. Cytokine secretion and cytolytic activity of PPD-specific T-cell clones generated from 3 allergic patients

Clone No.	CD	Proli-fer-ation (SI)	Cytokine secretion (pg/ml)									
			IL1α	IL4	IL5	IL6	IL8	IL 9	IL10	IL13	TNFα	IFNγ
PPD stimulation												
16	Dual	283.9	11.9	10000	7908.9	71.9	1550.6	2.4	8.7	13244	1383.3	6.8
26	Dual	38.4	13.6	2070.8	1564.5	69.4	191.1	0	10.6	8515.2	197.3	0
34	4	8.1	15.9	383.9	768.2	68.4	145.2	0	0	10607	79.48	29.9
44	Dual	8.4	10.1	20.64	160.5	35.3	213.53	0	0	3271.2	30.34	22.3
23	Dual	46.2	16.3	4472.9	2394.8	88	251.1	0	0	11115	346	31.4
29	4	74.5	12.7	1993	1653.1	71.8	308.5	0	10.8	11045	103.4	7.8
15	4	15.7	1.1	1969	7848.8	28.5	259.1	0	13.1	13122	410.4	24.2
21	4	8.1	2.6	44.5	1944.4	49.2	613.8	0	15	9532	75.1	17.6
39	4	50.1	0	122.9	4759.2	15.9	128.6	0	25.4	10683	87.8	0.5
49	4	26.6	12.7	91.9	211.5	38.7	424.9	0	9.49	3321.5	59.1	0
64	8	46.5	6.9	44.3	3217.3	37.8	264.2	0	8.57	11365	62.7	18.6
65	Dual	17.8	0	0	214	15.9	254.9	0	14.5	2802.2	45.1	0
PPD-modified HSA stimulation												
33	4	3.7	0	1.3	515.5	20.9	588.2	0	14.6	4953.2	28.3	0
40	4	2.4	0.6	28.3	294.8	45.8	841	0	16.8	3088.9	31.7	0
172	8	8.6	5.2	17.3	453.2	52.2	401.7	0	0	3664.3	18.9	8.1

