ATOPIC ECZEMA : A COMPARISON OF ZEMAPHYTE™, A TRADITIONAL CHINESE HERBAL TREATMENT AND CORTICOSTEROIDS ON CD23 EXPRESSION, CYTOKINE PRODUCTION AND CELL MEDIATED FUNCTION IN VITRO

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ABSTRACT
Atopic eczema (AE) is a chronic relapsing inflammatory skin disorder with multiple cellular and humoral defects. CD23, the low affinity IgE receptor is upregulated in the skin and peripheral blood mononuclear cells in patients with AE. The high affinity IgE receptor, together with CD23 may be important in the chronic T cell infiltration seen in the skin of these patients. The aetiology of the disease is not known thus treatment has focused on the reduction of inflammation in the skin by corticosteroids and other treatments. Two double blind placebo controlled trials of a decoction of 10 Chinese herbs, Zemaphyte™ (ZPT) have demonstrated the efficacy and safety of this treatment in AE. However, the mode of action of this treatment is still unknown and thus the effect of an aqueous extract of ZPT on interleukin 4 (IL-4) induced CD23 expression on the peripheral blood monocytes from non-atopic subjects and patients with AE was investigated. ZPT inhibited CD23 expression up to 60% whereas an aqueous placebo extract (PL) had no significant effect on this expression. This inhibition was not due to cell death as there is no change in cell viability and superoxide production by monocytes in peripheral mononuclear cell cultures (PBMCs). In comparison to prednisolone, ZPT showed a similar inhibitory activity on CD23 expression however cyclosporin, another treatment used in AE, had no significant effect on this expression. ZPT is a complex mixture consisting of many molecules, by mass spectroscopy two possible molecules were isolated which inhibited the expression of CD23.
Along with its action on CD23, ZPT decreased the production of IL-4 and increased TNF - α and IL-10 production by concanavalin A stimulated PBMCs. Treatment of patients with ZPT resulted in the reduction of activation markers for T cells and soluble adhesion molecules in the serum with no change in total IgE. Down regulation of the low affinity receptors for IgE on antigen presenting cells, adhesion molecules and activation markers on T cells in patients with atopic eczema may contribute to the benefit observed following treatment with ZPT. The purified molecule which downregulated CD23 will have to be further elucidated to determine its therapeutic benefit in AE.
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ABBREVIATIONS

AE Atopic eczema
BSA Bovine serum albumin
1% BSA-PBS 1% -bovine serum albumin-phosphate buffered saline pH 7.4
1% BSA-PBS-T 1% -bovine serum albumin-phosphate buffered saline pH 7.4 -0.05% tween
2% BSA-PBS-T 2% -bovine serum albumin-phosphate buffered saline pH 7.4 -0.05% tween
CD Cluster of differentiation
CD23 Low affinity IgE receptor
C_H Constant region of the immunoglobulin heavy chain
CLA Cutaneous lymphocyte-associated antigen
Con A Concanavalin A
DMSO Dimethyl sulfoxide
DSCG Disodium chromoglycate
HLA Human major histocompatibility complex
EBV Epstein-Barr virus
ECP Eosinophil cationic protein
EDTA Ethylenediamine tetra acetic acid
ELISA Enzyme linked immunosorbent assay
DEPC Diethylene pyrocarbonate
FACS Fluorescence activated cell sorter
FCS Foetal calf serum
FITC Fluorescein isothiocyanate
HBSS Hanks buffered saline
HDM House dust mite
H_2O_2 Hydrogen peroxide
H_2SO_4 Sulphuric acid
Ig Immunoglobulin
IL- Interleukin -
IFN- Interferon -
LPS Lipopolysaccharide
LT Leukotriene C
NBT Nitro blue tetrazolium
NOS  Nitric oxide synthase
iNOS  Inducible nitric oxide synthase
PBS  Phosphate buffered saline pH 7.4
PBS-T  Phosphate buffered saline pH 7.4 - 0.05% tween 20
PBMCs  Peripheral blood mononuclear cells
PDE  Phosphodiesterase enzyme
PG  Prostaglandin
PE  Phycoerythrin
PAF  Platelet activating factor
PHA  Phytohaemagglutinin
PL  Placebo extract
PMA  Phorbal 12-myristate-13-acetate
sCD23  Soluble low affinity IgE receptor
sECP  Soluble eosinophil cationic protein
sELAM  Soluble endothelial leucocyte adhesion molecule
sICAM 1  Soluble intercellular adhesion molecule 1
sIL-2R  Soluble interleukin 2 receptor
sVCAM  Soluble vascular adhesion molecule
TEMED  Tetramethyl ethylene diamine
TCHT  Traditional Chinese Herbal Therapy
TGF  Transforming growth factor
Th  T helper cells
TMB  Tetramethyl benzidine
TNF-  Tumour necrosis factor
Tx  Thromboxane
VLA  Very late antigen
UV  Ultraviolet
ZPT  Zemaphyte™ (PSE 222 BN 9240)
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Chapter 1

Introduction
1.1 Diagnosis and morphology of Atopic Eczema

Atopic eczema (AE) is a chronic relapsing inflammatory skin disorder with a genetic predisposition. It has been estimated that in the European population there has been an increasing prevalence, reaching about 12% in children aged between 0 - 7 years (Schultz Larsen 1986; Neame et al., 1993). The disease has a preponderance of females to males in the ratio of 1.4:1.0 and mainly occurs in childhood with remittance and the possibility of recurrences in adult life. The study of Schultz Larsen et al showed that a monozygotic twin had a concordance rate of 0.86 of developing the disease if the other twin already has the disease whilst dizygotic twins only had a risk of 0.21 (Schultz Larsen et al., 1986; Schultz Larsen 1993). This has been interpreted as providing evidence that AE has a genetic basis and several groups have studied the relationship between AE and HLA with no clear association emerging (Krain and Terasakin 1973; Goudemand et al., 1977).

In 1980 a series of guidelines were composed to characterize AE (Hanifin & Rajka 1980). The basis features included pruritus, lichenification, family or personal history of other atopic diseases and chronically relapsing course. Patients were diagnosed as having the disease if they had 3 out of the 4 basic features and 3 out of 23 minor features which included raised IgE, early onset and xerosis. These diagnostic criteria were re-evaluated by a UK working party in 1990 who suggested that AE could be separated from other dermatoses by 6 features instead of the previous 27 features (Williams et al., 1994). These were as follows:

- Pruritus with 3 or more of the following
  - a) History of involvement of skin creases
  - b) History of dry skin
  - c) A personal history of asthma or hay fever or a first degree relative who has either
  - d) Visible flexural eczema
  - e) An onset under two years of age

Although the diagnostic criteria are well known a reliable severity system has not yet been validated and there is a variation in scoring systems used by different dermatologists. Another variation is in the terminology; eczema is the Greek word for 'a boiling over' and referred to the weeping vesiculated dermatitis not always seen in the disease (Hanifin & Rajja 1980). However AE has become synonymous with
Atopic dermatitis with immunologists referring to the disease as the former and dermatologists the latter.

In younger children and infants the eczematous rash is usually found on the trunk, face, scalp and extensor surfaces of the extremities whilst in older children and adults the lichenified eczema is present in the face, neck, eyelids and flexural areas. Pruritus or 'itchy' skin is the hallmark of AE with a vicious circle of itching and scratching leading to the aggravation of the lesions. The lesions can be divided into acute and chronic by histology (Soter 1989). In the acute lesions the epidermis consist of intercellular oedema and a dermal infiltrate consisting mainly of lymphocytes. Macrophages and degranulated mast cells are found in the dermis but only occasionally are eosinophils, neutrophils and basophils seen. The venular endothelial cells are enlarged and slight thickening of the basement membrane is seen. The chronic lesions consist of lichenified plaques with the dermis infiltrated with monocyte-macrophages and lymphocytes. Endothelial cells of the venules are enlarged similar to that seen in the acute lesion but there is also demyelination and fibrosis of cutaneous nerves in the dermis. This last feature may be due to destruction of the nerves through repetitive scratching. Acute and chronic lesions can be present simultaneously as this disease is chronic and relapsing.

AE can be exacerbated by a number of factors including, secondary infections by organisms such as Staphylococcus aureus and herpes simplex virus. Staphylococcus aureus and the pityrosporum species colonize the skin in AE and have also been implicated in the pathogenesis of the disease (Neuber et al., 1991; Back et al., 1995). Stress is another cofactor in exacerbations and it has been suggested that a psychophysiological component is involved in the disease (Jordon & Whitlock 1972). Lastly irritants such as wool and especially soaps can add to the discomfort (Morren et al., 1994). Thus AE is a multifactoral disease whereby both genetics and environmental factors contribute to the disease.

1.2 Atopy and AE

Allergy, a term defined in 1906 by von Pirquet to mean 'changed reactivity' to an antigen on second exposure has over the years been confined to describe Type 1 hypersensitivity. The clinical aspects of type 1 hypersensitivity were characterized by Coca and Cooke 1923 and termed atopy. This reaction is initiated by the exposure of allergen to antigen presenting cells which activate allergen specific T cells. These
T cells stimulate B cells to produce specific immunoglobulin E (IgE) to the allergen. The specific IgE binds to mast cells via their high affinity receptor and on subsequent exposure to the allergen, crosslinking of the IgE molecules by the allergen occurs causing mast cell degranulation. This releases histamine and other inflammatory mediators which manifest as the characteristic wheal and flare reaction seen in allergic individuals. These individuals can be allergic to various proteins including pollens, house dust mites, animal danders and foods. Type 1 hypersensitivity can exhibit itself in hay fever, asthma, eczema and acute food allergy.

Wise and Sulzberg 1933 first used the term atopic dermatitis (AE) to show the similarity between this disease and other allergic diseases such as allergic rhinitis and allergic asthma. Juhlin et al found that one of the features of AE was raised IgE in the serum of 82% of patients (Juhlin et al., 1969). A Type 1 hypersensitivity reaction was also shown by 80% of the patients to environmental allergens with specific IgE to these allergens (Rajka, 1981). One of the most important aeroallergens is the house dust mite (HDM) with many patients having IgE anti HDM antibodies (Platts-Mills et al., 1983). Studies have elicited lesions of eczema after patch testing with HDM for 24 hours (Gondo et al., 1986; Langeland et al., 1989). However most of these patch tests become positive after 24 hours with only a few giving an reaction within 20 minutes (Langeveld-Wildschut et al., 1995). Similar results have been obtained for other allergens including pollens (Wuthrich et al., 1989; Clark & Adinoff, 1989) and animal dander (Clark & Adinoff, 1989). These patch tests show an infiltration of T cells which indicated that the disease may be a mixture of Type 1 and Type IV hypersensitivity where the T cell predominates.

Food allergy and its contribution to atopic eczema is still controversial. Most of the work conducted in this field has been carried out by Sampson and colleagues. In one study they tested 320 children with double blind placebo controlled food challenges and found that of the patients that reacted, 75% did so with skin eruptions (Sampson 1988). Those with positive food challenges also had a positive skin prick test to the food. The foods which cause the most reactions were eggs, peanuts, milk, fish and wheat. It is probable that IgE mediated food allergy is more important in children whilst HDM allergy is more prevalent in adults.

Genetic studies on atopy related genes have shown that there is a gene predisposing to atopy on chromosome 11q13 when asthmatics were used as a proband (Cookson et al., 1989; Young et al., 1992) and that this gene may be in close linkage with the 23
subunit of the IgE high affinity IgE receptor on chromosome 11 (Sandford et al., 1993). Variations in the subunit of this receptor were seen and it was thought that it is these variations that may be the link to atopy (Shirakawa et al., 1994). However this relationship with atopy and chromosome 11q13 has not been seen in a pure population of AE patients (Coleman et al., 1993) or when a mixed population of asthmatics, hay fever and AE patients were used (Hizawa et al., 1992). Thus the relationship between atopy and AE may be a complex interaction of different genes and environmental aspects.

1.3 IMMUNE DYSREGULATION IN AE

The inflammatory process seen in AE consists of a mixture of immunological and pharmacological abnormalities that are reflected in the serum and at the cellular level. This section discusses these defects and the proposed theories for the changes seen in the skin.

1.3.1 Serological Abnormalities

1.3.1.1 IgE

As mentioned previously, one of the prominent features of AE is a raised IgE, with patients having 100 times greater levels (200-20,000 ng/ml) than normal individuals (2-200 ng/ml). The relevance of this finding will be discussed later. Along with high IgE levels in AE, it has been demonstrated that the patients also have high levels of complexed IgE in the serum (Brostoff et al., 1977; Jacob et al. 1985; Kapp et al., 1986; Czech et al., 1995). The finding of IgG anti-IgE antibody in patients with AE lead to the belief that the IgE complexes consisted of IgE and this auto antibody (Nawata et al., 1985; Quinti et al., 1987), although Brostoff et al found that IgE could also be complexed with food allergens (Brostoff et al., 1979). The IgG autoantibody discovered was later shown to induce the release of histamine and other mediators from basophils and mast cells from one out of six AE patients (Marone et al., 1989) and has been isolated by repertoire cloning (Vogel et al., 1994). Stadler et al postulated that the anti-IgE autoantibody in a normal individual was produced to neutralise IgE response but that an allergic individual has a defect in this autoantibody which instead of neutralising IgE, potentiates its response and therefore a vicious circle is set up (Stadler et al., 1992). IgE auto antibodies have been shown to be directed at both the C_{H3} and C_{H4} regions of IgE in AE patients but
in controls only at the Cig3 region (Czech et al., 1995). This result may point to some evidence for Stadler's theory with the possibility that autoantibody directed at the Cig4 potentiates IgE responses. The importance of this autoantibody is still unknown as after therapeutic intervention with UV treatment which cleared the skin lesion there was no change in the autoantibody concentration.

1.3.1.2 Soluble CD23 (sCD23)

The low affinity IgE receptor (CD23) may play a role in IgE regulation as will be shown later. The soluble form of this receptor has been found in serum and urine (reviewed by Gordon, 1991). Studies have shown that patients with AE have increased levels of sCD23 in their serum compared to controls (Kim et al., 1989; Wuthrich et al., 1991; Kagi et al., 1992). These levels appeared to be age dependent with the highest being in infants and decreasing with age and with an overlap between the patient and control groups (Yanagihara et al., 1990; Kim et al., 1989; Matsumoto et al. 1991). Although the levels are raised there is no change in this elevation with treatment (Wuthrich et al., 1992; Kagi et al., 1992). Borres et al studied 64 children for the development of allergic diseases over a period of 18 months and found that although sCD23 was raised it showed no association with allergic disease or family history (Borres et al., 1995). However only 47% of their children developed allergic manifestations and only a third of these had AE. Thus the sCD23 levels in infants who developed only AE would have to be studied to determine the significance of an elevated sCD23 in relationship to the appearance of the disease.

1.3.1.3 Eosinophil Cationic Protein (ECP)

Patients with AE frequently have a raised eosinophil count and eosinophil-granule major protein are found in their skin (Leiferman, 1989; Leiferman et al., 1985). Eosinophil cationic protein (ECP) is a toxic protein released from eosinophil in the tissues and detected in the serum (Peterson et al., 1988). Elevated levels of ECP have been analysed in the serum of AE patients, however there was no correlation with blood eosinophil count (Kapp et al., 1991; Kagi et al., 1992). In nineteen patients undergoing different therapeutic regimens including corticosteroids and phototherapy there was a decrease in ECP after treatment (Czech et al., 1991). This was irrespective of the treatment given and ECP levels correlated with total clinical scores. The authors suggested that ECP may be useful for monitoring disease activity in AE.
1.3.1.4 Soluble IL-2 receptor (sIL-2R)

Along with activated eosinophils, patients with AE have activated T cells; the relevance of this will be discussed later. Activated T cells express the IL-2R and the $\alpha$ chain of this receptor is not only expressed on the surface but can be shed (sIL2R) and maintain its affinity for IL-2 (Rubin et al., 1986). Raised levels of sIL-2R have been reported in AE compared to controls (Kapp et al., 1988; Colver et al., 1989; Wuthrich et al., 1990; Kagi et al., 1992). This again correlates with disease activity and was reduced on treatment (Colver et al., 1989; Wuthrich et al., 1990; Kagi et al., 1992) but the decrease was seen best in patients treated with systemic steroids (Kagi et al., 1992). In a study of patients for 3-6 weeks on treatment it was demonstrated that a reduction in sIL-2R preceded a drop in ECP levels and it was suggested the activated T cells may be controlling eosinophil activity (Walker et al., 1992).

1.3.1.5 Soluble CD14 and adhesion molecules

Another cell type which may be important in AE is the monocyte. CD14 has been shown to be predominantly expressed on mature monocytes and macrophages (Todd et al., 1981). The soluble form was found in serum and was elevated in some pathological conditions and may be a reflection of increased monocytic activity. (Brazil et al., 1986). One report has shown that sCD14 is elevated in AE and there was a significant difference after treatment. (Wuthrich et al., 1992) The importance of this finding is unknown but may point to monocyte involvement.

Recently the intercellular adhesion molecule 1(ICAM-1) has also been implicated in AE. ICAM 1 is an adhesion molecule expressed on many cells and is a ligand for the lymphocyte function-associated antigen integrin on T cells, eosinophils and neutrophils (Marlin & Springer 1987; Gearing & Newman, 1993). A soluble form of this protein (sICAM) was detected in human serum by Seth et al (Seth et al., 1991). sICAM-1 was reported elevated compared to controls and decreased on treatment with topical steroids (Koide et al., 1994; Wuthrich et al., 1995; Kwalzick et al 1995). This was again predicted as a marker following clinical activity in AE. Soluble forms of the endothelial leucocyte adhesion molecule (sELAM) and E-selectin have also been detected and are raised in AE (Kwalzick et al .,1995; Czech et al., 1996 ). Although these proteins have been all shown to be elevated in AE, they are not exclusive to the disease. IgE auto antibody, sCD23, ECP, and ICAM-1 have also been shown in asthma (Nawata et al., 1984; Yanagihara et al., 1990 ; Hashimoto et al., 1993) and these manifestations may be the result of an ‘allergic response’ in two
different organs. However raised levels of sIL2- R and sCD23 have been reported in other inflammatory diseases such as rheumatoid arthritis (Symons et al., 1988; Chomarat et al., 1993) and myasthenia gravis (Bansai et al., 1993) thus some of the serum abnormalities seen in AE may be due to generalised inflammatory response rather than in the aetiology of the disease.

1.3.2 Cellular Defects
Evidence for a cellular component in AE came from the finding that atopy can be transferred through bone marrow transplantation (Agosti et al 1988). Also patients with Wiskott-Aldrich syndrome manifest eczema and thromocytopenia, the eczema being cleared following bone marrow transplantation. (Saurat, 1985). Thus the inflammation seen in AE may be due to an abnormality in one or more of the bone marrow cellular constituents.