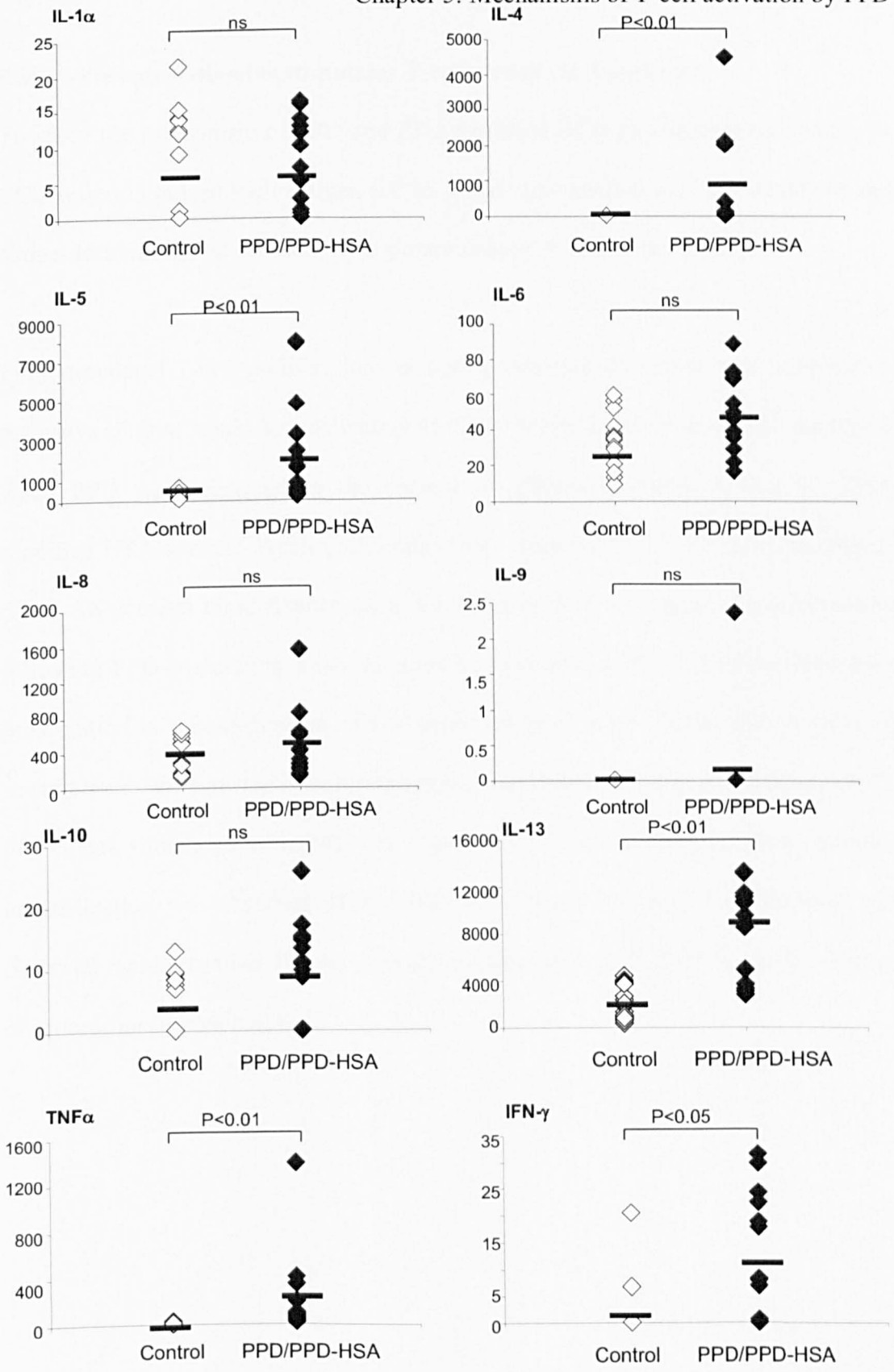
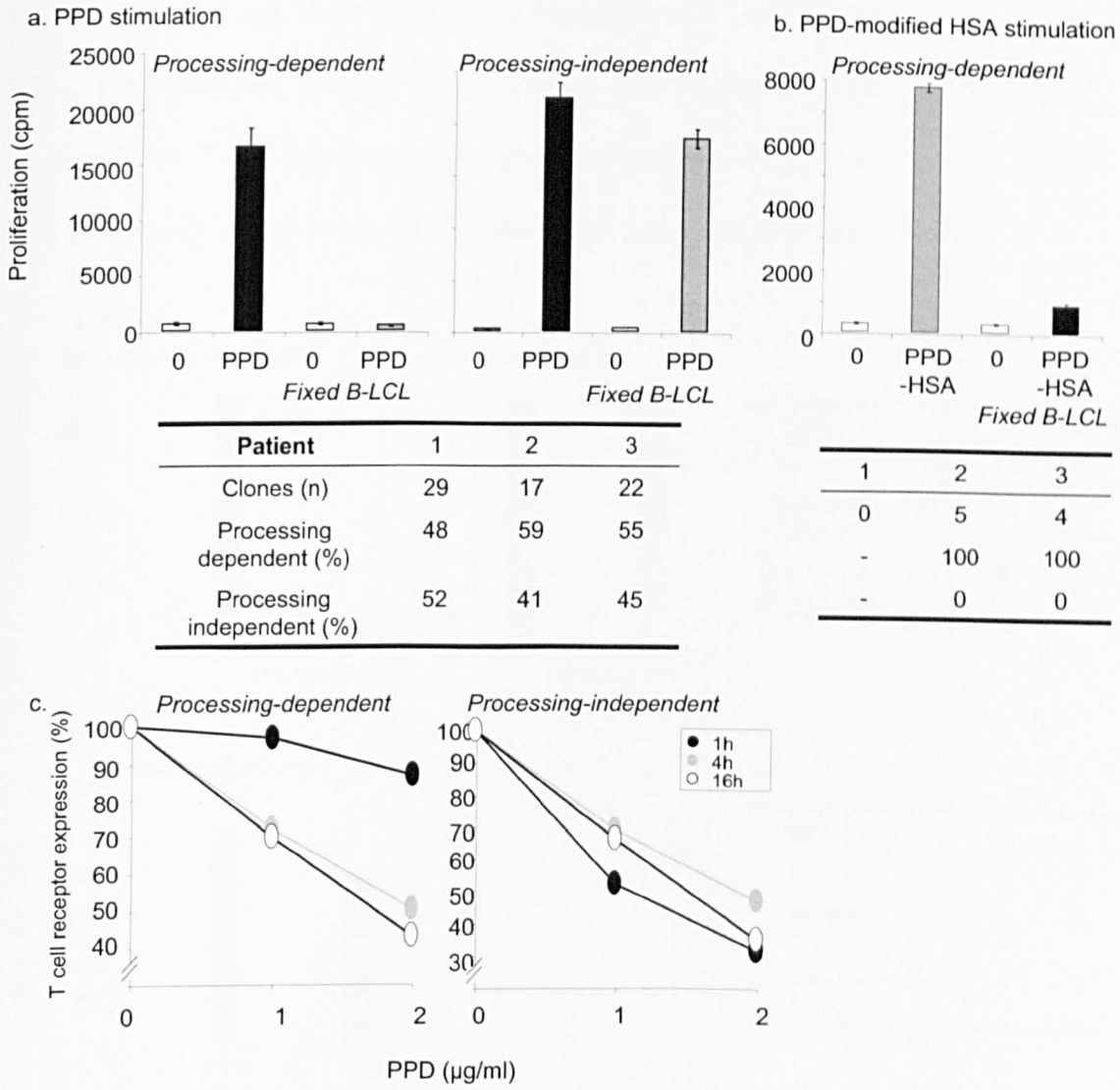


Figure 5.5. Cytokine secretion from PPD/PPD-modified HSA specific clones in response to PPD and PPD-modified HSA exposure. T-cells were incubated with PPD and PPD-HSA for the duration of the proliferation assay, prior to addition [3H] thymidine incorporation, aliquots of supernatant were taken and the sample concentration which provided the maximal proliferative response was analysed using the human cytokine LINCOplex multiplex assay kit. Statistical analysis was performed by comparing incubations of chemical with media.

5.3.6 *p*-Phenylenediamine stimulates T-cell clones via 3 pathways

To study the mechanism of PPD and PPD-modified HSA presentation to T-cells, B-LCL were (1) pulsed with antigen for 16 h (the time needed for PPD oxidation and adduct formation) and (2) fixed with glutaraldehyde to inhibit processing.

PPD stimulated T-cell proliferation via both processing dependent and independent pathways (Figure 5.6a). Approximately 45 % (n=68) of T-cell clones were stimulated when PPD was presented in the context of glutaraldehyde-fixed B-LCL. PPD-modified HSA-specific T-cell proliferation was inhibited by B-LCL fixation (Figure 5.6b). To confirm these fixation data, the kinetics of T-cell receptor internalisation following PPD stimulation was monitored by flow cytometry. Processing dependent presentation is time-dependent and receptor internalization occurs after a delay of several hours, the time required for antigen uptake and processing (Zanni *et al.*, 1997). For clones stimulated with PPD via a pathway dependent on processing, receptor internalization was observed after 4-16h. In contrast, receptor internalization was observed rapidly (within 1h) for clones stimulated with PPD directly, in the absence of processing (Figure 5.6c).



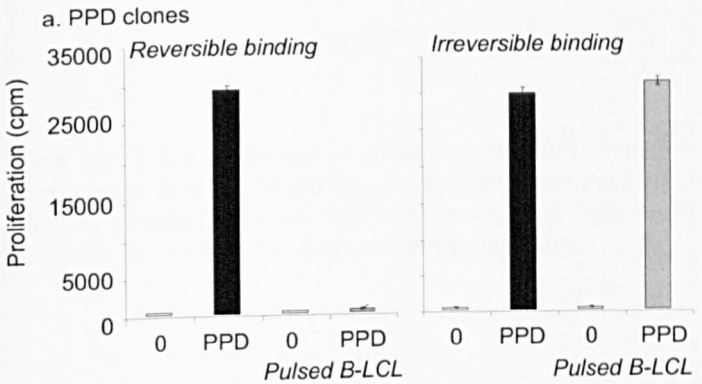
Patient	1	2	3
Clones (n)	29	17	22
Processing dependent (%)	48	59	55
Processing independent (%)	52	41	45

1	2	3
0	5	4
-	100	100
-	0	0

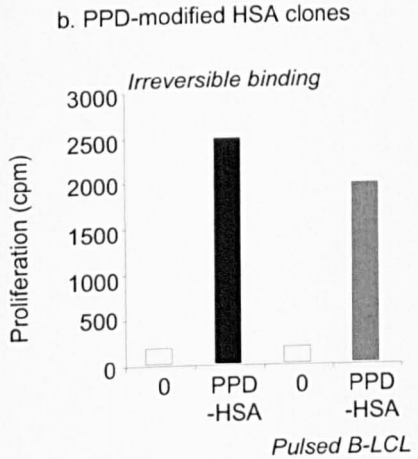
Figure 5.6. T-cell clones are stimulated with (a) PPD via processing dependent and independent pathways and (b) PPD-modified HSA via a pathway dependent on processing. T-cell clones were incubated with PPD and irradiated or glutaraldehyde-fixed antigen presenting cells. Proliferation was determined by incorporation of [^{3}H]thymidine. Coefficient of variation was consistently less than 20%. (c) Time-dependent internalization of T-cell receptor expression on PPD-specific T-cell clones.

T-cell clones stimulated with PPD via a processing dependent pathway were also stimulated with PPD-pulsed B-LCL, signifying a requirement for hapten complex formation. T-cell clones responding to PPD in the absence of processing were predominantly stimulated with PPD bound irreversibly to protein, presumably MHC itself, or a peptide embedded within (Figure 5.7a). Only 6 clones responding to

soluble PPD did not show specific proliferation with PPD pulsed B-LCL (Figure 5.7a). PPD-modified specific T-cell clones only responded with pulsed B-LCL (Figure 5.7b). T-cell responses were also demonstrable when B-LCLs were pulsed with PPD-modified HSA alone in the absence of other protein (Figure 5.8).

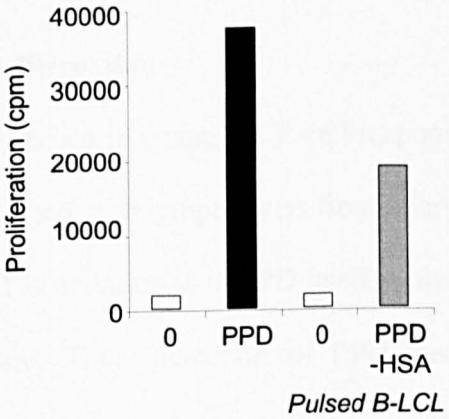


Patient	1	2	3
Clones (n)	12	13	23
Reversible binding (%)	33	15	0
Irreversible binding (%)	67	85	100



Patient	1	2	3
Clones (n)	0	3	5
Reversible binding (%)	-	0	0
Irreversible binding (%)	-	3	5

Figure 5.7. T-cell clones are stimulated with PPD predominantly via a pathway involving hapten protein complex formation. T-cell clones were incubated with soluble PPD and B-LCLs pulsed with PPD for 16h. Proliferation was determined by incorporation of [^{3}H]thymidine. Coefficient of variation was consistently less than 20%.



Patient	1	2	3
Clones (n)	0	13	23
Reversible binding (%)	-	4	8
Irreversible binding (%)	-	9	15

Figure 5.8. T-cell clones can be stimulated with PPD-modified HSA alone via a pathway involving hapten protein complex formation. T-cell clones were incubated with soluble PPD and B-LCLs pulsed with PPD-modified HSA for 16h. Proliferation was determined by incorporation of [^{3}H] thymidine. Coefficient of variation was consistently less than 20%.

5.4 Discussion

As shown in chapter 2 T-cell responses to the PPD oxidation product BB have been detected with lymphocytes from allergic patients and tolerant individuals; however, T-cell stimulation with PPD itself is discernable with lymphocytes from allergic patients alone. Thus, detection of PPD-specific T-cell responses represents an important discrimination between allergic and tolerant patient groups and is the reason the current study focuses on characterization of T-cell responses to PPD and primary products of auto-oxidation, but not synthetic BB.

Reactivity of chemical allergens towards nucleophilic amino acid residues on protein is coupled with its sensitising potential (Basketter *et al.*, 1997; Landsteiner & Jacobs 1935). Therefore binding of chemical allergens to specific loci on protein might be associated with activation of immune cells. In this respect work done with DNCB and penicillin bound to HSA (Pickard *et al.*, 2007; Brander *et al.*, 1995) has demonstrated a requirement for processing of antigen by APC's before presentation to T-cells.