1.3.2.1 Mast Cells and Basophils
Mast cells are the major initiating cells in the Type 1 hypersensitivity reaction and thus should be central in AE. In chronic lesions of AE mast cells are increased but in acute lesions the numbers are normal indicating they have a role in the continuation of the disease. (Soter 1989). Recently mast cells have been shown not only to release mediators such as histamine and prostaglandins but also cytokines (Okayama et al., 1995). These cytokines include IL-4, IL-5, IL-8 and tumour necrosis factor (TNF)-α. Horsmanheimo et al. studied the percentage of mast cells staining for IL-4 immunoreactivity in biopsies from patients with AE, nummular eczema and controls and found that 66% of the mast cells in AE were producing IL-4 compared to 23% in controls; in nummular eczema this percentage was 46 (Horsmanheimo et al., 1994). The authors suggested that mast cells are one source of IL-4 in the skin of AE patients and these cells could contribute to the development of the disease.

Basophils from AE patients have a hyperreleasibility of histamine when stimulated with Con A or anti IgE compared to controls (Butler et al., 1985; James et al., 1993) and this phenomena was related to the severity of the disease (Findly & Lichtenstein 1993). This hyperreleasibility has been linked to the increased phosphodiesterase hydrolytic activity, a defect which will further be discussed (Butler et al., 1985). IL-4 secretion has also been seen when basophils where stimulated by anti-IgE or IgE independent mechanisms. Therefore they may also be involved in the initiation
1.3.2.2 Langerhans Cells

Langerhans cells are known for their role in Type IV delayed type hypersensitivity such as contact sensitivity. In this reaction Langerhans cells internalize the antigen and travel to the regional lymph nodes where they activate CD4 + naive T cells. These T cells proliferate and return to the skin where on subsequent exposure to the antigen they are activated (reviewed by Hogan & Burks 1995). In AE the number of Langerhans cells was not significantly different from normals (Sillevis Smith et al., 1986; Bieber et al., 1988). However the finding in 1986 that IgE was bound to the surface of Langerhans cells suggested a new role for these cells in AE (Bruijnzeel et al., 1986). These Langerhans cells bearing IgE were found to be resident in inflammatory skin diseases where the circulating IgE levels were raised (Bieber & Braun- Falco 1991). It was first shown that normal Langerhans cell could express the low affinity Fc receptor for IgE, (CD23) (Torresani et al., 1991) and this expression was increased in lesional skin (Buckley et al., 1992). These results were soon followed by the discovery that Langerhans cells from normal and AE patients also expressed the high affinity IgE receptor ( Bieber et al., 1992; Wang et al., 1992; and Grabbe et al., 1993). Thus, the Langerhans cells in AE have the ability to bind IgE via a variety of IgE receptors and these receptors, the relevance of this binding, and the connection to the protein tyrosine phosphatase CD45 will be discussed later.

1.3.2.3 Eosinophils

Patients with AE are found to have a blood eosinophilia and these levels correlate with disease severity (Uehara et al., 1990; Walker 1992). Uehara et al also demonstrated that AE patients with a familial or personal history of respiratory allergy had elevated levels of eosinophils in their blood. Bruijnzeel et al performed patch test reactions with HDM on uninvolved skin of AE patients and investigated the role of eosinophils (Bruijnzeel et al. 1993). These experiments showed that lymphocytes and eosinophils infiltrated the dermis between 2-6 hours and within 24 hours they released ECP. After 48 hours the eczematous reaction decreased and the eosinophils disappeared. These authors postulated that eosinophil recruitment in lesional skin in AE was under the control of T cell derived cytokines such as IL-4 and IL-5. This theory has been indirectly confirmed by the finding that a decrease in activated T cells precedes a decrease in activated eosinophils following treatment (Walker et al., 1992). Hamid et al compared acute and chronic lesions in patients with AE and
found that acute lesions had more IL-4 producing cells than IL-5 whilst the reverse was seen in chronic lesions with more IL-5 producing cells (Hamid et al., 1994). The chronic lesions also had more eosinophils, therefore the maintenance of chronic inflammation may be associated with eosinophil infiltration.

1.3.2.4 Monocytes

One of the original findings on monocytes in AE patients was that there was a decrease in chemotaxis (Rogge & Hanifin, 1976). A decrease in natural killer cell activity has also been demonstrated and this was linked to a suppressive effect of monocytes (Jensen et al., 1984; Hall et al., 1985). In respect to cytokines, IL-1 production was found to be impaired in AE patients (Rasanen et al., 1987; Jakob et al., 1995) although others such as Thesstrup-Pederson et al have reported an increase (Thesstrup-Pedersen et al., 1990). Recently, overexpression of mRNA for IL-10 has been discovered in lesional skin of AE patients and this was mostly strongly expressed in CD14+ cells (Ohmen et al., 1995). This group also showed that the monocytes of these individuals produced more spontaneous IL-10 than controls when cultured for 24 hours and postulated that the increased IL-10 and IL-4 may be the cause of decreased interferon (IFN-γ) seen in AE. Increased prostaglandin E₂ (PGE₂) production from monocytes has also been implicated in lower IFN-γ levels as removal of monocytes increased IFN-γ production from T cells from AE patients (Chan et al., 1993). These authors also showed a correlation between increased PGE₂ levels and increased phosphodiesterase activity (PDE). PDE is involved in the hydrolysis of cyclic adenosine monophosphate (cAMP) and an increase of this enzyme is seen in AE leucocytes (Grewe et al., 1982; Chan et al., 1982; Sawai et al., 1995) and in particular in monocytes (Holden et al., 1986). It is thus been suggested that increased PDE activity leads to excessive PGE₂ secretion by monocytes and PGE₂ inhibits IFN-γ production by T cells. Thus PDE inhibitors specific for monocytes may be effective in the treatment of AE (Hanifin & Chan, 1995). The monocyte function may be crucial in initiation and continuation of the disease.

1.3.2.4 B cells

The two major defects found in AE with regard to B cells are that they have an increased spontaneous release of IgE in vitro (Vollenweider et al., 1991; Poulsen et al., 1995) and an increased CD23 expression (Spiegelberg et al., 1979; Nakamura et al., 1991; Muller et al., 1991). These findings are irrespective of the fact that the total number of B cells in AE has been found to be in the normal range (Walker et al.,
Both defects are under the control of IL-4 and IL-13 and therefore may be related to the activation states of T cells.

1.3.2.5 T Cells

Histologic studies in AE demonstrated that the cell infiltrate of the skin consisted of lymphocytes and monocytes (Mihm et al., 1976). This filtrate was further characterized to be predominantly T cells which were of the CD4+ subset (Braathen et al., 1979; Zachary et al., 1985; Sillevis Smith et al., 1986). In relationship to peripheral T cells in AE there have been reports to suggest that there was an increase in the ratio of CD4+ to CD8+ T cells compared to normals. This change in ratio has been attributed to an increase in CD4+ cells (Reinhold et al., 1990; Sowden et al., 1992) and a decrease in CD8+ cells (Leung et al., 1981; Reinhold et al., 1990; Sowden et al., 1992). The activation markers have also been investigated and Sowden et al have also showed that the percentage CD4+ T cells expressing HLA-DR and IL-2R were increased in patients with AE indicating that these cells were activated (Sowden et al., 1992).

The cutaneous lymphocyte-associated antigen (CLA) is the homing receptor on T cells for cutaneous sites (Picker et al., 1990). Interestingly allergen-specific Th2 clones isolated from the skin of AE patients expressed high levels of CLA and bound recombinant E-selectin (Rossiter et al., 1994). Isolated CLA+CD45RO+ T cells from AE who were skin prick test positive to HDM proliferated in response to this allergen whilst the CLA-CD45RO+ cells did not respond (Santamaria et al., 1994; Santamaria et al., 1995). In asthmatic patients who were allergic to HDM the reverse was seen with the proliferative response being in the CLA-CD45RO+ T cell population. These results indicate that in asthmatics there might be another homing molecule which is restricted to the lung. In a study of milk induced AE, there was a greater percentage of CLA+ T cells after stimulation with casein than in healthy controls or milk-induced gastroenteritis (Abernathy-Carver et al., 1995). The authors suggested that these results demonstrate the link between food allergy and the homing of T cells against foods to the skin.

In regard to T cell responses a depressed lymphocyte response to mitogens including phytohaemagglutinin (PHA), concanavalin A (Con A) and antigen has been reported by several workers (Lobitz et al., 1972; Elliot & Hanifin 1979; Jakob et al., 1990). This defect is more likely to be secondary to inflammation as the proliferation was normalised when the patients were in remissions (Lobitz et al., 1972) or when the
cells were preincubated for 2-4 days before testing (Elliot & Hanifin 1979). When peripheral blood T cells and autologous Langerhans cells were used in an autologous mixed leucocyte reaction (AMLR) there was decreased responsiveness compared to controls (Rasanen et al., 1987). Similar results were obtained with autologous mixed lymphocytes in AE patients and this was irrespective of whether the patients were in remission (Leung et al., 1983). Although these responses are indicative of decrease in cell mediated responses it is difficult to reconcile these findings with the increased activation state of the T cells in the skin and blood of AE patients.

1.4 IgE and Cytokine Profiles

1.4.1 Control of IgE Production

IgE production requires two signals, the first is IL-4 (Lebman et al., 1988) or IL-13 (Punnonen et al., 1993) and the second a cognate interaction between T and B cells which involves CD40 and its ligand (Jabara et al., 1990). These two signals lead to Ig-isotype switching to IgE. Recently, the interaction of CD23 and CD21 has also been shown to be involved in the control of IgE production (Aubry et al., 1992). IL-5 (Pene et al., 1988), IL-6 (Vercelli et al., 1989), TNF α (Gauchat et al., 1992), sCD23 (Sarfati et al., 1984a), hydrocortisone (Jabara et al., 1991) and β2 adrenoceptor agonists (Coqueret et al., 1995) have all been shown to enhance IL-4 induced IgE production in vitro. IFN-γ, IFN-α, PGE2 and IL-10 opposed the action of IL-4 and inhibits its induction of IgE (Pene et al., 1988; Punnonen et al., 1993). Depending on the system used cyclosporin seems to inhibit (Chang et al., 1993) or potentiate IL-4 induced IgE production (Wheeler et al., 1995).

In AE higher spontaneous in vitro release of IgE can be seen compared to controls (Vollenweider et al., 1991; Poulsen et al., 1995). The ability of IL-4 to increase this production in AE has yielded conflicting results with some groups showing an increase with IL-4 (Rousset et al., 1991; König et al., 1995; Poulsen et al., 1995); whilst others show no effect (Vollenweider et al., 1991). Dolecek et al also showed that although spontaneous pollen specific IgE was produced in allergic patients, the addition of anti CD40 with IL-4 or IL-13 did not increase this production although total IgE was increased (Dolecek et al., 1995). The higher spontaneous IgE production in vitro may be an indication that the B cells from AE patients have already been primed for IgE production in vivo. Recently it has been shown that the mean fluorescence intensity of CD40 on B cells isolated from patients with AE is increased.
compared to controls and the authors agree that this in part may lead to an increase in IgE production (Renz et al., 1994).

1.4.2 Cytokine imbalance in AE

Mossman et al. showed in the murine system that there were two types of CD4+ helper T cells, Th1 and Th2 (Mossman, 1986). Th1 on stimulation produced IL-2 and IFN-γ whilst Th2 produced IL-4, IL-5, IL-6, IL-10. Th0 was later discovered to produce a combination of Th1 and Th2 cytokines (Mosmann et al., 1991). In humans it is thought that these subsets exist however IL-10 may be produced by all three subsets (Wierenga et al., 1990; Del Prete et al., 1993). The Th2 subset provides help for B cells and antibody mediated responses; thus IgE production is favoured by this subset. The regulation of IgE production is normally controlled by a fine balance of cytokines and cell surface markers, however in AE there is a disruption of the balance and thus workers have looked for an imbalance in cytokines to throw light on this defect.

The first clue to a cytokine imbalance in AE was in the finding of Reinhold et al who showed that on PHA stimulation of peripheral blood mononuclear cells (PBMCs) from AE patients and controls, AE patients produced lower levels of IFN-γ (Reinhold et al., 1988; Reinhold et al., 1990). This was later confirmed by other workers using different mitogens (Rousset et al., 1991; Jujo et al., 1992; Tang et al., 1993). The percentage of IFN-γ producing cells in children with AE was found to be higher in unstimulated cultures than in control subjects (Tang & Kemp, 1994a). This defect in IFN-γ production was not overcome by the addition of IL-2 anti IL-4 and or calcium ionophore (Reinhold et al., 1990; Jujo et al., 1992) and these results lead Tang et al to investigate the mRNA for IFN-γ (Tang et al., 1994b). The group demonstrated that AE children had an increased mRNA for IFN-γ in unstimulated and stimulated cells compared to controls and concluded that AE patients had a post-transcriptional defect in IFN-γ production. IL-4 has also been investigated and an increased level was found compared to controls (Rousset et al., 1991; Jujo et al., 1992; Renz et al., 1992; Tang et al., 1993). An increase in mRNA for IL-4 receptor and spontaneous expression of IL-4 mRNA have also been demonstrated (Renz et al., 1992; Tang et al., 1994c). These results have indicated that in AE there is an imbalance of CD4+ cells with the Th2 subset prevailing. This theory is supported by the reports that have shown that low production of IFN-γ in cord blood (Rinas et al., 1993; Warner et al., 1994) and increased levels of IL-4 in the serum.
(Borres et al., 1995) is a predictor of atopic disease. Interestingly enterotoxin B produced by Staphylococcal aureus stimulated the peripheral blood mononuclear cells of AE patients to produce more IL-4 and IL-5 and less IFN-γ than controls (Neuber et al., 1995). Pityrosporum ovale induce IL-4 and IL-10 production from PBMCs from AE patients but IL-2 and IFN-γ from controls (Kroger et al., 1995). Thus these two organisms could contribute to the expansion of Th2 in the skin of some AE patients.

Another factor which might be connected to the cytokine imbalance is that increased IL-4 and hyper IgE production were both reversed when PDE inhibitors were added to AE cells in vitro (Chan et al., 1993; Cooper et al., 1985). However, a decrease in IFN-γ production inhibitor was also seen with a PDE inhibitor (Ostlere et al., 1995) although it was thought that excessive PDE activity was responsible for the decreased IFN-γ production. Thus the influence of secondary messengers and prostaglandins may be influential in the balance of cytokines which prevail in AE with Th1 and Th2 cells responding to different secondary messengers (Holden, 1993).

Other cytokines have also been shown to be impaired, IL-2 was decreased after stimulation with PHA compared to controls whilst increased serum IL-2R was seen (Kapp et al., 1991). These two findings seem contradictory to one another unless the serum sIL-2R is a reflection of the inflammation in the skin. TNF-α has been shown to be decreased after PHA stimulation (Kapp et al., 1990; Takahasi et al., 1992) in AE compared to controls but others have found no difference (Reinhold et al., 1989). Toshitani et al. reported that unstimulated T cells from AE patients produced more IL-6 than controls (Toshanti et al., 1993).

1.4.3 Cytokine production by T cell clones in AE

In all the studies mentioned above PBMCs or isolated T cells were used, Wierenga and his colleagues provided the first evidence from 2 AE patients that allergen specific T cells to HDM could be isolated and cloned. These clones produced IL-4, IL-5, IL-6 TNF-α and reduced levels of IL-2 and IFN-γ whilst allergen specific clones from non-atopics produced IFN-γ, IL-2, IL-6 and TNF-α (Wierenga et al., 1991). T cell clones specific for tetanus toxoid and Candida albicans from one patient showed a Th1 profile indicating that only the allergen specific clones were of the Th2 profile. This work was extended to show that it was not only HDM specific T cells which were of this profile but also grass pollen specific T cells (Parronchi et al., 1991). Although these clones were isolated from the blood of AE patients it was
not clear if they were significant to the lesional skin seen in AE. To address this Reinhold et al isolated T lymphocytes from skin biopsies of AE patients and found that stimulation of these cells with Con A induced significant concentration of IL-4 but only small amounts of IFN-γ and IL-6 (Reinhold et al., 1991). Pollen specific T cells and HDM specific T cells have been cloned from lesional skin (Ramb Lindhauser et al., 1991; van der Heijden et al., 1991). These all showed Th2 cytokine profiles and in the case of the HDM clones there was a higher frequency of these T cells in the skin compared to the peripheral blood. Allergen specific T cell lines obtained from biopsies after 24 hours patch testing with HDM (n=30) showed that 70% had the Th2 phenotype whilst 15% were of the Th0 phenotype (Van Reijsen et al., 1992). It is now established that allergen specific T cells occur in lesional skin and are of the Th2 subtype but their significance is not yet known.

As the criteria for diagnosis allows for AE patients who have no history of allergies, Kagi et al have proposed that AE patients can be subdivided into those who have an allergic basis for their disease and a non-atopic (intrinsic) group who have low IgE and negative skin tests. He compared these two groups and found that the PBMCs from the atopic group spontaneously produced high levels of IL-4 and IL-5 whilst the non-atopic group produced high levels of IL-5 and normal levels of IL-4 (Kagi et al., 1994). This was also revealed in the mRNA for the cytokines in the biopsies from the lesional skin of both groups. Thus this data demonstrated different immunological abnormalities could lead to the skin inflammation seen in AE.

In this disease there are many immunological defects and therefore it is difficult to distinguish which are involved in the pathogenesis of the disease and which are concomitant with inflammation. Recently, researchers in the field have suggested that IgE, its receptors and T cell control are the most important factors in patients with an allergic component.

1.5 IgE Receptors

IgE can bind to a variety of cells through three different receptors, the structure and function of two of these receptors and their relevance to AE will be outlined.

1.5.1 The High Affinity IgE receptor (FceR1)

The high affinity IgE receptor consists of four polypeptide chains α, (50-55kd) β (33kd) and γ2 (7-9Kd) (reviewed by Ravetch & Kinet 1991). The α subunit binds IgE whilst the β and γ2 are involved in the expression of the α chain and signal
transduction (Sutton & Gould 1993). The α chain is a member of the immunoglobulin supergene family and the binding of IgE is thought to be in the second domain of the α chain as predicted by inhibitory antibody binding (Riske et al., 1991) and chimeric Fc receptor binding (Hulett et al., 1993). The high affinity IgE receptor was thought to be only expressed on mast cells and basophils (Ravetch & Kinet 1991) until 1992 when it was showed that normal human epidermal Langerhans cells also expressed this receptor (Bieber et al., 1992; Wang et al., 1992). Since then monocytes and eosinophils have also been demonstrated to express the high affinity IgE receptor and the expression is related to the level of IgE (Maurer et al., 1994, Gounni et al., 1994). The receptor on Langerhans cells and monocytes only have the α and γ2 chains whilst eosinophil have the full complement. It has been shown that the three chain FceRI can be a functional receptor (Miller 1989). Also disruption of the α or γ chain on mast cells results in degranulation defects showing their importance in IgE-mediated responses (Dombrowicz et al., 1993; Takai et al., 1994).