It has also been shown that chemicals and drug metabolites can stimulate T-cells through direct covalent binding to MHC molecules expressed on APC surface, therefore bypassing the need for antigen processing (Moulon *et al.*, 1995; Schnyder *et al.*, 2000; Burkhart *et al.*, 2001). Also more recently a new hypothesis called the pharmacological interaction (p-i) concept has suggested that not all chemicals or drugs necessarily require covalent interactions with MHC molecules in order to trigger an immune response. It postulates that chemicals and drugs can activate T-cells directly in the absence of metabolism, covalent binding, and antigen processing, (Pichler, 2002; 2003) through a reversible interaction between the TCR, MHC, and

the chemical/drug. This interaction is believed to be stable enough to stimulate a cellular immune response.

PPD modification of HSA at cys-34 was characterized by the addition of 106 amu on the relevant tryptic peptide, corresponding to the addition of a single molecule of PPD (Figure 5.1; Chapter 4). PPD bound to the single cysteine residue on HSA existing in a reduced form (cys-34) (Figure 5.1a). Cys-34 is attached to the hydroxyl oxygen of Tyr84 by a hydrogen bond in unmodified HSA (Stewart *et al.*, 2005). PPD ligation will disrupt this hydrogen bonding interaction inducing a conformational change that alters functional activity (Kawakami *et al.*, 2006) and possibly generating new antigenic determinants.

The interaction of PPD with HSA is believed to be stable as no [^{14}C] PPD was detectable in supernatant samples analysed (data not shown; methods 5.3.1); therefore PPD does not dissociate.

Data from chapter 4 show that allergic patients' lymphocytes proliferate in the presence of PPD and PPD-modified HSA where as volunteer cells do not respond (Section 4.3.2). Interestingly, the response of lymphocytes from patients differed in terms of their preferred antigenic determinant (i.e., PPD or PPD-modified HSA; table 1); possibly highlighting alternative pathways of antigen presentation.

Therefore to explore in more detail the mechanisms of PPD presentation to specific T-cell receptors and the extent of cross reactivity between soluble and conjugated PPD, T-cells from 3 allergic patients were cloned by serial dilution using established methods (Naisbitt et al., 2003b). The occurrence of CD4+, CD8+ and CD4+CD8+ T-cell clones varied between the allergic patients even though each patient presented

with similar clinical symptoms (Table 5.1). These findings did not relate to variation in sample collection, methods employed, or materials used as these are all standardized in the authors' laboratory. In agreement with previous studies utilizing lymphocytes and clones from PPD allergic patients (Sieben *et al.*, 2002, Coulter *et al.*, in press), T-cells generated from allergic patients secreted high levels of the Th2 cytokines IL-4, IL-5 and IL-13 following antigen stimulation. The cytokine secretion profile did not differ when CD4+, CD8+ and CD4+CD8+ T-cells were compared or when PPD and PPD-modified HSA were used as antigens (Table 5.4). This in agreement with findings from Chapter 4 when comparison of purified CD4+ and CD8+ T-cells from allergic patients were found to secrete high levels of the type 2 cytokines IL-5 and IL-13 following PPD, BB and PPD-modified HSA stimulation, whereas in tolerant individuals, proliferation and low levels of cytokine secretion was only detected in the CD4+ T-cell population. Detection of antigen-specific, cytokine secreting CD8+ T-cells from allergic patients alone supports the hypothesis that CD8+ T-cells are important effectors in contact allergic dermatitis (Vocanson *et al.*, 2006; 2008).

Murine models of contact dermatitis suggest that CD8+ cells are effectors mediating cytotoxicity through FAS and perforin pathways (Kehren *et al.*, 1999; Saint-Mezard *et al.*, 2005; Vocanson *et al.*, 2006; Martin *et al.*, 2004). Although a detailed investigation of the involvement of different T-cell populations in PPD-mediated contact dermatitis was beyond the scope of the current study, several antigen-specific CD8+ and CD4+CD8+, but not CD4+, T-cell clones were found to display a transient increase in cell surface CD107a expression. CD107a is a membrane-associated protein that is transported to the cell surface during degranulation. Increased CD107a

expression following antigen exposure has recently been described as a sensitive marker of cytotoxic activity (Aktas *et al.*, 2008; Betts *et al.*, 2003).

Previous studies using cloned T-cells propose that specific T-cell receptors are predominantly stimulated with PPD bound directly to MHC molecules, therefore circumventing the requirement for antigen processing (Sieben *et al.*, 2002). Importantly, B-LCLs cultured with PPD for up to 4h failed to stimulate a specific T-cell response (Sieben *et al.*, 2002). In contrast, more recent studies indicate that a longer 16-24h pulse of B-LCLs with PPD, the time needed for oxidation of PPD and covalent binding (Coulter *et al.*, 2007; Aeby *et al.*, 2008), is associated with a PPD-specific proliferative response. Thus, PPD-protein complex formation seems to represent an additional antigenic signal to T-cells from allergic patients.

Serial dilution of PPD- and PPD-modified HSA-stimulated lymphocytes generated eighty antigen-specific T-cell clones (Table 5.2). Of 64 PPD-specific clones that were tested for additional reactivity with PPD-modified HSA, a proliferative response was detected with 31 clones (Table 4). All 10 T-cell clones initially deemed reactive towards PPD-modified HSA, displayed additional PPD reactivity. To delineate mechanisms of antigen presentation to specific T-cells, methods were employed that have previously been shown to identify pathways of drug and chemical presentation to T-cells (Wu *et al.*, 2006; Schnyder *et al.*, 2000; Pickard *et al.*, 2007; Naisbitt *et al.*, 2003; Burkhart *et al.*, 2003). Firstly clones were stimulated with gluteraldehyde-fixed or irradiated B-LCL to assess need for processing of protein antigens; fixed antigen presenting cells express MHC molecules and actively present antigenic peptides and chemicals that interact directly with MHC. However ability to process and present

protein antigens is completely inhibited (Zanni 1997, Schnyder, 2000). Secondly the role of irreversible binding was assessed by pulsing B-LCL overnight with PPD and PPD-modified HSA; 16h is the optimal time needed for PPD oxidation and complex formation (Coulter *et al.*, 2008; Aeby *et al.*, 2008). Thirdly the early parameters of T-cell receptor (TCR) engagement were assessed by measuring TCR down regulation by flow cytometry after antigen stimulation.