1.5.2 CD23 (low affinity IgE receptor)

CD23, the low affinity IgE receptor was initially found on B cells and several lymphoblastoid B cell lines (Lawrence et al., 1975, Gonzalez-Molina et al., 1976). It was independently discovered as marker on B cells infected with the Epstein-Barr virus (EBV) and on low levels on freshly isolated B cells (Kintner et al., 1981; Thorley-Lawson et al., 1985). It is a type II integral membrane 45kd glycoprotein i.e a reverse orientation of the molecule with a cytoplasmic N terminus (Kikutani et al., 1986; Ludin et al., 1987; Ikuta et al., 1987) and belongs to the calcium dependent lectin type family (Ikuta et al., 1987). From the sequence it was predicted that the region between the intracellular domain and C terminus would form α helical coils which would allow the formation of dimer or trimer molecules (Beavil et al., 1992; Dierks et al., 1993). There was a single copy of the human CD23 gene but two transcripts were derived from different transcriptional sites and alternative RNA splicing (Yokota et al., 1988). This gave rise to two different forms, CD23a and CD23b which differed by 6-7 amino acids in the N terminus. Type A is constitutively expressed on μδ B cells ((Kikutani et al., 1986) and type B can be induced by IL-4 on Langerhans cells (Bieber et al., 1989) and monocytes (Vercelli et al., 1988). IL-13 to a lesser extent can also induce CD23 on monocytes (de Waal Malefyt., 1993). IL-4 and IL-13 are shown to induce a nuclear factor called NF-IL-4 which interacts
with a DNA sequence in the promoter region of CD23b (Kohler & Rieber 1993; Kohler et al., 1994). In mice, type A is found on B cells and follicular dendritic cells (Waldschmidt et al., 1988, Maeda et al., 1992). However, whether type B form exists is still unclear with two groups obtaining conflicting results at the genomic level (Richard et al., 1991; Conrad et al., 1993).

CD23 is cleaved from the surface of cells first by a membrane protease to release a 37kd protein (Cairns and Gordon 1990) then autoproteolysis occurs to yield several fragments of 33, 29 and 25 kd (Nakajima et al., 1987, Letellier et al., 1988; Letellier et al., 1989; Letellier et al., 1990). However, some fragments can also be produced intracellularly (Lee et al., 1989). The most stable of these is the 25Kd form and this can be measured in the serum (sCD23) of individuals (Letellier et al., 1989). All the soluble forms retain their binding to IgE but the affinity is less than the surface molecule. Interestingly, Der p 1, the major allergen from HDM has protease activity and has been shown to cleave CD23 from the RPMI 8866 B cell line and it was suggested that this allergen may upregulate IgE production by cleaving CD23 from the surface of cells (Schulz et al., 1995; Hewitt et al., 1995).

1.5.2.1 Ligands for CD23

CD23 has two known ligands, the first, IgE is predicted to bind near the Ca2+ binding site (reviewed by Sutton & Gould 1993). On IgE binding, CD23 expression is increased and the rate of cleavage is reduced (Delespesse et al., 1991). The function of CD23 seems to be dependent on the expressing cell type. It has been shown to be spatially associated with HLA-DR antigens (Bonnefoy et al., 1988) on B cells and an anti-HLA-DR antibody decreases IL-4 induced CD23 expression (Kicza et al., 1989). Thus, it was speculated that CD23 on B cells may be associated with antigen presentation. This has been confirmed whereby human EBV transformed B cells and mouse B cells have been shown to present IgE complexed antigen to T cells via CD23 (Pirron et al., 1990; Kehry & Yamahita 1989). CD23 bearing monocytes have been found to be involved in phagocytosis of IgE coated particles (Spiegelberg 1984), IgE-dependent cytotoxicity and the release of mediators (Capron et al., 1983; Joseph et al., 1983). Macrophages when incubated with IgE dimers can also release superoxide and IL-1 (Dessaint et al., 1982) and IgE stimulated macrophages secrete IL-1α and TNF-α (Borish et al., 1991). This data presented for the function of CD23 has been strengthened by the data of Yokota and coworkers that type A bearing cells are involved in endocytosis whilst type B cells are involved in phagocytosis (Yokota et
al., 1992). The second ligand for CD23 is the complement receptor CD21. This interrelationship is calcium dependant and may be involved in the control of IgE synthesis (Pochon et al., 1992; Aubry et al., 1992). Henchoz et al reported that when comparing anti-CD21 monoclonal antibodies one was found which increased IL-4 induced IgE production by threefold (Henchoz et al 1994). This production was dependent on an anti CD40 antibody or the presence of T cells. Although the anti CD21 increased IL-4 induced $\varepsilon$ transcription levels, it did not induce emRNA by itself. The effects of HDM allergen and IL-4 on CD23 and CD21 on T and B cells of asthmatic patients and controls were investigated (Gagro et al., 1994). It was found that allergen and/or IL-4 induced CD23 expression on T cells of asthmatic patients and the allergen alone increased the mean fluorescence of both CD23 and CD21. This was not observed in the controls and it was concluded that the ability of allergen to induce CD23 expression on T cells may be the reason for their increased IgE synthesis. These experiments have not been so far performed in AE and thus it is not known whether the same observation would be made. CD21 expression on a basophilic leukemia cell line has recently been shown. Histamine release was seen from this cell line and human basophils either by triggering with CD23-liposome or anti CD21 antibody (Bacon et al., 1993). This showed another involvement of the CD21-CD23 interaction with the allergic response.

A role for sCD23 in IgE synthesis was first described by Sarfati and co-workers whereby they showed that sCD23 increased the spontaneous IgE production from B cells from allergic individuals (Sarfati et al., 1984a ; Sarfati et al.,1984b). sCD23 has also been reported to synergise with low levels of IL-4 to produce IgE (Pene et al., 1988; Chretien et al., 1990). Fragments $\geq$ 29kd have the ability to increase IgE synthesis (Sarfati 1992), this enhancing ability of larger sCD23 fragments may be due to the fact that they retain their trimer structure and can crosslink membrane IgE and CD21 on the surface of the B cell (Sutton & Gould,1994).

Surface CD23 and sCD23 have both been shown to have other properties not involved in IgE responses. The transfection and expression of CD23 into several cell types gave the first clue that CD23 might be involved in cell adhesion (Kikutani et al., 1989). Antibodies to CD23 and CD21 inhibited aggregation in human B cells and a variety of B cell lines (Bjorck et al., 1993). This group suggested that along with LFA-1/ICAM1 interaction, CD21 and CD23 may be important pairs in the homotypic aggregation of human B cells however this has not been shown in the
murine model (Davey et al., 1995). Bjorck et al also proposed that the CD23 /CD21 may be involved in the interaction between B cells and follicular dendritic cells. The finding that germinal centre B cells are prevented from apoptosis when cultured with anti CD21 antibodies has lead to a clue that this proposal may be correct (Bonnefoy et al., 1993).

sCD23 with IL-1 synergised to induce myeloid precursor cell growth, maturation of prothymocyte and the survival of germinal centre B cells (Mossalayi et al., 1990 Liu et al., 1991). The regulation of cytokine secretion is another feature of sCD23. It increased IL-2 and IL-12 induced IFN-α production from PBMCs but this effect was not seen when monocytes were removed, indicated that sCD23 was acting on monocytes (Armant et al., 1994). This group also showed that sCD23 by itself could induce TNF-α, IL-1α, IL-1β and IL-6 from PBMCs and monocytes but there was no production of IL-10 and IL-12. Another group demonstrated that sCD23 augmented IL-1 induced IL-6 and IL-1 receptor antagonist (IL-1ra) in whole blood cultures (Herbelin et al., 1994). When monocytes were isolated, sCD23 increased IL-1ra and IL-6 by itself as well as augmenting the effect of IL-1. sCD23 has also been shown to inhibit migration of the U937 monoblastic cell line (Flores-Romo et al., 1989). Thus sCD23 may have potentiating role in inflammation by the induction of proinflammatory cytokines.

### 1.5.2.2 In vivo manipulation of CD23

With the advance of gene manipulation, knockout mice and transgenic mice have been produced for CD23. In the knock out mice model there were no defects in T or B cell development (Yu et al., 1994; Stief et al., 1994; Fujiwara et al.,1994). In response to the parasite Nippostrongylus brasiliensis the knockout mice produced the same level of polyclonal IgE as the control littermates. Specific IgE antibodies to thymus-dependent antigens were raised on immunization in the knockout mice (Yu et al., 1994; Fujiwara et al., 1994). This lead to the conclusion by all the groups that CD23 was involved in downregulation of IgE responses. These results might explain the older observations that anti-CD23 antibodies were capable of suppressing IgE responses (Sarfati & Delespesse, 1988; Maggi et al., 1989; Bonnefoy et al., 1990;). It is interesting that these knockout mice were unable to enhance IgE mediated antibody response using an anti IgE antibody and challenging with DNP-OA (Fujiwara et al., 1994). This result indicated the role of CD23 in IgE dependent antigen presentation in vivo. In transgenic mice the overexpression of transmembrane
or soluble CD23 in their B or T cells showed no difference in basal IgE or IgG1 levels compared to littermate controls (Texido et al., 1994). Polyclonal IgE responses were reduced only in the transmembrane CD23 transgenic mice. This mouse data on CD23 however may be different from the counterpart seen in human as the overexpression in human is usually of the CD23b type.

1.6 The role of IgE receptors in AE

Patients with AE have all been shown to have elevated levels of CD23 on the surface of B cells (Spiegelberg et al., 1979; Nakamura et al., 1991; Muller et al., 1991), T cells (Takigawa et al., 1991), monocytes (Melewicz et al., 1981; Nakamura et al., 1991), Langerhans cells (Brijnzeel-Koomen et al., 1988; Bieber et al., 1989, Buckley et al., 1992) and RFD1+ dendritic cells (Buckley et al., 1995). This overexpression has been proposed to lead to an increased mediator response from monocytes (Ferreri et al., 1988) and increased B cell presentation (Mudde et al., 1995).

Langerhans cells in lesional skin and blood monocytes of AE patients show a higher expression of FceR1 than controls (Bieber et al., 1992; Grabbe et al., 1993; Mauer et al., 1994). There was an increase intracellular Ca^{2+} when FceR1 was ligated on Langerhans cells from patients with AE but not from controls (Jurgens et al., 1995). The CD45RO isoform has been identified on normal Langerhans cells, however when these cells are isolated from lesional skin they also expressed the CD45RB isoform (Bieber et al., 1995). The group also found that the Ca^{2+} mobilisation induced by activating FceR1 was reduced when an anti CD45 antibody was used and suggested that CD45 may be involved in the signalling of FceR1. In contrast normal monocytes show an increase in Ca^{2+} mobilisation when activated through FceR1 and anti CD45 antibody also blocks this increase (Reischl et al., 1995). PDE_{2} production has also been shown to be mediated through FceR1 on monocytes in AE (Takenaka et al., 1995). Thus FceR1 may also be involved in mediator release.

The link between the cell mediated response and high IgE levels was first demonstrated in 1990 when epidermal Langerhans cells were reported to present HDM antigen to autologous T cells via specific IgE and it was postulated then that this presentation was through CD23 (Mudde et al., 1990). However with the discovery of the FceR1 on Langerhans cells it is more likely that the presentation was via this receptor. FceR1 expression on monocytes of birch sensitive individuals has also been shown to have the ability to intervene in IgE dependent allergen presentation (Maurer et al., 1995).
These results with the high affinity IgE receptor have not negated the importance of CD23 in AE as Van der Heijden et al demonstrated that preincubation of purified antigen from house dust mite (Der p 11 protein) with serum from a house dust mite allergic individuals decreased the concentration of allergen needed by 1000 fold when EBV-B cells presented this complex to autologous T cells (Van der Heijden et al., 1993). This presentation was not seen with non allergic sera and was blocked with anti CD23 but not anti CD32. This work was extended to show that 10 AE patients all had the ability to present complexed HDM but none of the 13 atopic house dust mite allergic individuals without eczema showed this ability (Van der Heijden et al., 1995). There was a correlation between the concentration of anti Der p 11 antibody and presentation by CD23 and it was concluded this pathway may be involved in the pathogenesis of AE due to the high concentration of allergen specific IgE. The presentation of allergen by B cells via CD23/IgE has lead Mudde and co-workers to speculate that an activated naive B cell as well as presenting its specific antigen could also present allergen by the uptake through CD23 which could cause the activation of allergen specific Th2 cells which produce IL-4 (Mudde et al., 1995). IL-4 could be involved in the class switching of the B cell which would produce IgE antibodies to the specific antigen. Therefore there would be an increase of IgE antibodies to various antigens. In the in vivo model of IgE mediated antigen presentation there was only a potentiation of specific antibody responses and not nonspecific antibody responses (Gustavsson et al., 1994) therefore further experimental data will have to be done to justify this theory.

1.7 Hypothesis Relating To The Pathogenesis Of AE
The recent widely accepted theory for the pathogenesis of AE is that percutaneously absorbed allergens or digested allergens bound to allergen specific IgE on Langerhans cells play a crucial role in the activation of allergen specific Th2 cells. These Th2 cells which express the CLA+CD45RO+phenotype return to the skin and on subsequent activation release cytokines such as IL-4, IL-13 and IL-5. IL-4 and IL-13 induce CD23 on Langerhans cells, B cells and monocytes. Antigen presentation and mediator release is further achieved in the skin through FcεRI and CD23 on Langerhans cells and monocytes whilst B cells act through CD23. The increased IgE production causes the vicious circle to continue whilst IL-5 production causes proliferation and activation of eosinophils which continue the inflammatory response.
Mast cells may initiate the response through their release of mediators and cytokines such as IL-4 and also contribute to the pathogenesis of AE. This process will only apply to 80% of the patients who have a high IgE and are allergic to various aeroallergens and foods. As the patients with 'intrinsic' AE have high levels of IL-5 the pathogenesis of this disease may be related to eosinophils. Genetic defects in enzymes like PDE may be contributing factors to the disease.

1.8 Treatment of AE

Although the finer details of the immunological basis of this disease are being unravelled, the general treatment of AE is to reduce the inflammation in the skin.

1.8.1 Basic Treatment

This is achieved by the use of topical corticosteroids; they vary in potency and the general practice is to use the lowest strength which controls the eczema. Treatment is twice daily at the start of therapy to cause rapid remission of the lesions. Improvement is usually seen in 3-7 days and Przybilla et al suggested this basic therapy can control the majority of patients with AE (Przybilla et al., 1994). If this basic therapy is ineffective systemic corticosteroids are used but withdrawal usually has lead to rapid relapse and careful consideration has to be taken when giving these immunosuppressive drugs to children (David, 1988). Cyclophosphamide and azathioprine have also been used (Morrison & Schulz, 1978).

As mentioned before, the skin of AE patients is prone to be colonised with Staphylococcus aureus and Pityrosporum yeast. In these cases antibiotics or anti-yeast preparations can be administered systemically or topically (Lever et al., 1988; Williams & Mackie, 1993; Clemmensen & Hjorth, 1983).

1.8.2 Phototherapy and Photochemical therapy (PUVA)

Patients exposed to sunlight have been known to obtain an improvement in their AE and due to the side effects of steroids other methods of treatment have been investigated. Phototherapy uses long wavelength ultra violet radiation whilst photochemical therapy combines ultra violet radiation with a chemical photosensitizer such as 8-methoxy-psoralen. Ultra violet radiation (UVB) has been found to be effective in the treatment of AE (Hannuksela et al., 1985; Jekler et al., 1988) whilst a combination of UVB and UVA seemed to be more effective than UVB alone (Jekler et al., 1990). To further investigate this treatment Krutman et al compared high dose UVA 1 with the combination of UVA and UVB and found that high dose UVA 1
was even more effective (Krutman et al., 1992). This group also showed that ECP levels were reduced in patients who were treated with high dose UVA but not UVA and UVB. Grewe et al showed that 13/15 patients had mRNA for IFN-γ in lesional skin and with high UVA treatment this mRNA was decreased in 9/13 patients. Only 4 patients had an increased mRNA for IL4 and this was unchanged with treatment (Grewe et al., 1994). The mechanism of this modulation was unknown but the authors have suggested that high UVA act on eosinophil and T cell function.

PUVA has been used in adults and children with successful outcomes (Morison et al., 1976; Atherton et al., 1988; Sheehan et al., 1993). In children it was found that treatment for an average of 37 weeks lead to a large number of them remaining in remission for up to year (Sheehan et al., 1993). Although successful, the risks involved in this treatment have to be considered as the long term effects are unknown.

1.8.3 IFN-γ therapy

The rational behind using IFN-γ as treatment for AE came from the fact that it is known to reduce IL-4 induced IgE production (Pene et al., 1988). Boguniewicz et al conducted a pilot study with 22 patients administrating subcutaneous injections of IFN-γ between 0.01-0.1mg/m³ (Boguniewicz et al., 1990). There was clinical improvement in patients treated but this was followed by a rapid relapse. The serum IgE level was unaffected but there was a significant decrease in spontaneous IgE synthesis by PBMCs of the treated patients. Similar results were obtained with 14 patients treated for 6 weeks (Reinhold et al., 1993). There was marked clinical improvement (57%) but again there was no change in serum IgE levels. A randomized placebo controlled double blind trial was conducted by Hanifin et al and 40 patients received 50μg/m³ of rIFN-γ whilst 43 received placebo injection over a period of 12 weeks (Hanifin et al., 1993). There was the greatest improvement in erythema and excoriation although a placebo effect was seen. A significant decrease in eosinophil count was seen but the IgE level was increased. A study of patients treated has shown a decrease in basophil histamine release but no reduction in eosinophil proteins or IgE (Weindel Nelson et al., 1994). From this data it is seen that IFN-γ has some efficacy in AE.

1.8.4 Cyclosporin

The initial report that two severe AE patients improved after treatment with cyclosporin lead to the investigation of this effective immunosuppresant in the treatment of AE (van Joost et al., 1987). In a double blind placebo controlled crossover
trial 33 adult patients were given 5mg/kg/day of cyclosporin for 8 weeks. The patients showed a marked improvement (Sowden et al., 1991). In the multicentre placebo controlled trial, 23 started the treatment and 14 completed and there was over 75% improvement in the severity of disease in 9/14 patients, whilst in the placebo group there was a 4% decrease. Two patients developed hypertension and there was an increase in serum creatinine and bilirubin in the cyclosporin group. After treatment these levels all returned to normal. Reducing the levels of cyclosporin to 1mg/kg/day still retained its therapeutic effect but the relapse was quicker once the treatment was discontinued. (Munro et al., 1994). A follow up study of patients who were treated with cyclosporin showed that half the patients had relapsed after 2 weeks and 90% after 6 weeks (Granlund et al., 1995). However 5 out of the 43 patients showed no relapse after 1 year the authors suggested that in some patients cyclosporin treatment may improve the long term outcome of AE. In lesional biopsies CD14+cells, CD25+ and IL-8 + cells were all reduced after treatment with cyclosporin (van Joost et al., 1992). There was no change in TNF-α, IFNγ or IL-1 producing cells. Surprisingly, the immediate and late phase reaction in patients on skin testing with HDM was increased during the treatment with cyclosporin but the delayed responses to HDM were unchanged (Munro et al., 1991). This lead to the speculation that allergic responses are not directly relevant to the pathogenesis of AE. Irrespective of the mechanism of action, cyclosporin has proved successful for short term use but fears about its toxic effects to the liver and kidneys has reduced its use in children.

1.8.5  ZEMAPHYTE™ (ZPT)

However, even with these effective treatments which have significant side effects there is still a proportion of patients who are recalcitrant to all forms of Western treatment. To fill this therapeutic void Zemaphytc™ a traditional Chinese Herbal Therapy (TCHT) has recently been successfully used to treat the severer forms of eczema.

1.8.5.1  History and Theory of TCHT

Traditional Chinese medicine predates its current interest in the West by over two thousand years. Its pharmacopoeias contains a variety of medicines from plant, animal and mineral origin (Needham, 1970). Traditional Chinese medicine seeks to treat the whole person not just the disease; thus the emotional and environmental aspects of the patients are considered. The essential theme behind this approach resides in the two forces Yin and Yang. Yin and Yang literally translated represent...
two banks of a river, one is the shade and the other is the sun. Yin can also portray the female unresisting nature and Yang the masculine controlling side of the universe. These aspects of Yin and Yang may appear antagonistic, however in Chinese philosophy they are complementary to each other. In healthy individuals the Yin and Yang are in perfect balance, thus illness only occurs when there is an inequality between them. The objective of the Traditional Chinese medicine practitioner is to detect the imbalance and to treat the patient to achieve harmony. There is an exchange between the environment and the body: food, drink, air entering and waste leaving and this interchange occurs through the Zang Fu (Chinese organ systems). Transport within the body is carried out by the blood vessels and other parts of connective tissues (Jing Luo, Xue Mai) to various organs (Ross, 1985). All these factors are taken into account and it is likely that a Traditional Chinese doctor will also examine the tongue, iris and pulse of the individual. The treatment of a patient may be mixed, including herbal remedies, massage and acupuncture. This concept leads to individual cures for each patient embracing physiological and psychological aspects. In China TCHT and conventional medicine are practised together, however in the West practitioners find the TCHT difficult to comprehend. The astonishing results achieved in AE have lead most Western doctors to believe that the Chinese have perfected the science or art of choosing specific traditional Chinese herbs to treat specific disease, rather than as they do to redress the balance between the Yin and Yang.

1.8.5.2 What Diseases are treated by TCHT?
There are a variety of herbal treatments for a collection of diseases in China. These include rheumatoid arthritis, asthma and psoriasis. At present the only TCHT studied with rigorous double blind placebo controlled trials (Sheehan et al., 1992a; Sheehan et al., 1992b) has been Zemaphyte™ (ZPT) and this will be discussed further.

1.8.5.3 Clinical evidence for the efficacy of ZPT
The first double-blind placebo controlled trial of this preparation for AE was carried out in children at The Hospital for Sick Children, London (Sheehan et al., 1992a). Chinese practitioners prescribe a personalised prescription for each patient. Thus the initial challenge for this trial was to formulate a standard preparation for all patients, a concept alien to Chinese Herbal practitioners. However with the help from Dr Luo, a Chinese doctor with vast experience in the area of AE, ten individual herbs were recommended. The ten herbs were: Ledebouriella seseloides, Potentilla chinensis,
Clematis armandii, Rehmannia glutinosa, Paeonia lactiflora, Lophatherum gracile, Dictamnus dasycarpus, Tribulus terrestris, Glycyrrhiza uralensis, Schizonepeta tenuifolia. A placebo herbal mixture was needed and one was comprised with a similar smell, taste and appearance. This consisted of Homulus lupulus, Hordeum distichon, Hordeum distichon ustum, baker's bran, sucrose, Salvia spp, Thymus vulgaris, Rosmarinus officinalis, Mentha piperita and clove oil. To standardised each treatment both ZPT and placebo were ‘fingerprinted’ by thin-layer chromatography and compared to a reference standard. The herbs were ground and placed in porous paper sachets and these ‘teabags’ were given to the patient who then boiled them and approximately 100ml of the decoction was drunk. The structure of the trial was that of a double blind placebo controlled study with a washout period between treatments. 47 children entered the study and 37 completed. There was a significant decrease in erythema and surface damage with the active but not placebo formula. In the later adult study (Sheehan et al., 1992b) there was an even more significant improvement in erythema and surface damage (Fig 1.9.7.3a). The median decrease in erythema was 91.4% compared to 10.6% with placebo. Surface damage scores were decreased by 85.7% (active) and 17.3% (placebo). Many of the patients reported that they were less itchy and had a greater ability to sleep. In both studies renal function, full blood count and liver function test were all measured and were all found to be normal. In the long term study of one year 37 children were followed with ZPT (Sheehan et al., 1994). 18 of the children showed a 90% decrease in severity scores while 5 showed an improvement to a lesser degree. 14 children withdrew from the study due to lack of response or unpalatability of the treatment. By the end of the follow up period, 7 children no longer required treatment without relapse and the other 16 reduced their dosage. The eosinophil count which was raised in the group at the start of the study was reduced to normal levels at the end. In all these studies the palatability and preparation of ZPT proved to be problematic for patients thus freeze dried granules of a water extract was prepared by Phytopharm. This new formulation was efficacious in an open study where 22 patients received the original decoction and 20 received the granules (Banerjee & Rustin 1994).

However, patients who used Traditional Chinese Herbs unrelated to ZPT have been reported with hepatotoxicity (Davies et al., 1990) and recurrent facial herpes (Russell-Jones, 1991). With these incidents in mind research on the active components of ZPT is desirable.
Figure 1.9.7.3a Sequential total body scores (geometric means) for erythema (A) and surface damage (B) of patients with AE treated with ZPT. The body was divided into 20 equal parts and scored for severity and percentage affected. The sum of the severity score multiplied by the area score gives a total body score, maximum being 180. Kindly reproduced by the permission of The Lancet Ltd. (Sheehan et al., 1992b).
1.9 **Aim of this project**

Due to the success of ZPT in recalcitrant AE and the fact that it was speculated to be as effective as corticosteroids and cyclosporin it was thought that ZPT warranted enquiry. As there is no suitable animal model for AE a variety of immunological systems had to be examined. CD23 was thought to be implicated in the inflammation seen in the skin with the overexpression seen on monocytes, T and B cells. The aim of this project was to investigate the following:

1) The mechanism of action of ZPT on CD23 induced by IL-4 on monocytes and other immunological parameters relevant to AE.

2) Comparison of the mechanism of ZPT, prednisolone and cyclosporin on CD23 expression in vitro.

3) Immunological changes when AE patients are treated with ZPT.

4) Isolation of the active component(s) from the mixture with a view for therapeutic use in AE.

5) An insight to further the understanding of the immunological defects in AE.
Chapter 2

Materials and Methods
2.1 Reagents

All chemicals were AnalaR grade

<table>
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<tr>
<th>Reagent</th>
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<tr>
<td>Absolute Ethanol</td>
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<td>Agarose</td>
<td>Sigma Chemical Co, St Louis, USA</td>
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<tr>
<td>Agarose (low melting point)</td>
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<td>Cambridge Bioscience, Cambridge, USA</td>
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<tr>
<td>Ampicillin</td>
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<td>Anti TNF-α antibody (neutralising)</td>
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Biotinylated anti TNF-α Mab 11

Pharmingen, San Diego, CA USA

Bromophenol blue

BDH Chemical Ltd, Poole, UK

BSA

Sigma Chemical Co, St Louis, USA

Buffy Coat Units (N-82)

South Thames Blood Transfusion Service, London, UK

Caesium chloride

BDH Chemical Ltd, Poole, UK

Control IgG

Beckton Dickinson, Mountain View, CA, USA

Control IgG<sub>2b</sub>-phycoerythrin

Dako Ltd, High Wycombe, UK

Con A

Sigma Chemical, St Louis, USA

Cyclosporin

Sandoz, Basel, Switzerland

DEPC

Sigma Chemical Co, St Louis, USA

Dextran sulphate

BDH Chemical Ltd, Poole, UK

DMSO

Sigma Chemical Co, St Louis, USA

Dowex-AG1-X8 (hydroxide form)

Biorad, Hemel Hempstead, Herts, UK

Dowex AG3-X4 (free base form)

Biorad, Hemel Hempstead, Herts, UK

Dowex AG50-X12 (hydrogen form)

Biorad, Hemel Hempstead, Herts, UK

EBV transformed B cells

Kind gift from Ms S Wilson, UCSM, UK

Ecoscint A

National Diagnostics, Altanta, Georgia, USA

Ethidium bromide

Sigma Chemical Co, St Louis, USA

Enzyme Labelling Kit

Amersham, Amersham, UK

Foetal Calf Serum (FCS)

Sigma Chemical Co, St Louis, USA

Ficoll 400

Sigma Chemical Co, St Louis, USA

FITC-conjugated rabbit F(ab)2 anti mouse immunoglobulin

Dako Ltd, High Wycombe, UK

Formaldehyde

Sigma Chemical Co, St Louis, USA

Formamide

BDH Chemical Ltd, Poole, UK

Franz competent cells E.coli JS5

Kind gift from Dr T Lund, UCLMS, UK

Glutamine

Life Technologies, Paisley, UK

Glycine

BDH Chemical Ltd, Poole, UK

Glycyrrhetinic acid

Aldrich/Sigma Chemical Co, St Louis, USA

Glycerol

BDH Chemical Ltd, Poole, UK

H<sub>2</sub>O<sub>2</sub> (30% w/v and 6% w/v)

BDH Chemical Ltd, Poole, UK

Herring Sperm DNA

Sigma Chemical Co, St Louis, USA

Hexane

BDH Chemical Ltd, Poole, UK

Hexan-1-ol

Sigma Chemical Co, St Louis, USA

Human gamma globulin

Sigma Chemical Co, St Louis, USA

HBSS

Life Technologies, Paisley, UK

50
Hybond N+ transfer papers
IL-2
IL-4
IL-10
IL-13
IFN-γ
Leupeptin
LPS (E coli Serotype 055:B5)
Lymphoprep
Magnesium chloride IM solution
Methanol
[35S] methionine
Mono Mac 6 cell lines
Mouse serum
MOPS
NBT
N-butanol
Non-essential amino acids
NP-40
NuncTrapp™ push columns
Octan-2-ol
Oxalacetate
Paeoniflorin
Pefabloc
Penicillin
Peroxidase labelled rabbit anti human IgE (polyclonal)
PE conjugated anti CD14
PL
PMA
Polyethylene glycol 6000
Polymyxin B sulphate
Polyvinylpyrrolidone
Prednisolone disodium phosphate
Protein A-Sepharose
PUC18 plasmid containing CD23

Amersham, Amersham UK
NBSB, Potters Bar, UK
Genzyme, Cambridge, MA, USA
Biosource International, Camarillo CA, USA
R & D Systems, Abingdon, Oxon UK
Genzyme, Cambridge, MA, USA
Sigma Chemical Co, St Louis, USA
Sigma Chemical Co, St Louis, USA
Nycomed, Oslo, Norway
Sigma Chemical Co, St Louis, USA
Fluka Gillingham, Dorset, UK
ICN Pharmaceuticals Inc, Irvine, CA, USA
Kind gift from Dr Ziegler-Heitbrock, Munich
(Dr Ziegler-Heitbrock et al., 1988)
Dako Ltd, High Wycombe, UK
Sigma Chemical Co, St Louis, USA
Sigma Chemical Co, St Louis, USA
Fluka, Gillingham, Dorset, UK
Life Technologies, Paisley, UK
Sigma Chemical Co, St Louis, USA
Sigma Chemical Co, St Louis, USA
Stratagene, La Jolle, CA, USA
Sigma Chemical Co, St Louis, USA
Life Technologies, Paisley, UK
Sigma Chemical Co, St Louis, USA
Sigma Chemical Co, St Louis, USA
Sigma Chemical Co, St Louis, USA
Aldrich/Sigma Chemical Co, St Louis, USA
Pentapharm AG, Basel, Switzerland
Life Technologies, Paisley, UK
Dako Ltd, High Wycombe, UK
Beckton Dickinson, Mountain View, CA, USA
Phytopharm Ltd, Borough, UK
Sigma Chemical Co, St Louis, USA
BDH Chemical Ltd, Poole, UK
Sigma Chemical Co, St Louis, USA
Sigma Chemical Co, St Louis, USA
Glaxo Laboratories Ltd, Middlesex, UK
Sigma Chemical Co, St Louis, USA
Kind gift from Prof Gould, UK
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### 2.1.1 Tissue culture plastics and Equipment

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2.1.2 Buffers and solutions

Bicarbonate Buffer pH 9.6 - 16mM anhydrous sodium carbonate and 34mM sodium bicarbonate.

Biotin Labelling Buffer - 0.1 M Sodium hydrocarbonate and 0.1 M Sodium chloride adjusted to pH 7.4 with concentrated hydrochloric acid.

Citrate/Acetate Buffer (5x) - 0.5M sodium acetate adjusted to pH 6.0 with 0.5M citric acid.

100 x Denhardt's Solution - 5g Ficoll 400, polyvinylpyrrolidone, 5g BSA dissolved in 250ml of water.

Electrophoresis Buffer (PAGE) (10x) - 0.25M Tris base and 1.9 M glycine adjusted to pH 8.5.

LB medium - 10g tryptone, 10g NaCl and 5g yeast extract in 1 litre of distilled water.

Loading buffer (DNA separation) - 20% Ficoll, 10mM Tris, 1mM EDTA and orange G.

Loading buffer (RNA separation) - 1mM EDTA pH 8, 0.25% bromophenol, 0.25% xylene cyanol and 50% glycerol.

Lysis Buffer (Cell labelling) - 150mM sodium chloride, 50mM Tris-Cl pH 8.0, pefabloc 240 μg/ml, leupeptin 100μg/ml and 1% NP -40.

Lysis Buffer (MINI/MAXI PREP FOR PLASMID DNA) - 0.2M NaOH and 1% SDS.

MAXI Buffer - 50mM glucose, 25mM Tris-Cl pH 8.0 and 10mM EDTA.

10x MOPS Buffer - 20.9g of MOPS was dissolved in 400ml of DEPC treated water and adjusted to pH 7 with NaOH. 8.3ml of 3M DEPC treated sodium acetate and 10ml of 0.5M DEPC treated EDTA was added, the solution adjusted to 500mls and filtered.

PBS (10x) - 1.5M sodium chloride, 27mM potassium chloride, disodium hydrogen phosphate,14mM potassium dihydrogen phosphate adjusted to pH7.4.

Prehybridization Solution - 6x SSPE, 5x Denhardts solution, 1% SDS, 50% formamide and Herring sperm DNA 5μg/ml. The hybridization solution was the same as the prehybridization solution except dextran sulphate is added at a final concentration of 5%.

Pyridine buffer - 7.67ml pyridine , 2.56ml of acetic acid adjusted to 1L of distilled water.

Removal of radioactive probe buffer - 5mM Tris-Cl pH 8, 2mM EDTA pH 8 and 0.1% Denhardt solution.
Sample buffer 2X - 420μl 3M Tris-Cl pH 6.8, 4ml 10% SDS, 2ml glycerol, 0.005% bromophenol blue and 2.5ml of distilled water.

5x Sodium acetate /citrate buffer - 0.5M sodium acetate was adjusted to pH 6 with 0.5M citric acid.

20x SSC - 3M NaCl and 0.3M Na₂citrate, adjusted to pH 7 with NaOH.

20x SSPE - 3M NaCl, 0.2M sodium phosphate and 0.002M EDTA adjusted to pH 7.4 with NaOH.

1x STE - 100mM NaCl, 20mM Tris-CL pH 7.5 and 10mM EDTA.

50x TAE Buffer - 40mM Tris-acetate and 2mM EDTA adjusted to pH 8.5

10x TBE Buffer - 89mM Tris base, 89mM boric acid and 2mM EDTA.

TE Buffer- 25mM Tris-Cl pH 8.0 and 10mM EDTA.

Tris-saline Buffer (Dialysis Buffer) - 0.1M Tris.Cl pH 7.4, 0.2M NaCl and 0.1% sodium azid. The solution was adjusted to pH 7.4 with concentrated sodium hydroxide.

Veronal buffered saline - 0.7M NaCl, 9mM Sodium barbitone, 15mM Barbitone.

2.1.3 Substrates

Peroxidase - 5mg of TMB was dissolved in DMSO and added to 50ml of 1x sodium acetate/ citric buffer. Just prior to use 37μl of 6% (w/v) H₂O₂ was added. The reaction was stopped with 12.5% H₂SO₄.

Phosphatase - 4 Sigma 104 phosphatase substrate tablets (Nitrophenyl phosphate 20mgs) were added to 20ml of bicarbonate buffer pH 9.6 containing 0.5 mM MgCl₂. The reaction was left in the dark and read after 1hour.
2.2 METHODS FOR CHAPTER 3

2.2.1 Zemaphyte™ (ZPT) and Placebo (PL)

ZPT consisted of a preparation standardized by botanical identification and thin layer chromatography containing plant material widespread in China which have been identified by their botanical names as:

- Ledebouriella seseloides (4)
- Potentilla chinensis (6)
- Clematis armandii (3)
- Rehmannia glutinosa (6)
- Paeonia lactiflora (4)
- Lophatherum gracile (4)
- Dictamnus dasycarpus (6)
- Tribulus terrestris (4)
- Glycyrrhiza uralensis (2)
- Schizonepeta tenuifolia (2)

The figures in brackets refer to the relative weight of each herb in the mixture. The placebo was comprised of Homulus lupulus, Hordeum distichon, Hordeum distichon ustum, baker's bran, sucrose, Salvia spp, Thymus vulgaris, Rosmarinus officinalis, Mentha piperita and clove oil. ZPT and placebo were supplied as water extracts taken to dryness. The yield from the extraction of ZPT was 5.75 kg of dried powder from 18.25 kg of raw herbs (31.3%). The extracts were then redissolved in RPMI 1640 and filtered before use.

2.2.2 Peripheral blood mononuclear cells (PBMCs) separation and culture

Heparinized venous blood or buffy coat cells from the South Thames blood transfusion service was diluted 1:1 with RPMI 1640 and layered onto Lymphoprep. The gradients were centrifuged at 800g for 25 mins at room temperature. The peripheral blood mononuclear cells (PBMCs) were separated from the interface and washed twice in Hanks buffered Saline (HBSS). The cells were resuspended at a concentration of $4 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100µg/ml streptomycin and 100U/ml penicillin. 200µl (1 x $10^6$ cells) were cultured with various concentrations of recombinant IL-4 either alone or with various drugs or other cytokines at 37°C, 5% CO₂ for 18 hours. Similar experiments were carried out with recombinant IL-13.