The results have suggested that PPD can activate T-cells via 3 different mechanisms.

(1) Classical hapten mechanism (Pickard *et* al., 2007) in which chemically modified protein is taken up and processed by antigen presenting cells and subsequently presented as modified self peptides on the MHC to T-cells. 53% of the PPD-clones did not proliferate in the presence of fixed B-LCL's highlighting the requirement for processing (Figure 5.5), which is further confirmed the by slow internalisation of TCR of these PPD-specific clones (4-16h; Figure 5.5). Furthermore processing-dependent clones proliferated after B-LCL's were pulsed with PPD for 16h (Figure 5.6), demonstrating the importance of irreversible binding in hapten-protein complex formation and T-cell activation. Presentation of PPD-modified HSA to T-cell clones was dependent on processing and irreversible binding, which was expected for a hapten-protein complex. Studies underway in our laboratory have been designed to characterize the structure of MHC eluted immuno-stimulatory peptides deriving from PPD-modified HSA and site of PPD modification.

(2) Direct chemical modification of peptide presented in MHC groove in which irreversibly binding is neccessary but processing of antigenic protein is avoided (Zanni *et al.*, 1997). 47% of PPD-specific clones were able to proliferate in the presence of fixed antigen presenting cells and rapid TCR down regulation was also observed (Figure 5.5), providing evidence of this processing independent pathway.

The majority of these clones proliferated in the presence of pulsed B-LCL's were able to stimulate specific T-cell receptors by binding irreversibly to both MHC-associated protein (Figure 5.6).

(3) p.i-mechanism (Pichler, 2003) involving a direct reversible interaction of PPD with MHC molecules. As seen with the direct modification of the peptide in the MHC groove, this pathway is processing independent and TCR is rapidly internalised (Figure 5.5). However in this case the T-cells were stimulated with PPD bound reversibly to MHC through non-covalent interactions as no response was seen when the clones were pulsed with B-LCL's; however, this form of T-cell stimulation seems to be less-favoured since it was only detected with low numbers of clones (6 out of 48).

In conclusion it would appear that there are multiple clones that can be observed which can recognise various types of antigens derived from PPD in vitro. However formation of a PPD hapten protein complex is an important mechanistic step in the stimulation of T-cells from allergic patients as this step will be the initial trigger in development of a reaction to PPD, the other mechanisms will play a role in the elicitation phase.

CHAPTER 6

Final Discussion

Allergic contact dermatitis (ACD) is a common and important occupational health issue associated with exposure to certain low molecular weight chemicals. These contact allergens cause a specific T cell mediated, delayed type hypersensitivity reaction that results in skin erythema and oedema at the site of challenge in certain susceptible individuals (Smith-Pease, 2003). The chemical and cellular mechanisms associated with development of tissue damage and individual susceptibility factors are ill defined.

Development of ACD can be divided into two stages defined as: sensitisation, a priming response to an unknown antigen; and elicitation, a recall event in response to secondary exposure to the same antigen. Due to their low molecular weight, contact allergens are incapable of initiating an immune response by themselves as they as they do not occupy a sufficient molecular volume. It is generally thought that they are incomplete antigens that must bind to proteins to form stable associations known as hapten-protein complexes (Park *et* al., 1998) in order to initially sensitise an individual during primary exposure. This results in the immunological priming of the hosts cell mediated immune system by generating a circulating population of antigen-specific memory T cells that can be activated upon subsequent exposure to the same chemical.

The aim of these studies was to use a model contact allergen para-phenylenediamine to explore the mechanisms of hapten-protein complex formation and its presentation to T cells to gain better understanding of the chemical and cellular basis of ACD.

Initial experiments addressed the question; is PPD or one of its oxidation products the antigen responsible for the associated allergic reactions (Chapter 2). PPD-induced ACD is believed to be associated with PPD being intrinsically unstable and its ability to undergo auto-oxidation either in solution or on the skin, leading to the formation of an electrophilic primary quinonediimine intermediate, which is susceptible to sequential self-conjugation reactions and further oxidation, resulting in the formation of a trimer Bandrowski's base (BB; Picardo et al 1990; Coulter *et al.*, 2007). PPD and BB have previously demonstrated stimulatory capacity in lymphocytes from allergic individuals (Sieben *et al.*, 2002; Krasteva *et al.*, 1993), whilst animal models have acknowledged the autooxidation product BB as exhibiting a greater immunogenic potential than the parent compound in terms of LLNA assessment (i.e. approximately 10 times more potent than PPD; White *et al.*, 2006). In our laboratory, we explored the immunogenic potential of PPD and BB in a mouse model. BALB/c strain mice were immunized with either PPD or BB via a single sub-cutaneous injection (to avoid potential PPD air (auto) oxidation processes on the skin or during cutaneous absorption, which is thought to be important for obtaining concentrations of PPD derivatives needed to induce skin sensitization (Aeby et al., 2008)), and antigen-specific T-cell proliferation was analyzed after 7 days. Exposure of mice to BB resulted in marked T-cell stimulation as assessed by measurement of spleen cell proliferation following in vitro BB stimulation, whereas a single sub-cutaneous PPD

injection did not induce an immune response in the mouse; no proliferation was observed with spleen cells following in vitro stimulation with PPD (Farrell *et al.*, 2008).