2.2.3 Double-staining FACS analysis

After culturing with IL-4 or IL-13 the cells were washed twice in 200 µl of 1% bovine serum albumin in phosphate buffered saline (1% BSA-PBS). The cells were incubated with 20 µl of anti CD23 antibody H107 at a concentration of 50 µg/ml and human gamma globulin at 20mg/ml to saturate the IgG receptors. Due to the
unavailability of H107 in later experiments an anti CD23 antibody from Beckton Dickinson was used at the same concentration as H107. The cells were washed twice with 1% BSA-PBS and then stained with FITC-conjugated rabbit F(ab)_2 anti mouse immunoglobulin. After washing, the unreactive sites were blocked with 2% normal mouse serum in PBS-BSA. The final step after further washings was an incubation with 10μl (50μg/ml) of PE-conjugated anti CD14. All incubations were carried out at 4°C for thirty-five minutes. The cells were finally resuspended in PBS-1% formaldehyde for fixation and analysed by a FACScan using the program Consort 30. Negative control cells were stained with isotype matched antibodies IgG_1 and IgG_2-PE. All assays were carried out in duplicate using double staining for CD14 and CD23. When analysing HLA-DR expression on monocytes, an anti HLA-DR antibody was substituted for the anti CD23 antibody in this procedure. For the analysis of B cells a PE-conjugated anti CD19 antibody was used instead of an anti-CD14 antibody.

2.2.4 Analysis of Tissue Culture Supernatant for soluble CD23 (sCD23)
PMBCs were cultured as above and 150μl was taken after 18 hours and stored at -70°C for sCD23 analysis.

2.2.5 Biotinylation of anti CD23 (m135Ab) monoclonal antibody
1.3mg of m135Ab was dialysed against biotin labelling buffer at 4°C with four changes over a period of two days. After dialysis 5μl of amino-hexanoyl-biotin N-hydroxysuccinimide at a concentration of 10 mg/ml in DMSO was added to 0.5 mg of antibody. The reaction was left for one hour at room temperature and then dialysed for two days against a Tris-saline buffer.

2.2.6 sCD23 ELISA
Maxisorp ELISA plates were coated overnight at 4°C with an anti CD23 monoclonal antibody m176Ab at a concentration of 20 μg/ml in bicarbonate buffer. The plates were washed three times with phosphate buffered saline - 0.05% tween 20 (PBS-T) and blocked with 1% BSA-PBS-T for 1 hour at room temperature. After three washes a sCD23 standard ranging from 128U/ml to 1U/ml and the tissue culture supernatants were added for 2 hours at 37°C. The sCD23 standard was diluted in...
RPMI 1640 supplemented with 10% FCS. Biotinylated m135Ab at a dilution of 1:1000 in 1% BSA-PBS-T was added for 1 hour at room temperature after four washes. The final step was streptavidin-peroxidase at a dilution of 1:400 in 1% BSA-PBS-T. After six washes the samples were detected with TMB as a substrate. The reaction was stopped with 12.5% sulphuric acid and read at 450nm on a Dynatech MR500 ELISA plate reader. The volume per well was 50μl except for the stopping solution which was 100μl/well.

2.2.7 Assessing cell death

PBMCs were cultured in triplicate with ZPT or placebo for a period of three days. The cell counts were assessed using acridine-orange /ethidium-bromide under fluorescent microscopy using a standard technique (Lee et al., 1975). The viability of the cells was also determined using propidium iodide. 20μl of propidium iodide (50μg/ml) was added to 200μl of PBMCs (1 x 10⁶) and incubated for ten minutes. The cells were washed twice with PBS and resuspended in 200μl of PBS. The samples were analysed by FACScan within an hour of the incubation (Yeh et al., 1981).

2.2.8 Assessing Superoxide production by monocytes cultured with ZPT or PL

PBMCs were cultured as previously described with IL-4 (200U/ml) and ZPT or PL (1mg/ml). After culturing, the cells were induced to release superoxide by incubating with Phorbol 12-myristate-13-acetate (PMA) and this was measured by the reduction of nitrobluetetrazolium (NBT) to formazan according to the method of Rook et al (Rook et al., 1985). Briefly the cells were washed twice with 1% BSA-PBS and then 100 μl of RPMI 1640 warmed at 37°C was added. NBT and PMA were added to give a final concentration of 1mg/ml and 10μg/ml respectively in a volume of 200 μl. The cells were incubated for 30 mins at 37°C, 5% CO₂ and then centrifuged for 10 mins. The supernatant was removed and the cells were washed four times with methanol to remove the unreduced NBT. The wells were left to dry and the formazan precipitate was then redissolved in 120μl of 2M potassium hydroxide and 140 μl of DMSO. The optical density of the wells was read at 630nm in an ELISA reader. Known amounts of NBT were reduced to construct a standard curve beginning from 10nM of NBT per well. Thus the amount of unknown NBT reduced by the monocytes could
be determined. Negative wells without cells but undergoing the same procedure were used as blanks for the optical density. All assays were performed in triplicate.

### 2.2.9 Criteria for the diagnosis of AE

Patients were diagnosed accorded the criteria of Hanifin & Rajka (Hanifin & Rajka 1980). Erythema and surface damage was determined quantitatively using a standardised scoring system (Heddle et al., 1984). Briefly, the body was divided into 20 equal zones and each area scored for erythema and surface damage on a score of 0 (none) to 3 (severe). For both clinical features the percentage area affected was also assessed with a score of 1 corresponding to <33% affected, 2 = 34-66% and 3 > 67%. Each zone score was multiplied by the severity score and then by 20 to provide a total body score for both of the aforementioned clinical parameters.

### 2.2.10 Measurement of IgE

Maxisorb ELISA plates were coated with rabbit anti human IgE at a concentration of 10 μg/ml. After overnight incubation at 4°C the plate was washed three times with PBS-T and blocked with 1% BSA-PBS-T for 1 hour. Serum samples were diluted 1 in 100 in 1% BSA-PBS-T and placed on the plate for two hours. The final stage involved a peroxidase labelled rabbit anti human IgE. The samples were detected with TMB as a substrate and read at 450nm on an Dynatech ELISA reader. A standard curve was constructed from the VII International IgE reference sample starting with a concentration of 100 IU/ml. The normal range of IgE in serum is 10 - 150 IU/ml.

### 2.3 METHODS FOR CHAPTER 4

#### 2.3.1 Prednisolone and Cyclosporin

Prednisolone was dissolved in RPMI at a concentration of 100μg/ml and filtered through a 0.2μ filter. Cyclosporin was dissolved in ethanol and then diluted in RPMI 1640 to the desired concentration.

#### 2.3.2 Glycyrrhetic acid and Paeoniflorin

Glycyrrhetic acid and paeoniflorin were dissolved in a 50% ethanol-water mixture, filtered and then diluted in RPMI 1640 to the desired concentration.
2.3.3 Monocyte Isolation
PBMCs were isolated as previously described. The cells were resuspended at a concentration of $2 \times 10^6$ to $4 \times 10^6$/ml in RPMI 1640 supplemented with 15% FCS, 2mM L-glutamine, 100µg/ml streptomycin and 100U/ml penicillin in 250cm$^2$ flasks. The flasks were incubated for 2hrs at 37°C, 5% CO$_2$. Non-adherent cells were removed and the flasks were washed twice with HBSS which had previously been warmed to 37°C. Monocytes were removed by scraping with a rubber policeman and resuspended at a concentration of $2 \times 10^5$ to $4 \times 10^5$ cells/ml. Cell viability was greater than 95% by acridine-orange /ethidium-bromide staining. The purity of the cell population was assessed by anti-CD14, anti-CD19 and anti-CD3 staining using FACScan analysis.

2.3.4 The human monoblastic/monocytic leukemia cell line U937
U937 cells were maintained in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100µg/ml streptomycin and 100U/ml penicillin and seeded at a concentration of $5 \times 10^5$ cells/ml at 37°C, 5% CO$_2$.

2.3.5 Mono Mac 6 cell Line
Mono Mac 6 cell Line were maintained in with 10% FCS, 2mM L-glutamine, 100µg/ml streptomycin, 100U/ml penicillin, 1mM sodium pyruvate, 1mM oxalacetate insulin 9µg/ml and non-essential amino acids and seeded at a concentration of $2 \times 10^5$ cells/ml at 37°C, 5% CO$_2$.

2.3.6 Analysis of CD23 expression on semi-purified Monocytes, U937 and Mono Mac 6 cells
Semi-purified monocytes ($2 \times 10^6$ cells/ml), U937 cells ($5 \times 10^5$ cells/ml) and Mono Mac 6 (($5 \times 10^5$ cells/ml) were cultured with IL-4 (400U/ml) and various concentration of drugs for 18 hours at 37°C, 5% CO$_2$ and then assessed for CD23 expression using a single staining technique.

2.3.7 EBV transformed B cells
EBV transformed B cells were maintained in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100µg/ml streptomycin and 100U/ml penicillin at a concentration of $5 \times 10^5$ cells/ml at 37°C, 5% CO$_2$. 

60
2.3.8 Transformation of Competent cells
Franz competent cells were thawed on ice for 5 minutes and 600 ng of the PUC18 plasmid containing CD23 was added to 200 µl of the cells. 1 ng of a control plasmid was also added to 200 µl of cells to determine the efficiency of transformation. The cells were placed on ice for half an hour and then heat shocked at 42°C for 2 minutes and then returned to ice for a further 5 minutes. 800 µl of LB medium was added and incubated at 37°C for 45 minutes. The cells were grown on LB- ampicillin agar plates overnight at 37°C. The efficiency of transformation was determined to be 2.2 x 10^9/µg from the control plasmid.

2.3.9 Minipreparation of plasmid DNA
Six colonies were chosen from the transformation plates and grown overnight in 5 ml of LB supplemented with 100 µg/ml of ampicillin. The cells were spun at 2000 g at 4°C for 20 minutes. The pellet was resuspended in 300 µl of MAXI buffer and transferred to an Eppendorf tube. 600 µl of lysis buffer and then 300 µl of 3M potassium acetate were added. The precipitate was obtained from spinning for 10 minutes and the supernatant was removed and RNAsé A treated for 90 minutes at 37°C. Equal volume of phenol/chloroform was added and mixed and centrifuged for 3 mins. The top layer was removed and equal volume of chloroform was added and the centrifugation repeated. The top layer was again removed and 3M sodium acetate and 0.6 volume of isopropanol was added. After centrifugation for ten minutes the plasmid DNA was washed with 70% ethanol and resuspended in TE buffer.

2.3.10 Isolation of cDNA for CD23 from plasmid
cDNA for CD23 was isolated from the plasmid DNA by an enzyme Sty 1 which cuts at EcoR1 sites. The reaction was performed overnight at 37°C and then run on a 0.6% agarose gel containing ethidium bromide in TBE buffer to identify the digested fragment of 900 base pairs.

2.3.11 Maxi prepraration of cDNA for CD23
Transformed colonies were incubated for eight hours in 5 ml of LB medium supplemented with ampicillin at 37°C. After the incubation period the cells were transferred to a litre of LB medium with ampicillin and left shaking overnight at 37°C. The cells were spun at 2000 g for 30 minutes at 4°C. The supernatant was
removed and the pellet resuspended in 40ml of MAXI buffer. The cells were lysed with 80 ml of lysing buffer and then precipitated with 40 ml of 3M potassium acetate. The solution was spun at 2000g for 30 minutes at 4°C and then filtered through nylon into Sorval tubes. The DNA was precipitated with 0.6 volume isopropanol, spun and washed with 70% ethanol and resuspended in TE buffer.

2.3.12 Caesium Chloride Gradient

The plasmid DNA prepared from the MAXI PREP was ultracentrifugated on a caesium chloride gradient with ethidium bromide at 150,000g for 48 hours. The supercoiled DNA was removed and placed in 5ml of sterile water. The plasmid was precipitated with 2.5% ethanol and spun at 2000g for 15 mins and washed with 70% ethanol. The precipitate was resuspended in TE and two chloroform/phenol extractions, followed by a chloroform extraction was performed as previously described in the MINIPREP section. The DNA was precipitated with 3M sodium acetate and isopropanol, spun and washed with 70% ethanol and resuspended in TE buffer. A further precipitation with 3 M sodium acetate and 2.5% ethanol was followed by washing with 70% ethanol. The precipitate was resuspended in TE buffer. 1mg of plasmid DNA was obtained from this procedure.

2.3.13 Preparation of the cDNA for CD23

The enzyme Sty 1 was used to isolate the cDNA for CD23 from 33µg of the plasmid DNA as previously described. The mixture was prechecked on a 0.6% agarose gel before running on a 0.8% low melting agarose gel in TAE buffer at 30V for 1.5 hours. The cDNA for CD23 was removed from the gel with a scapel and melted at 70°C. The DNA was eluted from the agarose using Wizard™ PCR Preps DNA columns as directed by the manufacturers. Briefly 1ml of resin was added to the melted agar and mixed well for 20 seconds. The mixture was added to the column and then 2ml of 80% isopropanol to wash the column. The column was centrifuged at 15,000 rpm for 20 secs to dry and 50µl of TE buffer was added and left for one minute. Centrifugation of the column recovered the DNA. This procedure was repeated with a MAXIPREP of a plasmid containing cDNA for actin except the enzyme PSTl was used and the cDNA was 1.8 Kb.
2.3.14 Diethyl pyrocarbonate (DEPC) treatment of solutions

Solutions used in RNA isolation were treated with DEPC to remove any RNases. 500 µl of DEPC was added to 500ml of the solution to be treated and left overnight. After this incubation period the solution was autoclaved.

2.3.15 RNA isolation

Monocytes were isolated as previously described. 2.5x 10⁶ monocytes /ml were cultured with IL-4 (400U/ml) without or with ZPT 1mg/ml or PL 1mg/ml or prednisolone 10µg/ml for 18 hours at 37°C, 5% CO₂. The cells were washed twice with DEPC treated PBS. To achieve at least 10 µg of RNA for each observation monocytes from two individuals were pooled just prior to RNA isolation. An RNA isolation kit which utilised the procedure of Chomczynski & Sacchi was used (Chomczynski & Sacchi, 1987). The pooled cells were lysed with 1ml of guanidinium isothiocyanate supplemented with β-mercaptoethanol (solution D) and 100µl of 2M sodium acetate was added. 1ml of water saturated phenol was added and mixed thoroughly by inversion. 250 µl of chloroform:isoamyl alcohol mixture was added, the solution was vortexed for 20 seconds and then left on ice for 15 minutes. The tubes were centrifuged at 3000g for 20 minutes and the upper layer was removed and transferred to an eppendorf. An equal volume of isopropanol was added. Although the RNA could be precipitated after one hour at -20°C, it was found that an overnight precipitation procedure gave a better yield of RNA. After overnight incubation the tubes were centrifuged at 10,000g for 20 minutes and the supernatant removed. The pellet was redissolved in 300µl of solution D and an equal volume of isopropanol was added and left overnight at -20°C. After centrifugation as previously described, the supernatant was removed and 400µl of 70% ethanol was added for 15 minutes. The RNA pellet was obtained by centrifugation and allowed to dry. 300µl of DEPC treated water was added and the RNA frozen at -70°C. Just prior to analysis, the RNA was thawed and the OD obtained at A₂₆₀ to determine the concentration. The ratio of OD of A₂₆₀/A₂₈₀ showed the purity of the RNA. The RNA was dried by a speed vacconcentrator and redissolved in DEPC treated water between 6-10µg/10µl. All pipette tips used in RNA isolation were autoclaved and baked at 80°C and glassware at 200°C.
2.3.16 Northern Hybridisation

2.3.16.1 RNA separation

The gel apparatus was soaked in 3% H$_2$O$_2$ solution for 30 minutes. Then a 1.2% agarose gel for separation of the RNA was prepared using the following method. 3g of agarose was boiled in 217.4ml of DEPC treated water to dissolve and then placed at 60°C to cool. After cooling, 25mls of 10x MOPS buffer and 7.6 ml of 37% formaldehyde were added. The gel was left to set for 30-45 minutes and prerun for ten minutes at 40V.

2.3.16.2 RNA Sample Preparation

6-10|ig of RNA was resuspended in 10|μl of DEPC water, and 4|μl of 10x MOPS, 7|μl of formaldehyde and 20 |μl of formamide were added. The sample was vortexed and incubated in at 55°C for 15 minutes and then cooled on ice for five minutes. 6 |μl of sample buffer was added and the samples were loaded onto the agarose gel. The gel was run overnight at 35V. After rinsing three times with DEPC treated water the gel was left in 10x SSC for 45 minutes.

2.3.16.3 Blotting

3MM Whatman filter paper was used to form a wick soaked in 20x SSC. The gel was placed face down on the filter paper and Hybond N+ transfer paper 3mm smaller than the gel was placed on top. Five pieces of Whatman filter paper 7mm smaller than the gel was placed on top. The first piece of filter paper was soaked in 20x SSC. Air bubbles were removed by rolling a glass pipette over the Whatman paper. A stack of paper towels were placed on top with a 500g weight. The gel was blotted overnight at 4°C. After transfer, the Hybond N+ was rinsed twice in 2x SSC and dried for 30 minutes between two sheets of Whatman filter paper. The RNA was crosslinked to the Hybond N+ by a UV Stratalinker.

2.3.16.4 Hybridization

The Hybond N+ filters were placed in prehydridization solution for 4 hours at 42°C in a water bath. cDNA for CD23 and actin were radiolabelled using an enzyme labelling kit. 50ng of the cDNA(10|μl) was heated to 96°C for 5 minutes, cooled on ice and then added to 10 |μl of enzyme buffer, 5 |μl of the primer solution and 2 |μl of the enzyme Klenow. To this solution 3 |μl of αdCT$^{32}$P was added and the mixture incubated for 30 minutes at 37°C. The radiolabelled probe was removed from the unbound probe using a NucTrap™ push column. 70|μl of 1xSTE was used to
preshot the column and then the sample was added in a volume of 70µl. The sample was pushed through the column and a further 70µl of 1x STE added. The radiolabelled probe was heated to 96°C for 5 minutes and cooled on ice. The prehybridization solution was removed from the hybond N+ filters and hybrization solution added with the radiolabelled probe. The filters were hybridized overnight at 42°C and then washed with decreasing concentrations starting at 2x SSPE and 0.1% SDS. The filters were dried and then exposed to Kodak X-ray film with intensifying screens at -70°C and developed. The filters were stripped with a Tris buffer at 75°C for two hours and then reprobed with a radiolabelled probe for actin. The bands on the autoradiograms were read on an Imaging Densitometer and analysed using a Molecular Analyst programme on a personal computer.

2.3.17 Biosynthesis labelling of proteins

2.3.17.1 Short term labelling with [³⁵S] Methionine

5 x 10⁵ monocytes were cultured overnight with IL-4 (400U/ml) without or with ZPT 1mg/ml and then transferred to conical shaped tubes and washed twice with HBSS. The cells were resuspended in methionine free RPMI containing 1% FCS for 45 minutes at 37°C, 5% CO₂ and then centrifugated. The cell pellets were resuspended in 1ml of methionine free RPMI containing 0.25miCi of [³⁵S] methionine in a water bath at 37°C for three hours with the lids of the tubes tightly screwed on. After this incubation time the cells were washed once with RPMI and then twice with HBSS. 50µl of lysis buffer was added and the cells left on ice for 30 minutes. The cell lysate was transferred to eppendorf tubes and centrifuged at 10,000g for 30 minutes. The supernatant was removed and frozen at -70°C.

2.3.17.2 Immunoprecipitation

Protein A-Sepharose beads were swollen in PBS for an hour and then washed with PBS. The solution was stored at 4°C in PBS-0.01% sodium azide prior to use. The beads were washed twice in lysis buffer and then a 50% solution in lysis buffer was prepared. Rabbit anti mouse IgG was added to the beads at a concentration of 10µg/50µl of beads and incubated for half an hour at 4°C. The rabbit anti mouse coated beads were washed four times at 10,000g for 20 secs at 4°C and 50µl added to 50µl of the cell supernatant obtained from short term cell labelling. This step was to preclear the cell lysate. After an incubation of 1 hour at 4°C with the tubes rotating,
the tubes were centrifuged and the supernatant removed and 5µg of an anti CD23 antibody mAbs 64 was added at 4°C for 45 minutes. The beads were washed as previously described and 10µl of 2x sample buffer added. 50µl of rabbit anti mouse coated beads was added to the supernatant and the same procedure was used as previously described for preclearing the supernatant. The procedure was repeated again with an anti HLA-DR antibody at a concentration of 5µg/ml.

2.3.17.3 Polyacrylamide gel electrophoresis (PAGE)

A Hoefer SE 245 minigel apparatus was used for preparing the gel. The glass plates (10cm x 8 cm) and 0.75mm spacers were thoroughly washed and cleaned with 70% alcohol and assembled in the casting apparatus. The 7.5% acrylamide separating gel was prepared by adding 5ml Protogel (30% acrylamide, 0.8% bisacrylamide stock solution), 7.5ml distilled water, 7.5ml 1.5M Tris pH 8.7, 200µl 10% SDS, 100µl 10% ammonium peroxidisulphate and 10µl TEMED. The separating gel solution was added to the glass sandwich with a syringe to 6 cm and a layer of H₂O saturated butanol was added. The gel was allowed to polymerize for 45 minutes. The H₂O saturated butanol was removed and the top of the gel rinsed three times with distilled water. The stacking gel was prepared by adding 3.5ml Protogel, 13.5ml distilled water, 2.5ml 1M Tris pH 6.8, 200µl 10% SDS, 100µl 10% ammonium peroxidisulphate and 20µl TEMED. A 0.75mm Teflon 10 well comb was inserted and the stacking gel poured using a syringe. The stacking gel was allowed to polymerize for 30 minutes. As the stacking gel was polymerising the samples were prepared by boiling 5 minutes at 100°C and then centrifuged at 10,000g for 1 minute. Rainbow markers were used as protein molecular weight standards. The combs were removed from the gel and the gel was rinsed with electrophoresis buffer. The gels was transferred to the electrophoresis tank and the wells and the buffer chambers were filled with electrophoresis buffer. 10 µl of the radioactive supernatants from immunoprecipitation were added to the wells. The gel was run at 100V for 1.5 hours. The gel was removed from the glass sandwich and the stacking gel was discarded using a scalpel blade. The separating gel was fixed in 40% methanol, 10% acetic acid and 50% distilled water mixture for three hours and then soaked in a 5% acetic acid solution containing 0.1% glycerol for fifteen minutes. The gel was placed on a sheet of 3MM chromatography paper with a sheet of clingfilm on the top and dried on a gel drier for 2 hours at 55°C. The gel was exposed to a Kodak X-ray film.
with intensifying screens at -70°C and developed.

2.4 METHODS FOR CHAPTER 5

2.4.1 Concanavalin A (Con A) proliferation of PBMCs

PBMCs were isolated as previously described. The cells were resuspended at 1 x 10^6 cells in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100μg/ml streptomycin and 100U/ml penicillin. Duplicate 2ml cultures were set up with Con A without or with ZPT (0.5mg/ml) or PL (0.5mg/ml) for three days at 37°C, 5% CO₂. Supernatants were taken each day and frozen at -70°C for cytokine assay. Cells were also cultured without any stimulus but with ZPT or PL.

For proliferation studies cells were cultured at a concentration of 2 x 10^5 /well in U bottomed 96 well microtiter plate as described above except the cultures were set up in triplicate. The cells were pulsed with ³H thymidine at 0.4μCi/well for the final 16 hours of culture and harvested onto glass fibre disks using a Titertek harvester. The disks were placed in Ecosint A scintillation fluid and counted using a Packard β counter.

2.4.2 IL-2/IL-4 ELISA

Maxisorb ELISA plates were coated with the anti cytokine antibody diluted in PBS and incubated overnight at 4°C. The plates were washed three times with PBS-T and blocked with 2% -BSA-PBS-T for two hours at room temperature. After washing the cytokine standard and samples were added to the plate together with the biotinylated anti-cytokine antibody and left for two hours at room temperature shaking. After the incubation the plates were washed four times and a streptavidin -peroxidase conjugate diluted in 2% -BSA-PBS-T was added for 30 minutes at room temperature. After six washes the samples were detected with TMB as a substrate and stopped with 12.5% sulphuric acid. The plates were read at 450nm on a Dynatech MR500 ELISA plate reader.

The coating anti IL-2 antibody and anti IL-4 antibody were used at a concentration of 2.5 μg/ml. The biotinylated anti IL-2 and IL-4 antibodies were diluted 1/1000 according to the manufactures instructions. The IL-2 standard ranged from 1430pg/ml to 11 pg/ml and IL-4 from 2500pg/ml to 19pg/ml. The standards and samples were diluted in 10% FCS-RPMI 1640. The coating, sample volumes and substrate volumes were 100μl/well whilst the biotinylated antibody was added in a volume of 25μl/well.
2.4.3 IFN-γ ELISA
ELISA plates were coated with an anti IFN-γ monoclonal antibody at a concentration of 1μg/ml in PBS overnight at 4°C. After coating the plates were washed with PBS-T and blocked with 2% -BSA-PBS-T for two hours. The IFN-γ standard and samples were added to the plates and left for two hours at 37°C and then washed three times. A streptavidin-alkaline phosphatase conjugate was added at a dilution of 1: 1000 in 2% -BSA-PBS-T and incubated for one hour at room temperature. The plates were developed with p-nitro-phenyl-phosphate and read at 405nm on a Dynatech MR500 ELISA plate reader. A volume of 100μl/well was used throughout the assay.

2.4.4 IL-10/TNF-α ELISA
The anti cytokine antibodies were diluted in PBS and absorbed onto maxisorb ELISA plates overnight at 4°C. After washing twice the plates were blocked with 2% -BSA-PBS-T for four hours at room temperature. The standards and samples were left overnight at 4°C and then the plates washed four times. The biotinylated anti cytokine antibodies were added for 45 minutes at room temperature. A streptavidin-peroxidase conjugate was added for 30 minutes after six washings. The plate was developed with TMB and stopped with 12.5% sulphuric acid after a further eight washings and read at 450nm on a Dynatech MR500 ELISA plate reader. The anti IL-10 antibody and anti TNF-α were used at 2μg/ml. The biotinylated antibodies were also used at this concentration. The IL-10 standard was used at a range from 20,000 pg/ml to 156 pg/ml and the TNF-α standard from 1000pg/ml to 7.8 pg/ml. A volume of 50μl/well was used throughout the assay except for the stopping solution where the volume was 100μl/well.

2.4.5 Cytokine production from monocytes by ZPT and PL
200μl of semi-purified monocytes (2 x10⁶ cells/ml) were cultured in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100μg/ml streptomycin and 100U/ml penicillin with ZPT at different concentrations, PL (1mg/ml) or without a stimulus for 18 hours at 37°C, 5% CO₂.150 μl of supernatant was harvested and stored at -70°C.
2.4.6 LPS and ZPT induction of cytokines

PBMCs were isolated and cultured with various concentrations of LPS (100ng/ml - 0.16ng/ml) with or without polymyxin sulphate B (20μg/ml) at 37°C, 5 % CO₂ for 18 hours. Supernatants were collected and analysed for IL-10 and TNF-α. PBMCs were also cultured with ZPT or PL and polymyxin sulphate B (20μg/ml) and supernatants collected and analysed.

U937 cells (2 x 10⁵) were cultured with PMA (50ng/ml) without or with LPS (100ng/ml) or ZPT(1mg/ml) at 37°C, 5 % CO₂ for 18 hours. Cells were also cultured with LPS and ZPT alone. Supernatants were harvested and measured for IL-10 and TNF-α.

2.4.7 Neutralising cytokine production by ZPT

PBMCs were cultured as previously described except anti-IL-10 and anti TNF-α neutralising antibodies were added. The antibodies were used at concentrations between 10 - 0.1 μg/ml. The cells were assayed for CD23 expression on monocytes and the supernatants for IL-10 and TNF-α production.

2.4.8 Cytokine effect on IL-4 induced CD23 expression on monocytes

PBMCs were isolated and cultured with IL-4 (200U/ml) without and with different concentrations of IL-10 and TNF-α ranging from 10ng/ml - 0.1 ng/ml for at 18 hours at 37°C, 5 % CO₂. After the incubation period the cells were stained for CD23 expression on monocytes.

2.5 METHODS FOR CHAPTER 6

2.5.1 IgE complexes

IgE complexes were isolated by a standard method described elsewhere (Jacob et al., 1988). The serum was mixed with polyethylene glycol 6000 and left overnight at 4°C. Complexes were centrifuged to a pellet at 2000g, washed twice and resuspended in veronal buffered saline. The immune complexes were then assayed for IgE content as previously described.
2.5.2 Soluble Interleukin 2 receptor (sIL-2R)
sIL-2R was detected in serum using a Biosource Cytoscreen™ sIL-2 R Kit. The assay was conducted according to the manufacturer's instructions. The microtiter plates were supplied precoated with an anti sIL-2R antibody. Standard and serum were added with a biotinylated anti sIL-2R antibody and incubated for two hours at 37°C. The plates were washed and a streptavidin-peroxidase conjugate added for 45 minutes at 37°C. The plates were developed with the substrate and read at 450nm. The minimum detectable level of sIL-2R was 32 pg/ml.

2.5.3 Soluble Vascular Cellular Adhesion Molecule (sVCAM)
sVCAM was measured in the serum using a kit supplied by R&D systems. The serum was diluted 1:50 and then added to the precoated anti sVCAM plate. Peroxidase labelled anti sVCAM was added and the plates incubated for 1.5 hours at room temperature. The plates were washed six times and then developed with the substrate and read at 450nm. The minimum detection level was 4 ng/ml.

2.5.4 Soluble Intercellular Adhesion Molecule 1 (sICAM1)
sICAM was measured in the serum using a commercially available kit. Standards and serum diluted 1:20 and an peroxidase labelled anti sICAM were added to the precoated anti sICAM plates for 1.5 hours at room temperature. The plates were washed six times and then developed with the substrate and read at 450nm. The sensitivity of the assay was 7ng/ml.

2.6 METHODS FOR CHAPTER 7

2.6.1 Individual herbs of ZPT
Water extracts of the ten herbs were made at Phytopharm and then dissolved in RPMI at concentrations equivalent to that in the whole mixture as shown below:
Table 2.6.1a  Concentration of individual herbs in ZPT

<table>
<thead>
<tr>
<th>Herb</th>
<th>Concentration mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZPT</td>
<td>1.000</td>
</tr>
<tr>
<td>Ledebouriella seseloides</td>
<td>0.080</td>
</tr>
<tr>
<td>Potentilla chinensis</td>
<td>0.081</td>
</tr>
<tr>
<td>Clematis armandii</td>
<td>0.050</td>
</tr>
<tr>
<td>Rehmannia glutinosa</td>
<td>0.300</td>
</tr>
<tr>
<td>Paeonia lactiflora</td>
<td>0.110</td>
</tr>
<tr>
<td>Lophatherum gracile</td>
<td>0.061</td>
</tr>
<tr>
<td>Dictamnus dasycarpus</td>
<td>0.124</td>
</tr>
<tr>
<td>Tribulus terrestris</td>
<td>0.070</td>
</tr>
<tr>
<td>Glycyrrhiza uralensis</td>
<td>0.062</td>
</tr>
<tr>
<td>Schizonepeta tenuifolia</td>
<td>0.050</td>
</tr>
</tbody>
</table>

2.6.2 Paper Chromatography

500 µl ZPT (10mg/ml) was spotted at the origin of Whatman 3MM chromatography paper (40 x 3cm) as prepared in Fig 2.6.2a and eluted by descending paper chromatography using butanol: ethanol: water (4:1:1).

```
descending elution
2cm 11cm 2cm 41cm
```

Figure 2.6.2a  Schematic diagram of a paper chromatography strip

The paper was air dried and then cut in 2 cm intervals for the first 18 cm from origin and then 1 cm thereafter (origin corresponds to -1 to +1cm). Paper segments were eluted with methanol (40µl/cm²), centrifuged and then eluted twice more. The paper segments were subsequently eluted with water (3x at 40µl cm²). The samples
were evaporated to dryness and then redissolved in 500μl of RPMI. The different fractions were analysed for their ability to inhibit IL-4 induced CD23 on monocytes.

2.6.3 Solvent Extraction

400μl of ZPT or the individual herbs were extracted with hexane (100μl), vortexed and then centrifuged to separate the layers. The hexane layer was removed, hexane (100μl) added and the extraction repeated twice more. 100μl of the aqueous was removed for assaying and the remainder was then extracted with butan-1-ol. Aqueous and solvent extracts were evaporated to dryness and assessed for their ability to inhibit IL-4 induced CD23 on monocytes.

2.6.4 Large scale ZPT extraction followed by paper chromatography

101.8mg of ZPT was dissolved in 5ml of distilled water, extracted with hexane (3 x 1ml). Aliquots from hexane and aqueous phase were removed for the CD23 inhibition assay. The hexane phase was then evaporated to dryness and weighed (drying until constant weight). The aqueous phase was extracted with hexanol (3 x1ml) and again aliquots were taken for assaying. The hexanol and aqueous phase were then evaporated to dryness and weighed. Distilled water (3x200μl) was added to aqueous phase and spotted onto the origin of Whatman 3MM chromatography paper as previously described. The paper strips were air dried and cut into fractions (5cm). Fractions were eluted with distilled water (20μl/cm²), centrifuged for 10 minutes. Elution was repeated twice more and then the procedure repeated with methanol. Aliquots were taken of the aqueous and methanol fractions for assaying. Fractions were evaporated to dryness and weighed.

2.6.5 Analysis of ZPT on various columns and charcoal

ZPT (100mg in 5ml) was extracted with hexane and then hexanol as previously described. The aqueous extract (100μl) was applied to the following columns:

- Dowex AG50 X12 (H⁺) eluted with water (5 x 500μl)
- Dowex AG1 X8(OH⁻) eluted with water (5 x 500μl)
- Dowex AG3 X 4 (OH⁻) eluted with water (5 x 500μl)
- Dowex AG3 X4 (Acetate) eluted with 4M acetic acid (5 x 500μl)
- Sep-Pak® C-18 cartridge (1ml capacity) eluted initially with water (5x1 ml) then 10 % methanol in water (5 x 1ml) and finally methanol

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Column eluates were dried by evaporation and when reconstituted were assayed for CD23 inhibition.

The aqueous extract (100μl) was also added to a suspension of activated charcoal (500μl) in water, mixed by vortexing centrifuged and the supernatant collected. The charcoal layer was re-extracted with water (2 x 500μl) and aqueous extracts were combined. The charcoal layer was extracted with pH 5.4 pyridine buffer (3 x 500 μl). The aqueous and pyridine buffer extracts were dried by evaporation and assayed for their ability to inhibit CD23 expression.

2.6.6 Purification of ZPT
ZPT (1.211g) was dissolved in 25mls of distilled water and extracted with hexane. The aqueous phase was then extracted with hexanol. The aqueous extract was evaporated to dryness and applied to a mixed bed column of AG1X8(OH)/AG50X12(H+) in water and eluted in water. The column eluate was evaporated to dryness and applied to Sep-Pak® C-18 cartridge (5ml capacity) in water and eluted with water. The aqueous elution through Sep-Pak® C-18 cartridge was repeated twice more. The aqueous eluant was applied to a mixed column of AG1X8(OH)/AG50X12(H+) in water and washed with water. This was taken to dryness and assayed for CD23 inhibition.

0.1367g of dried aqueous phase was dissolved in 1ml water and 1ml of methanol added. The mixture was heated to 50°C and then slowly allowed to cool to room temperature, then to 4°C and finally on ice. A further 2ml of methanol was added and left on ice for 30 minutes and then centrifuged at 3000 rpm. The supernatant was removed and the precipitate was washed with 1ml of methanol, spun and the methanol removed. Samples were evaporated to dryness and assayed for CD23 inhibition. The precipitate from the methanol extraction was sent for negative ion electrospray mass spectroscopy.

2.7 Calculation of results
All the results are presented as the mean ± SEM except where n is less than 5.

2.8 Statistical analysis
The paired Student's t-test was used to analyse the effect of drugs on IL-4 induced CD23 expression on monocytes. The Wilcoxon ranked signed test was used to
compare the various parameters before and after treatment. The unpaired Student’s t-test was used to analyse differences between controls and patients. Results were analysed using Statworks Version 1.1 and Instat Version 2.01 programmes for the Macintosh.
Chapter 3

Effect of ZPT on IL-4 induced CD23 surface expression on monocytes from normal subjects and patients with atopic eczema
3.1 INTRODUCTION

ZPT has been shown to have beneficial effects in recalcitrant AE. Many anecdotal cases have testified to this and the efficacy has recently been confirmed by two double-blind placebo-controlled crossover studies carried out in both children (Sheehan et al., 1992) and adults (Sheehan et al., 1992). These results have stimulated investigation into the mode of action of ZPT. CD23, the low affinity IgE receptor (FcεR II) has been implicated as having a role in AE. B cells (Nakamura et al., 1991; Muller et al., 1991), T cells (Takigawa et al., 1991) monocytes (Melewicz et al., 1981; Nakamura et al., 1991) and Langerhans cells (Brynzeel-Koomen et al., 1988; Bieber et al., 1989a, Buckley et al., 1992) from patients with AE have all been shown to express elevated levels of CD23 on their surface. The overexpressed CD23 is of type B which is inducible by IL-4 (Yokota et al., 1988). In particular, the levels of CD23 on monocytes have been correlated with superoxide production (Polla et al., 1992) and induction of nitric oxide (Mossalayi et al., 1994). The skin in AE resembles a delayed type hypersensitivity reaction indicating T cell involvement (Braathen et al., 1978). The initiation of such a reaction has been the proposed binding of percutaneously absorbed aeroallergens onto IgE bound to the surface of cutaneous antigen presenting cells.