Therefore to fully determine the nature of the chemical antigen involved in human ACD we assessed the T-cell stimulatory capacity of PPD and BB using lymphocytes isolated from allergic patients, tolerant and naive volunteers. T-cells from allergic patients and both sets of volunteers proliferated in the presence of BB, however patient lymphocytes additionally responded to PPD highlighting an important discrimination between the allergic and non-allergic groups. As previous hair dye exposure seemed to have no effect on the response to BB, its stimulatory capacity was assessed further using cord blood cells (collaboration with Southampton University). No BB-specific T-cells were detected in these experiments, suggesting an acquired immune response to BB, which has not translated into an allergic reaction. In agreement with these data, spleen cells from naïve mice tested as part of our animal model (Farrell *et* al., 2008) were not specifically stimulated following *in vitro* BB stimulation, indicating that BB exposure is not associated with mitogenic T-cell stimulation. The use of glutathione in certain experiments, which has previously been shown to block BB formation (Coulter *et al.*, 2007), proved that patient cells are specifically stimulated with PPD (or a primary oxidation product). Furthermore, PPD-specific T cell clones that were not stimulated with BB, adding further evidence to our hypothesis that PPD is the immunogenic factor in ACD.

Patch test studies support the argument that PPD is the antigenic determinant associated with the development of allergic contact dermatitis as a high frequency of

patch test reactivity to PPD in allergic individuals has been shown (Patel *et al.*, 2007), whereas only 16% of PPD patch test positive patients are responsive to BB (White *et al.*, 2006). However, since the patch test response is time-dependent (Basketter *et* al., 2006) the immuno- stimulatory effects of PPD are likely to be dependent upon cutaneous oxidation. Aeby *et al*, (2008) recently discussed oxidative abilities of PPD, noting there is a difference in what skin will be exposed to during *in vivo* use and *in vitro* testing. They suggest that for periods of prolonged exposure (temporary henna tattoos, LLNA, human diagnostic patch testing) formation of BB that can ultimately penetrate the skin barrier is likely. However, during oxidative hair dyeing, the skin is exposed to formulations containing PPD and couplers for a short period (30 minutes). The chemistry (pH4.9) here is designed to favor reactions with the couplers, and therefore the formation of di and trimeric PPD auto-oxidation products such as BB is prevented (Goux *et al.*, 2007; Brody and Burns, 1968; Bracher *et al.*, 1990). Despite this low levels of non-consumed PPD reach the epidermis (~1.1%; Rastogi *et al.*, 2006). This could explain why BB is classed as a more potent sensitiser in the LLNA as the conditions of prolonged exposure favour formation and penetration of BB. Also the difference seen with the mouse model in our laboratory may be explained by the direct injection of BB into the skin, bypassing the initial penetration barrier, resulting in high reactivity, something not seen in the case of the humans with the patch test.

It is a theoretical possibility that everyday exposure to BB provides tolerance to the chemical; however this concept requires experimental validation. Also the patient population used in these studies developed contact dermatitis following exposure to hair dye thus, it would be interesting for future experiments to test henna tattoo-allergic patients to see whether PPD or BB is the antigenic component in these cases.

The main finding from studies described in Chapter 2 was that PPD is the antigenic factor related to the development of contact dermatitis. Therefore the next component of the project was to explore the binding of PPD to protein. Preliminary experiments (Chapter 2) showed that PPD is capable of forming complexes with cellular and serum proteins with or without GSH, demonstrating the potential for a hapten-protein complex formation. Work performed by Hopkins *et al.*, (2005) suggests that distribution of antigen formation with chemical allergens segregates with the type of cytokines that will ultimately be secreted. The authors found that chemicals bound preferentially to cellular protein secreted a type 1 cytokine profile, whereas those that bound to serum proteins provoked a type 2 profile. In agreement with this work, PPD bound preferentially to serum protein and PPD stimulated T cell clones were found to secrete type 2 cytokines.

Experiments were carried out to characterise binding of PPD to specific amino acid residues in designer peptides, a model intracellular protein glutathione-*S*-transferase-pi (GSTP) and model extracellular protein human serum albumin (HSA), and to determine the specific site of modification within the proteins (Chapter 3&4). Using small designer peptides containing single nucleophilic amino acids, PPD was found to significantly deplete cysteine and lysine containing peptides. However in the larger synthetic peptide (DS3), that has each of the 20 common amino acids known represented in the sequence, mass spectrometry techniques revealed PPD bound exclusively to the cysteine residue. In the model intracellular protein (GSTP), PPD selectively modified the cysteine 47 residue of this protein. Similarly PPD was able to modify the only free cysteine residue (34) of HSA. Modification of this cysteine is known to disrupt the hydrogen-bonding interaction between this residue and Tyr84,

resulting in a conformational change that alters functional activity (Kawakami *et al.*, 2006), possibly leading to new antigenic determinants.

The pKa of most cysteine residues in protein is around 8.5, however the Cys47 of GSTP has a low pKa value (3.5-4) due to its deprotonation by the nearby lysine 54 residue, making it highly nucleophilic and subject to modification by Michael addition (Benesch *et al.*, 1955; LoBello *et al.*, 1993). The same could be said for the Cys34 of HSA as this also has a lower pKa of 6.7, due to the ability of the Tyr84 to both lower the pKa of the thiol and contribute to its high redox potential (Stewart *et al.*, 2005). The selective binding of PPD to a single cysteine binding site in an isolated protein suggests that PPD protein binding *in vivo* might also display some degree of selectivity and pKa value could play a pivotal role.

These data highlight a role for this amino acid in hapten-protein complex formation.

To assess the T cell stimulatory capacity of PPD conjugated protein, a new patient and volunteer cohort (Chapter 4) were identified and proliferation of lymphocytes with PPD, BB and PPD-modified protein determined. HSA was selected as the model protein for conjugation as it is an abundant protein in the skin and a likely target for PPD modification. In agreement with data described in Chapter 2, BB stimulated proliferation in lymphocytes from patients and volunteers, whereas only patients cell responded to PPD. Likewise PPD-modified HSA only elicited a response in patient cells, proving that a hapten-protein complex is capable of inducing an immune response. These results also highlight a potential role for cysteine-selective protein modification in PPD-mediated immune reactions. Work carried out by Eilstein *et al.*, (2006; 2007; 2008) using the structurally similar compound 2,5- dimethyl-*p*-benzoquinonediimine showed that it would react with multiple nucleophiles, and in a

complex peptide environment bound specifically to lysine residues. The authors conclude that lysine should be considered an important amino acid for conjugate formation for allergenic *p*-benzoquinonediimines. However, these studies were carried out in a cysteine free environment and the T cell stimulatory capacity of the lysine-modified 2,5-dimethyl-*p*-benzoquinonediimine was never tested: thus, no definite conclusions can be made about the role of lysine in PPD-mediated ACD. Interestingly when 2,5-dimethyl-*p*-benzoquinonediamine was used in our studies we found no evidence of a lysine modification and patients lymphocytes did not proliferate in response the chemical.

The pattern of cytokine secretion from patient and volunteer lymphocytes following antigen stimulation was assessed (Chapter 4), as previous studies have found significantly higher levels of type 2 cytokine secretion and gene expression associated with PPD and BB exposure to patients' cells (Coulter *et al.*, submitted). Animal models of PPD-mediated contact allergic dermatitis found Th2 cytokines and IgE antibodies were important in the development of tissue pathology (Yokozeki *et al.*, 2003). Patient cells were found to secrete a predominant Tc2/Th2 cytokine profile as measured after PPD and PPD-modified HSA exposure to patient lymphocytes (Chapter 4) and to specific T cells (Chapter 5). Significantly higher levels of IL-5, IL-13, IL-9 and TNFα were associated with stimulation of lymphocytes by PPD and PPD-modified HSA. Proliferation of PPD and PPD-modified HSA T cell clones resulted in high levels of IL-4, IL-5 and IL-13 being secreted. No increase of type 2 cytokines was noted after PPD/PPD-modified HSA stimulation of volunteers' cells. These data confirm an important role of type 2 cytokine secretion in the pathogenesis of allergic inflammation as secretion of IL-4, IL-5 and IL-13 has previously been

demonstrated after PPD exposure (Coulter *et al.*, submitted, Sieben *et al.*, 2002; Yokozeki *et al.*, 2003). Newly described is the increase of IL-9 following antigen stimulation, although this has recently been implicated in development of other allergic diseases. Produced by T cells, eosinophils and mast cell (Kajiyama *et al.*, 2007), IL-9 is thought to promote IgE production by B cells and to synergise with IL-13 to increase eotaxin release and IL-5 to drive eosinophil maturation (Fawaz *et al.*, 2007). Perhaps increase of this cytokine in patients and not volunteers is a possible discriminating factor for immune responses to PPD as it exerts effects on IL-5 and IL-13. The increase noted with TNFα may be associated with the recent finding that TNFA-308 G/A polymorphism is a susceptibility factor for contact allergy to chemicals such as PPD (Blomeke *et al.*, 2008). Further research needs to be carried out to assess the role of Type 2 cytokines in allergic contact dermatitis.

It is theorised that only one cell type is the effecter cell in allergic contact dermatitis, possibly $CD8^+$, which infiltrate the skin rapidly after allergen exposure are responsible for the inflammation, while $CD4^+$, which arrive later abrogate the effect (Vocanson *et al.*, 2006). To address this, purified $CD4^+$ and $CD8^+$ T-cells from allergic patients and tolerant individuals were stimulated with PPD and BB and proliferation and cytokine secretion assessed. $CD4^+$ and $CD8^+$ T-cells from allergic patients proliferated and secreted high levels of the Type 2 cytokines IL-5 and IL-13 following PPD and BB stimulation. In contrast, in tolerant individuals, proliferation and low levels of cytokine secretion was only detected in the $CD4^+$ T-cell population. Detection of antigen-specific, cytokine secreting $CD8^+$ T-cells from allergic patients supports to hypothesis that $CD8^+$ T-cells are important effectors in contact allergic dermatitis (Vocanson *et al.*, 2006; 2008). However little is known about Tc2 cells.

They have been detected in chronic infections, cancer, and autoimmune diseases and their presence correlates with disease severity and progression. It has been hypothesised that Tc2 cells are able to modify DC function to favour differentiation of naive T cells to type 2 (Iezzi *et al.*, 2006). However, further work needs to be performed on these cells to understand their importance in ACD.

Finally, the mechanisms involved in the presentation of soluble and conjugated PPD to T cells by generating PPD specific T cell clones from 3 PPD allergic patients (Chapter 5). 80 clones (70 PPD-specific; 10 PPD-HSA specific) were generated in total with the occurrence of CD4+, CD8+ and CD4+CD8+ T-cell clones varying between the allergic patients even though each patient presented with similar clinical symptoms. As regards to activation of T cells by soluble PPD, results collected found 3 different mechanisms of PPD presentation – (1) classical hapten mechanism in which chemically modified protein is taken up and processed by antigen presenting cells and subsequently presented as modified self peptides on the MHC to T-cells; (2) direct chemical modification of peptide presented in MHC groove in which irreversibly binding is neccessary but processing of antigenic protein is avoided (Zanni *et al.*, 1997); and (3) p.i-mechanism involving a direct reversible interaction of PPD with MHC molecules (Pichler, 2003).