Since it has been demonstrated that lymphocytes from patients with AE produce higher levels of IL-4 when stimulated with mitogens (Vollenweider et al., 1991; Jujo et al., 1992; Renz et al., 1992), any compound that might downregulate CD23 cell surface expression or block IL-4 production may be effective in decreasing the afferent pathway of the cell mediated reaction in AE. Various groups have studied the regulation of IL-4 induction of CD23 on monocytes. IFN-α, IFN-γ (Velde et al., 1990), TGF-β (Delespesse et al., 1991), TNF-α (Gessl et al., 1993, Hashimoto et al., 1995), IL-6 (Willheim et al., 1991), IL-10 (Spittler et al., 1995) and 1,25 (OH)₂ Vitamin D₃ (Fargeas et al., 1990) all downregulate this induction whilst there is augmentation by β₂ - adrenoceptor agonists (Braquet et al., 1991) and PAF (Paul-Eugene et al., 1990). IL-6 (Willheim et al., 1991), TNF-α (Gessl et al., 1993) and IL-10 (Spittler et al., 1995) increase IL-4 induced CD23 expression on the human monoblast cell line U937 indicating that blood monocytes and this cell line may have different regulatory pathways for CD23. On B cells IFN α, IFN γ (Defrance et al., 1987; Delespesse et al., 1989), TGF β and anti CD19 antibody (Gordon et al., 1991) have been all shown to inhibit IL-4 induced CD23 expression. The inhibition
on B cells was blocked by engaging CD40 (Gordon et al., 1991).

From this information, it was decided to use a model system to study the effect of ZPT on CD23 on monocytes under the influence of IL-4. This chapter will concentrate on its effects on surface expression of CD23 on monocytes from normal subjects and patients with AE.
3.2 RESULTS

3.2.1 Modulation of IL-4 induced CD23 expression on monocytes in normal individuals

Samples were obtained from laboratory workers or supplied by the South Thames Blood Transfusion Service. None of normal individuals had a history of AE.

3.2.1.1 Induction of CD23 on monocytes by Interleukin 4 (IL-4)

PBMCs from normal individuals were incubated with three concentrations of IL-4 and the surface CD23 expression on monocytes measured after eighteen hours by flow cytometry. The cells were analysed by a FACScan using the forward scatter and side scatter to optimize for monocytes (Fig 3.2.1.1a). CD14 was used as a marker for monocytes and the percentage CD14+ cells in the monocyte gate was always greater than 80%. The results were expressed as percentage positive cells (CD14+ CD23+) in the presence of IL-4, minus the double stained cells in the absence of IL-4. Initial experiments with ten normal individuals showed that a dose of 200U/ml of IL-4 gave an expression of approximately 50% CD14+CD23+ cells which was considered optimum for the assessment of the effect of ZPT (Fig 3.2.1.1b). This dose was used in all subsequent experiments except where a dose range of IL-4 was used. The background expression of CD14+CD23+ cells in the absence of IL-4 was < 1.0%.
Figure 3.2.1.1a Forward (FSC) and side scatter (SSC) of PBMCs as analysed by FACScan. [ ] represents the monocyte gate.

Figure 3.2.1.1b The dose-response of IL-4 induced CD23 expression on monocytes after 18 hrs (n= 10)
3.2.1.2 The effect of ZPT and PL on IL-4 induced CD23 expression on monocytes

ZPT and PL at a concentration of 1 mg/ml (dried weight of extract) were added to PBMCs to study the effect on IL-4 induction of CD23 on monocytes. ZPT inhibited the expression of CD23 on monocytes whilst the PL had little effect as seen in the three representative FACScan profiles from controls (Fig 3.2.1.2a). The CD14 molecule was slightly downregulated in the presence of IL-4 as previously shown (Lauener et al., 1990). However, this did not interfere with this assay as the maximum downregulation of CD14 by IL-4 is seen after three days (Lauener et al., 1990) and these experiments were performed after 18 hours. In all experiments, cells from the same individual were always incubated with both preparations i.e. ZPT and PL.
Figure 3.2.1.2a Three representative dot plot FACScan profiles of the monocyte gate from controls. A) Cells with no stimulus B) Cells with IL-4. C) Cells with IL-4 + PL. D) Cells with IL-4 + ZPT. CD14+ cells (red) CD23+ cells (green), CD14+CD23+ cells (blue) and CD14-CD23- (black). There is a reduction in CD14+CD23+ cells when cultured with IL-4 +ZPT.
3.2.1.3 Time course of inhibition of ZPT and PL on CD23 expression

PBMCs from three different individuals were cultured with IL-4 and ZPT or PL for a period of 18, 42 and 66 hours. The percentage expression of CD14+CD23+ cells was analysed and the inhibition calculated from the following formula.

The percentage inhibition of CD14+CD23+ cells =

\[
\frac{(\text{CD14}^+\text{CD23}^+ \text{with IL-4 only}) - (\text{CD14}^+\text{CD23}^+ \text{with IL-4 + ZPT/PL})}{(\text{CD14}^+\text{CD23}^+ \text{with IL-4 only})}
\]

Although inhibition was long lasting up to 66 hrs, the maximum inhibition of CD23 expression was found to be at 18 hours (Fig 3.2.1.3a), thus successive experiments were carried out at this time point.

---

**Figure 3.2.1.3a** (A) The time course of the effect of ZPT and PL extracts on the expression of CD23 on monocytes. IL-4 alone (□), IL-4 + PL (●) and IL-4 + ZPT (○).

(B) The inhibition of CD23 by ZPT (□) and PL (●).
3.2.1.4 The effect of ZPT on CD23 expression using varying concentrations of IL-4

The inhibitory effect of ZPT on IL-4 induced CD23 expression was examined using three concentrations of IL-4 (50, 100 and 200 U/ml). Twenty normal individuals (12 female, 8 male; mean age 29 ± 8.9) were studied. In Fig 3.2.1.4a the percentage CD14+CD23+ cells and the mean inhibition of expression ± SE shows that ZPT significantly inhibits CD23 expression on monocytes at all concentrations of IL-4 used (p < 0.001). The mean fluorescence intensity of CD23 also reflected the inhibition by ZPT but not by PL. The level of CD14+ cells was not influenced by either ZPT or PL. The percentage ± SD of CD14+ cells in the monocyte gate incubated alone was 88.44 ± 4.28; with ZPT 84.6 ± 6.87; with PL 88.36 ± 5.27, this difference is not significant.

Figure 3.2.1.4a (A) The percentage of CD14+CD23+ cells using various concentrations of IL-4 in twenty normal subjects. IL-4 alone (□), IL-4 + PL (○) and IL-4 + ZPT (●).

(B) The inhibition of CD23 expression on monocytes with ZPT (■) and PL (□). ZPT significantly inhibits CD23 expression at all doses of IL-4 used (p < 0.001).
3.2.1.5 The effect of varying the concentrations of ZPT and PL on CD23 expression

The effect of decreasing the concentration of ZPT was investigated. Using concentrations of 1.0, 0.5, 0.25 and 0.125 mg/ml of ZPT or PL coincubated with 200U/ml of IL-4, ZPT inhibited in a dose dependant fashion. The mean inhibition ± SE of the twelve individuals studied was significant (p = 0.001) down to a concentration of 0.25 mg/ml (Fig 3.2.1.5a).

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 3.2.1.5a (A)** The percentage of CD14+ CD23+ cells using various concentrations of ZPT and PL in twelve normal subjects. IL-4 only (□) IL-4 + PL (○) and IL-4 + ZPT (●).

(B) The inhibition of CD23 on monocytes by ZPT (□) and PL (○). ZPT inhibits in a dose dependant manner and is still significant at a dose of 250 μg/ml.

3.2.1.6 Time Course of Addition of ZPT and PL

ZPT and PL were added at time 0, 0.5, 1, 2, 3, 4, 8, 12, 18 and 24 after the addition of IL-4. CD23 expression on monocytes was inhibited when added up to 12 hours.
after IL-4 addition (Fig 3.2.1.6a). Again, PL had little effect on CD23 expression on monocytes. The increase in CD23 expression between twelve and eighteen hours is seen because the cells were cultured for a further 24 hours.

Figure 3.2.1.6a The ability of ZPT or PL to inhibit IL-4 induced CD23 expression on monocytes when added at intervals after the addition of IL-4. IL-4 alone (□), IL-4 + PL (○) and IL-4 + ZPT (●). Inhibition was still present even when the ZPT was added 12 hours after the addition of IL-4. This result represents the mean of duplicate experiments.

### 3.2.1.7 The effect of ZPT on sCD23 production

PBMCs from seven controls were cultured with IL-4 and PL or different concentrations of ZPT. Supernatants were taken after eighteen hours and assayed for sCD23. There was a significant increase in sCD23 production when the cells were cultured with IL-4 (p = 0.001). This increase was only significantly inhibited at the highest concentration of ZPT (p = 0.006) but not at other concentrations or with PL.
However the surface expression of CD23 on monocytes in these cultures was significantly inhibited down to concentration of 250 μg/ml (Table 3.2.1.7a). After induction with IL-4, B cells, T cells and monocytes contribute to the sCD23 production in these cultures thus analysis of pure monocyte populations might give different results.

Figure 3.2.1.7a The effect on ZPT and PL on sCD23 production from PBMCs from seven normal individuals. No IL-4 ( ), IL-4 alone (■), IL-4 + PL (□) and IL-4 + ZPT (▲). IL-4 significantly increased sCD23 production (p = 0.001) but ZPT only significantly inhibited this production at the highest concentration (p = 0.006).

Table 3.2.1.7a Inhibition of CD23 on monocytes with different concentrations of ZPT in relationship to sCD23 production.

<table>
<thead>
<tr>
<th></th>
<th>% Inhibition of CD14+CD23+ cells</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>(1mg/ml)</td>
<td>3.6 ± 1.49</td>
</tr>
<tr>
<td>ZPT</td>
<td>(1mg/ml)</td>
<td>50.4 ± 9.18</td>
</tr>
<tr>
<td></td>
<td>(0.5mg/ml)</td>
<td>40.6 ± 11.17</td>
</tr>
<tr>
<td></td>
<td>(0.25mg/ml)</td>
<td>37.2 ± 9.76</td>
</tr>
<tr>
<td></td>
<td>(0.125mg/ml)</td>
<td>23.0 ± 9.80</td>
</tr>
</tbody>
</table>
3.2.2 Cell Death

3.2.2.1 Viability studies

To examine whether the inhibition of IL-4 induced CD23 expression by ZPT was due to cell death, PBMCs from normal individuals were cultured with varying concentrations of ZPT or PL for three days. The results using acridine-orange/ethidium-bromide are expressed as the mean of four experiments (Fig 3.2.2.1a). There was no difference in cell death between control and ZPT/PL cultures up to 2.5mg/ml. However, ZPT became toxic when used at 5mg/ml, whereas, this was not reflected with PL until a concentration of 10 mg/ml was reached. In all our experiments the dose of ZPT used never exceeded 1mg/ml at which the viability was 97%.

Figure 3.2.2.1a Viability of PBMCs cultured with ZPT (●) and PL (○) for three days using acridine orange/ethidium bromide. As the percentage of live cells was the same in the ZPT and PL cultures at a concentration of 1mg/ml the inhibition of CD23 seen in these experiments cannot be due to cell death. → indicates the concentration used (1mg/ml).
3.2.2.2 Confirmation of Cell Death Studies

PBMCs were cultured with ZPT and PL overnight and the cells in the monocyte gate were assessed for cell death with propidium iodide. The cells fluoresce red when they are dead or dying. In three separate experiments there was no significant difference in the percentage viability ± SD in control cultures 95.6 ± 2 compared with ZPT 90.1 ± 2.6 and PL 95 ± 3.

3.2.2.3 Superoxide Production

The possibility that ZPT or PL was affecting cell metabolism but not killing cells was considered. The reduction of NBT by superoxide produced by monocytes is a clear measure of cell viability. PBMCs from five individuals were activated with PMA and the NBT reduction was measured. As can be seen in Table 3.2.2.3a there is no variation in NBT reduction by PMA stimulated monocytes whether in the presence of ZPT or PL. This result clearly shows that there was not an impaired superoxide production at the dose of ZPT previously shown to reduce the expression of CD23. This indicates that the alteration of CD23 expression was neither due to cell death nor a toxic effect on cell metabolism.

Table 3.2.2.3a NBT reduction by PMA stimulated monocytes cultured in the absence or presence of IL-4 with ZPT or PL (n=5). There was no difference in the superoxide production and reduction in NBT between the control cultures or cells cultured with ZPT or PL. However there was a significant inhibition of CD23 by ZPT in the parallel cultures of the same individuals (mean ± SD). The results are expressed as nM of NBT reduced per well.

<table>
<thead>
<tr>
<th></th>
<th>nM NBT reduced per well</th>
<th>% INHIBITION OF CD14+23 CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No IL4</td>
<td>IL4 (200 U/ml)</td>
</tr>
<tr>
<td>CELLS</td>
<td>2.11 ± 0.8</td>
<td>2.04 ± 0.7</td>
</tr>
<tr>
<td>ZPT</td>
<td>2.09 ± 0.9</td>
<td>2.08 ± 0.63</td>
</tr>
<tr>
<td>PL</td>
<td>2.08 ± 0.8</td>
<td>1.86 ± 0.50</td>
</tr>
</tbody>
</table>

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3.2.3 Stability of ZPT

As ZPT undergoes a crude extraction, the interbatch variation and stability of different ZPT preparations was tested to ensure that the inhibitory effects obtained were not seen only in one preparation. Dried aqueous extracts were prepared by Phytopharm and stored at room temperature and samples were tested after 1, 6 and 12 months to see the effect on CD23 expression on monocytes. As experiments were performed at different time intervals the results were compared to the original extract used in all previous tests. The results were expressed as follows:

\[
\text{Ratio compared to control} = \frac{\% \text{ Inhibition of CD14+CD23+ cells by extract}}{\% \text{ Inhibition of CD14+CD23+ cells by ZPT (PSE222/9240)}}
\]

Each time point was tested on five different individuals and there was a slight interbatch variation between the different preparations of ZPT (Fig 3.2.3a). In three out of the four batches tested there was a decrease in activity after 12 months however, this decrease was not significant. These results show that the active component in the mixture which inhibits CD23 inhibition is reasonably stable and is extracted in all the preparations of ZPT. ZPT (PSE 222 BN 9240) was the preparation used throughout the studies.

![Graph showing stability and interbatch variation of ZPT](image)

**Figure 3.2.3a** The stability and interbatch variation of ZPT. There was a slight interbatch variation and decrease in activity after 12 months but these changes were not significant.
3.2.4 The effect of ZPT on HLA-DR expression on monocytes

As IL-4 also upregulates the expression of HLA-DR on monocytes (Gerard et al., 1990) the effect of ZPT was investigated to determine if the inhibition was specific for the CD23 molecule. PBMCs from seven individuals were cultured with different concentrations of IL-4 and ZPT and PL (1mg/ml), and then double stained with antibodies to HLA-DR and CD14 (Fig 3.2.4a). ZPT and PL had no significant effect on HLA-DR expression although both mixtures seem to slightly increase the basal expression. The cells were also double stained with CD23 and CD14 and the % CD14+23+ cells was 49.16 ± 9.06, with % inhibition by ZPT 44.76 ± 10.66 and PL 1.97 ± 1.07.

Figure 3.2.4a (A) Effect of ZPT and PL on HLA-DR expression on monocytes. IL-4 only , (□) ZPT (●), PL (○). There was no significant effect of either ZPT or PL on HLA-DR expression. (B) There was no dose effect of ZPT (●) on IL-4 induced HLA-DR expression. PL (○) was used as a control.

3.2.5 Modulation of IL-4 induced CD23 expression on monocytes in atopic eczema patients

As ZPT is an effective treatment in AE, its effect was examined using peripheral mononuclear cells from patients with AE.
3.2.5.1 Comparison of IL-4 induced CD23 expression in AE patients and control individuals

The atopic eczema patients were diagnosed according to the criteria described in the methods. Their IgE levels ranged from 270 - 30,000 (mean 6759.61± 2417.3 IU/ml). The data from the twenty normal individuals (Fig 3.2.4.1a) and twenty AE patients were compared for the ability of IL-4 to induce CD23 expression on monocytes. Although at all concentrations of IL-4, AE patients expressed slightly higher levels of CD23 this increase was not significant (Fig 3.2.5.1a).

![IL-4 induction of CD23 expression](image)

*Figure 3.2.5.1a IL-4 induction of CD23 expression on monocytes from normal individuals (□) and AE patients (■). There was no significant difference in CD23 expression between the two groups.*

3.2.5.2 The effect of ZPT and PL on IL-4 induced CD23 expression on monocytes from AE patients

In twenty AE patients using the same techniques as previously described, ZPT inhibited the expression of CD23 in the same manner as in the controls. At different concentrations of IL-4 the inhibition in AE patients was greater than in normals. However, the PL effect was increased by a factor of two (Fig 3.2.5.2a) and therefore
the overall net effect of inhibition was the same in both groups. The effect of the PL may be non-specific and due to the activation state of the AE monocytes.

![Graph A](image1.png)

**Figure 3.2.5.2a (A)** The percentage of CD14+CD23+ cells using various concentrations of IL-4 in twenty AE patients. IL-4 alone (□), IL-4 + PL (∆) and IL-4 + ZPT (●).

**Figure 3.2.5.2a (B)** The inhibition of CD23 expression on monocytes with ZPT (□) and PL (□) in AE patients. The expression of CD23 was significantly inhibited by ZPT (p < 0.001).