In contrast to soluble PPD, the conjugated PPD required processing to become immunogenic, as previously seen with penicillin (Brander *et al.*, 1995). HSA was used as a model protein for these experiments as it is an abundant protein in the skin, therefore it is likely that a HSA-derived peptide modified by PPD could be responsible for T cell proliferation. Another possibility is that PPD modification could

alter the normal processing of HSA, resulting in formation of a neo-epitope to which T cells are reactive, instead of the usual epitope that allows tolerance. Recent studies by Kawakami et al (2006) support this latter hypothesis as they found modification of the free cysteine in HSA can disrupt hydrogen bond interaction, which they state can alter functional activity of the protein.

Final Conclusions

While predictive animal tests, such as the local lymph node assay (LLNA), have been useful in identifying the sensitizing properties of chemicals, they cannot identify the nature of the chemical that stimulates immune cells. For some time there has been a recognized need to move away from *in vivo* skin sensitisation testing and develop novel *in vitro* assays to replace animal tests (Smith Pease 2003) and with the forth coming European Union ban on *in vivo* testing of cosmetics and toiletry ingredients the need to develop non-animal based methods to assess sensitising potential of new chemicals is vital.

In vitro methods such as DCs and lymphocyte proliferation assays and use of cell lines such as THP-1 (Ashikaga *et al.*, 2002; Yoshida *et al.*, 2003) have also been recognised as potentially important assessment methods for predicting the skin sensitisation potential of chemicals and irritants. Using DC and cell line based assays several groups demonstrated the potential of PPD to provide co-stimulatory signalling to activate T cells via increased expression of co-stimulatory receptors such as CD40 and CD86 (Coulter *et al.*, 2007; Toebak *et al.*, 2006; Hulette *et al.*, 2005). The LTT has been used to assess potential of a chemical to stimulate proliferation of T cells *in*

vitro (Sieben *et al.*, 2002; Pichler and Tilch, 2004; Wu *et al.*, 2006; Pickard *et al.*, 2007; Naisbitt *et al.*, 2003). Predictive T cell tests have been developed (i.e.: Engler *et al.*, 2006) with limited success. One reason for this is the lack of knowledge regarding the nature of the antigenic determinants.

Recently, work has focused on using proteomic methods to assess reactivity of chemicals in vitro. In particular several groups have been working on chemical reactivity screening methods to assess a chemicals skin sensitisation potential (Aptula *et al.*, 2006; Divkovic *et al.*, 2005; Gerberick *et al.*, 2004; Kato *et al.*, 2003). These methods involve measuring a chemicals' reactivity with peptides containing single reactive nucleophilic amino acid residues. Peptide reactivity with the test chemicals is reported as percent peptide depletion and data from these assays are proving useful indicators of contact sensitisers (data is in agreement with previously known LLNA data). As shown in this work, more advanced methods are also being used (MALDI and QSTAR) to identify potential binding sites of possible allergens, with the hope of eventually modifying chemicals to avoid such interactions. Work carried out for this thesis used new proteomic techniques to identify possible antigenic determinants by characterising possible binding sites of a chemical on protein and combined this knowledge with cell assays to determine immunogenic potential of this complex. In the future combining chemical reactivity with a biological outcome may prove beneficial in predicting adverse reactions.

One question that remains unanswered in the development of allergic contact dermatitis to PPD is why only a small proportion of hair dye users develop these side effects.

PPD is a primary intermediate required to construct oxidative dyes; primary intermediates are themselves colourless materials capable of being oxidised to give coloured reaction products. Also present in hair dye formulations is a coupler (which when present during the oxidation of primary intermediates result in the production of coloured species), an oxidising agent, (almost always hydrogen peroxide; H_2O_2) and an alkalising agent (usually ammonia). In the first step of dye formation, PPD undergoes an oxidation reaction to form the highly reactive p-benzoquinone diimine which in turn rapidly reacts with the coupler to form polynuclear dyes that are ultimately trapped in the hair shaft revealing what is essentially permanent colour. A study carried out by Rastogi *et al.*, (2006) showed a significant decrease in the content of precursors and couplers in the colour formulation at the zero time point, indicating that the colour forming reaction starts immediately after the two components of the oxidative hair dye product are mixed. However, this study also revealed low level (1.1%) of unconsumed PPD reaches the epidermis, allowing a potential for skin sensitisation to occur.

This unconsumed PPD that arrives in the living epidermis (Hueber-Becker *et al.*, 2004; White *et al.*, 2007) is considered to predominantly undergo dermal N-acetylation as a major detoxification pathway; the majority of PPD is diacetylated when reaching the plasma (Dressler and Appelqvist, 2006) and more than 80% are mono- or diacetylated when excreted in the urine following hair dyeing (Nohynek *et al.*, 2004).

This allows potential for genetic factors to play a role in the development of allergic contact dermatitis and polymorphisms affecting enzyme functions have been elucidated for genes encoding NAT-1 and NAT-2 (Grant *et al.*, 1997). However it is also possible that the sensitisation to PPD is dose dependent, with sufferers of PPD-

associated allergy being susceptible to lower concentrations of PPD, possibly due to the expression of specific, as yet un-defined HLA haplotypes. If this were the case, higher levels of PPD exposure may generate sufficient antigen to overcome the preferred HLA-restriction and stimulate contact sensitization. This theory argues that everyone has the potential to develop allergic contact dermatitis to PPD; susceptibility being dependent on the concentration of PPD on skin.

In conclusion, data presented throughout this thesis has enhanced our understanding of the cellular and chemical basis of PPD sensitisation. By combining proteomic and cellular techniques, we have demonstrated that the presence of PPD circulating T cells is associated with susceptibility to contact dermatitis. PPD bound to cysteine residues on protein stimulates T cells from allergic patients via a processing dependent hapten mechanism and secretion of type 2 cytokines plays an important role in the manifestation of the allergic reaction. Future work now aims to identify the actual haptenic determinants presented in vivo. Work has begun on this by eluting peptides directly from MHC molecules on antigen presenting cells and analysing these elutants using proteomic methods to further understand the importance of protein modification in ACD.

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