### 3.5.2.3 The effect of varying the concentrations of ZPT and PL on CD23 expression in AE patients

In the controls inhibition was seen at a concentration of 0.25 mg/ml while in the AE patients, ZPT significantly inhibited at a concentration of 0.125 mg/ml (Fig 3.5.2.3a). This was significantly different from the effect seen by PL and suggest that the molecule(s) in ZPT that inhibit CD23 expression may be more potent on AE monocytes.
Figure 3.2.5.3a (A) The percentage of CD14+ CD23+ cells using various concentrations of ZPT and PL in twenty AE patients IL-4 only (□) IL-4 + PL (○) and IL-4 + ZPT (●).
(B) The inhibition of CD23 on monocytes by ZPT (■) and PL (□). The inhibition by ZPT is significant down to a concentration of 125 μg/ml.
3.3 DISCUSSION

In this chapter it has been shown that an aqueous extract of ZPT significantly inhibited the expression of CD23 induced by IL-4 on normal and atopic eczema monocytes. An extract of placebo used in the two double blind clinical trials (Sheehan et al., 1992a, 1992b) had little effect on this IL-4 driven CD23 expression. The rationale for using this placebo extract was to give an indication of whether a water extract from any plant material would be active in vitro as this was not the case it was thought that PL would be a suitable control in all experiments. The inhibitory effect of ZPT was concentration dependent in both groups studied however, it was independent of the concentration of IL-4 used. sCD23 production was also increased by IL-4 but only significantly inhibited at the highest concentration of ZPT. The sCD23 levels were a reflection of the production by PBMCs and not just monocytes and this may indicate that this mixture may not be targeting B and T cells. The effect of ZPT on surface CD23 and sCD23 production from purified monocytes will have to be investigated to justify this statement. The expression of CD14 or HLA-DR on monocytes was not affected by ZPT or PL. HLA-DR has been shown to be spatially associated with CD23 (Bonnefoy et al., 1988) on B cells and an anti-HLA-DR antibody decreased IL-4 induced CD23 expression (Kicza et al., 1989). This presumably indicates that ZPT’s effect may be specific for CD23 and does not cause a general decrease in cell surface markers on monocytes. As the superoxide production by monocytes was not impaired, the assumption must be made that these cells are metabolically viable and that one or more components of the mixture must be acting on the pathway to the induction of CD23.

The inhibitory property was long lasting and the time course study showed that ZPT could be effective after the addition of IL-4 and this may be realistic in view of the in vivo situation. This also suggests that the active component is acting after IL-4 binds to its receptor. ZPT may be acting on a pathway after IL-4 induction possibly at the mRNA or translation of the protein. The stability studies indicated that the inhibitory factor(s) was hot water extractable and not easily degraded. These properties may give clues the types of molecules that may be responsible for the inhibition seen by ZPT.

An artificial system of CD23 induction was used where the levels of CD23 induced by IL-4 (200U/ml) are above the expression seen on the monocytes of patients with
AE. The results indicate that ZPT is more effective at lower levels of CD23 expression and this may be comparable with those seen in vivo. The levels of CD23 on monocytes induced by IL-4 were no different between controls and AE patients thus the overexpression seen in vivo in AE is likely to be due to the overproduction of IL-4 rather than an inherent defect in the modulation of CD23 or the monocyte. There was no significant difference in the effect of ZPT on monocytes from AE patients and control subjects suggesting its immunosuppressive activity is on CD23. Thus, the effectiveness of ZPT in AE may be due to the fact that it is able to target newly induced CD23 molecules i.e. the type B form of CD23 was inducible by IL-4 rather than the form constitutively expressed on B cells (type A). These subtypes may have different functions (Yokota et al., 1992) and similar experiments with Langerhans cells and B cells may give a clearer view to which form of CD23 is being inhibited. So far the data has only shown that CD23 expression can be inhibited, however the overall efficacy of the decoction is likely to be multifactorial and further studies will have to be performed to analyse the modulatory functions on the inflammatory process in the skin.

It has recently been shown that IL-13 acts in a similar manner to IL-4 in respect to the induction of CD23 on monocytes (de Waal Malefyt et al., 1993) and IgE production by B cells (Punnonen et al., 1993). Although overproduction of IL-13 in AE has not yet been shown, this cytokine has to be given some consideration. If the hypothesis is that CD23 overexpression is important in the immunopathology of AE then the treatments used should all in some way lead to the downregulate of CD23 expression. Therefore the mode of action by various treatments on CD23 should be investigated.
Chapter 4

Comparison of prednisolone, ZPT and cyclosporin on IL-4 and IL-13 induction of CD23 on different cell types
4.1 INTRODUCTION

One of the basic therapies for AE is topical or systemic corticosteroids which reduce the inflammation of the skin. Glucocorticoids such as prednisolone and dexamethasone act by first diffusing through the plasma membrane and binding to specific receptors in the cytoplasm. This complex is then translocated to the nucleus where it has been shown to regulate gene expression (Baxter & Faunder 1979). One of the major actions of these molecules is the synthesis of lipocortin which inhibits the enzyme phospholipase A2. This causes a reduction in release of arachidonic acid from the membrane phospholipids and leads to an interference in the generation of inflammatory mediators from the prostaglandin/leukotriene cascade. Various effects of glucocorticoids have been found on the immune system, in particular, receptors have been found on monocytes (Werb et al., 1978) and glucocorticoids have been shown to inhibit TNF-α (Waage and Backko 1988) and IL-1 (Kern et al., 1988) production from these cells. Although the effects of glucocorticoids are usually inhibitory, it has been demonstrated that dexamethasone augments IFN-γ induced HLA -DR expression (Shen et al., 1984) and IgG Fc receptors (Girard et al. 1984) on monocytes.

In relation to CD23, prednisolone has been shown to inhibit its surface expression and production of sCD23 when induced by IL-4 and IL-2 on peripheral blood lymphocytes (Fischer et al., 1990). Similar results have been obtained with prednisolone (Katira et al., 1993) and dexamethasone (Kaufman Paterson et al., 1994) on purified B cells. In the former case, engaging CD40 and in the latter infection with EBV counteracted the inhibitory effect of corticosteroids on B cells. Dexamethasone has also been shown to reduce IgE binding (Naray-Fejes-Toth et al., 1985) and IFN-γ induced expression (Naray-Fejes-Toth et al., 1984) on the monoblast cell line U937. However, the influence of corticosteroids on CD23 expression on purified monocytes in vitro has not been investigated. Along with in vitro data showing the modulation of CD23 by corticosteroids, Bieber et al has demonstrated that there is a reduction of IgE+ Langerhans cells in AE patients treated with corticosteroids which may indicate that this treatment affects CD23 expression or the high affinity IgE receptor in vivo (Bieber et al., 1989).

Newer forms of treatment for AE include cyclosporin (Sowden et al., 1991, Joost et al., 1994, Granuland et al., 1995), and ZPT (Sheehan et al., 1992a, 1992b; Sheehan et al., 1994). Cyclosporin, a major immunosuppressant for activated T cells acts by
binding to calcineurin which is a calcium and calmodulin dependant phosphatase enzyme. This directly or indirectly blocks the nuclear factor of activated T cells (NF-ATp), a transcription factor involved in pathways leading to the induction of various cytokine genes including IL-2, IL-4 and IFN-γ (Rao, 1994). Although most of the data on cyclosporin relates to T cells, it has been shown to have effects on antigen presentation by Langerhans cells (Furue and Katz 1987) and monocytes (Palay et al., 1986). Its effect on CD23 expression has not been investigated.

ZPT has been used in clinical trials but the mechanism of action on the immune system is unknown. In the previous chapter it was shown that ZPT inhibits IL-4 induced CD23 expression and this might in part account for some of its action in vivo.

This chapter focuses on the comparison of prednisolone, cyclosporin, and ZPT on IL-4 induced CD23 expression on monocytes and the human monoblast cell line U937. The downregulation of IL-4 induced CD23 may be through the modification of the pathway for CD23 expression and therefore the mRNA for CD23 and translation of protein are also studied. IL-13 has recently been shown to have similar actions to IL-4 thus the in vitro action of the three treatments on the IL-13 induction pathway of CD23 expression was also investigated.
4.2 RESULTS

4.2.1 The effect of Prednisolone and Cyclosporin on CD23 expression on monocytes

Prednisolone and cyclosporin were investigated to see if these two compounds had similar effects as ZPT on IL-4 induced CD23 expression on monocytes. This information might point to similar mechanisms of action on CD23 by these three treatments in AE. PBMCs were isolated from six individuals and cultured with IL-4 and different concentrations of prednisolone and cyclosporin. ZPT and PL at a concentration at 1 mg/ml were also included in this series of experiments for comparison.

4.2.1.1 Prednisolone

Prednisolone inhibited CD23 expression on monocytes in a dose dependant manner (Fig 4.2.1.1a). The most substantial inhibition was seen at the highest concentration used of 10μg/ml (2 x 10^{-3} M) of prednisolone. The inhibition of ZPT at a concentration of 1 mg/ml was comparable with 1μg/ml of prednisolone. Even at the highest concentration there was minimum cell death with prednisolone.

**Figure 4.2.1.1a (A)** The percentage CD23+ monocytes with different concentrations of prednisolone. 

**Figure 4.2.1.1a (B)** The inhibition of IL-4 induced CD23 expression on monocytes by prednisolone (■), ZPT (□) and PL extract (■).
4.2.1.2 Cyclosporin

There was a slight enhancement of IL-4 induced CD23 expression on monocytes but this was only significant at 0.01µg/ml (Fig 4.2.1.2a)

![Graph showing CD23 expression vs Cyclosporin concentration](image)

**Figure 4.2.1.2a** (A) The percentage CD23+ monocytes with different concentrations of cyclosporin. (B) The effect of cyclosporin (■), ZPT (□) and PL (▲) on IL-4 induced CD23 expression. There was enhancement of CD23 expression by cyclosporin which was significant at 0.01 µg/ml.

As prednisolone and ZPT gave a significant inhibition of IL-4 induced CD23 expression these were studied for their ability to inhibit the IL-13 pathway.

4.2.2 The effect of ZPT and Prednisolone on IL-13 induction of CD23

4.2.2.1 Induction of CD23 on monocytes by IL-13

IL-13 like IL-4 has been shown to induce CD23 expression on monocytes. (de Waal Malefyt et al., 1993). Mononuclear cells from six normal individuals were cultured...
with different concentrations of IL-13 to establish the optimum concentration for investigating the effects of prednisolone and ZPT. As seen in Fig 4.2.2.1a 50\(\mu\)g/ml of IL-13 gave a similar expression 200U/ml of IL-4 (Fig 3.2.1.1b). Thus this concentration of IL-13 was used for further studies.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure.png}
\caption{Dose response of IL-13 induction of CD23 on monocytes (n = 6)}
\end{figure}

\subsection*{4.2.2.2 Comparison of inhibition by ZPT of IL-4 and IL-13 induced CD23 expression on monocytes}

PBMCs from seven individuals were cultured with IL-4 (200U/ml) or IL-13 (50\(\mu\)g/ml) and different concentrations of ZPT ranging from 1 mg/ml-0.125 mg/ml to substantiate if there was any differences in the inhibition obtained. There was a slight but significant difference in the percentage expression of CD23 on monocytes induced by IL-4 (66.91 ± 4.4) and IL-13 (59.086 ± 4.36) (\(p = 0.04\)). ZPT inhibited IL-13 induction of CD23 at all concentrations. However there was no difference between the inhibition on IL-13 and IL-4 pathway of induction of CD23 (Fig 4.2.2.2a).
Figure 4.2.2.2a Inhibition of ZPT on IL-4 and IL-13 induction of CD23 expression. IL-4 + ZPT (■), IL-13 + ZPT (□), IL-4 + PL (▲), IL-13 + PL (▲). There was no difference in inhibition by ZPT on IL-4 or IL-13 induction of CD23.

4.2.2.3 Comparison of inhibition by Prednisolone on IL-4 and IL-13 induced CD23 expression on monocytes

As previously shown prednisolone inhibited IL-4 induced CD23 expression. Inhibition was also seen with IL-13 induced CD23 (n = 8)( Fig 4.2.2.3a). However the influence of prednisolone on the IL-13 pathway was less pronounced than observed with IL-4.
4.2.3 Comparison of the inhibitory effects of ZPT and Prednisolone on CD23 expression and sCD23 production from semi-purified monocytes

4.2.3.1 ZPT

The inhibitory effects of ZPT and prednisolone on monocytes were examined in a mixed cell culture whereby monocytes were only a small percentage of the cells. Monocytes from seven individuals were isolated by adherence to plastic and then cultured with IL-4 and different concentrations of ZPT and prednisolone. sCD23 was also measured in the supernatants of the cultured monocytes. The cells obtained by plastic adherence were 72 ± 3.3 % CD14+, 6.61 ± 2.85 % CD19+ and 13.95 ± 3.6 % CD3+. An increased concentration of IL-4 (400U/ml) was required to induce a substantial expression of CD23 expression (32.95 ± 5.4 %) on this preparation of monocytes. In this semi purified monocyte preparation ZPT inhibited down to the same concentration as seen with the PBMC preparations (Fig 4.2.3.1a).
Figure 4.2.3.1a (A) The percentage of CD23+ cells in semi purified monocyte preparations using various concentrations of ZPT (●) and PL 1mg/ml(○).

(B) The inhibition of CD23 on monocytes by ZPT (□) and PL (○). ZPT inhibited in a dose dependant manner as previously seen in PBMCs.

ZPT also showed a dose dependant inhibition of sCD23 production but as previously described with PBMCs, this was only significant at its highest concentration. (Fig 4.2.3.1b).
Figure 4.2.3.1b The effect on ZPT and PL on sCD23 production from semi purified monocytes from seven normal individuals. IL-4 + PL (1mg/ml) (Ο) and IL-4 + ZPT (■). ZPT significantly inhibited this production down to 500μg/ml.

4.2.3.2 Prednisolone

Prednisolone showed similar effects as previously described on monocytes in PBMCs preparation with a dose dependant inhibition on IL-4 induced CD23 expression (Fig 4.2.3.2a).
Figure 4.2.3.2a (A) The percentage of CD23+ semi purified monocytes with different concentrations of prednisolone. (B) The inhibition of IL-4 induced CD23 expression on monocytes by prednisolone. CD23 expression was inhibited by prednisolone in a similar manner as seen with PBMC preparations.

Prednisolone significantly inhibited sCD23 production but like ZPT the inhibition of surface expression was greater than on the production of the soluble form of CD23 (Fig 4.2.3.2b).
4.2.4 The effect of ZPT and prednisolone on CD23 expression on U937 cells

4.2.4.1 ZPT

As monocyte isolation by adherence contained contaminating T and B cells, the human monoblastic/monocytic leukaemia cell line U937 was used to further investigate the inhibition of CD23. U937 cells have been shown to constitutively express CD23 (Anderson & Spiegelberg et al., 1981) and this can be upregulated by IL-4 (Willheim et al., 1991). The effect of ZPT on constitutively expressed CD23 and IL-4 induced expression was investigated as a model for human blood monocytes (n=6). 60% of the U937 cells expressed CD23, this expression or IL-4 induced expression was not influenced by ZPT (Fig 4.2.4.1a). There was also no change in sCD23 concentration (Fig 4.2.4.1b). This was in contrast to human blood monocytes where CD23 was inhibited by ZPT.

Figure 4.2.3.2b Inhibition of sCD23 production on semi purified monocytes by prednisolone
Figure 4.2.4.1a  The effect of ZPT and PL on U937 cells. U937 cells cultured with ZPT but without IL-4 (○) and with IL-4 (●). PL at 1mg/ml was used as a control without IL-4 (□) and with IL-4 (■). There was no effect of ZPT on constitutively expressed CD23 or IL-4 induced CD23.

Figure 4.2.4.1b  sCD23 concentration in supernatants from U937 cells cultured with ZPT but without IL-4 (○) and with IL-4 (●). PL at 1mg/ml was used as a control without (□) and with IL-4 (■). There was no effect of ZPT on sCD23 production from U937 cells.
4.2.4.2 Prednisolone

U937 cells were cultured without and with IL-4 and the effect of different concentrations of prednisolone on CD23 expression was investigated. As seen in Fig 4.2.4.2a prednisolone downregulated both the constitutively expressed and IL-4 induced CD23.

![Graph](image)

**Figure 4.2.4.2a** The effect of prednisolone on constitutively expressed (○) and IL-4 induced CD23 expression (●) on U937 cells. Prednisolone significantly inhibited the expression of both at 10 and 1 μg/ml.

This inhibitory effect was not limited to IL-4 induced CD23 but IL-13 induced CD23 expression on U937 cells was also inhibited (Fig 4.2.4.2b). This effect on IL-4 and IL-13 induced CD23 expression was equipotent. This was in contrast to monocytes where the inhibitory effect was less pronounced on the IL-13 pathway.
Figure 4.2.4.2b The effect of prednisolone on IL-13 induction of CD23 on U937 cells. Prednisolone significantly inhibited constitutively expressed □ and IL-13 induced ■ CD23 expression.

sCD23 production in the supernatants of IL-4 and IL-13 stimulated U937 cells was also analysed. There was a decrease in sCD23 production when cells were cultured with IL-4 and IL-13 and prednisolone (Fig 4.2.4.2c). The unstimulated sCD23 production was also inhibited by prednisolone.
As no effect of ZPT was found on U937 cells, although blood monocytes were inhibited, it was decided to investigate the effect on a Mono Mac 6, a monocyte cell line which was considered to be similar to mature monocytes (Ziegler-Heitbrock et al., 1988). Although these cells were very proliferative, they did not express CD23 nor could they be induced to express this marker by IL-4, thus these experiments were not continued.

4.2.5 Comparison of ZPT on CD23 expression on B cells and monocytes

Lawrence et al demonstrated that human peripheral B cells expressed CD23 and later it was shown that IL-4 increased this expression (Lawrence et al., 1975; Kitutani et al., 1986; Defrance et al., 1987). As ZPT has been shown to inhibit the CD23 expression on monocytes, the influence of this mixture on constitutively expressed (a) and inducible (b) CD23 on B cells was investigated. PBMCs (n=4)
were cultured with IL-4 and doubled stained with CD19 and CD23 for the expression on B cells and CD14 and CD23 for monocytes. ZPT or PL had no effect on constitutively expressed CD23 on B cells but there was a slight increase by both mixtures on IL-4 induced expression (Fig 4.2.5a). This was in contrast to ZPT's effect on IL-4 induced CD23 expression on monocytes.

**Figure 4.2.5a** The influence of ZPT and PL on constitutively expressed and IL-4 induced CD23 expression on B cells and monocytes. Medium only (□), PL alone (■), ZPT alone (■), IL-4 (■), IL-4 + PL (■) and IL-4 + ZPT (■). ZPT had no effect on CD23 on B cells but inhibited its expression on monocytes.

### 4.2.5.1 The effect of ZPT on EBV transformed B cells

EBV infection has been shown to increase the expression of CD23 on B cells (Thorley-Lawson & Mann 1985) and this has been linked to EBNA-2, an EBV-encoded nuclear antigen. EBV transformed B cells from five normal individuals were stained for their surface expression of CD23 and their ability to respond to IL-4. The five EBV B cells expressed CD23 ranging from 35% to 71.8%. The addition of IL-4 (1000U/ml) increased this range from 60% to 80 CD23+. The highest
expressor of CD23. The highest expressor of CD23 (71.8%) EBV B cells lines was chosen to study the effect of ZPT. The cells were cultured overnight with ZPT or PL with or without IL-4. Table 4.2.5.1a shows that both ZPT and PL slightly decreased the expression of CD23 when the cells were unstimulated but neither had a significant effect on the CD23 expression of this EBV transformed B cell line. This was repeated with the lowest expresser of CD23 + (35%) and again the same result was obtained.

Table 4.2.5.1a Effect of ZPT and PL on CD23 expressed on EBV transformed B cells. There was no significant effect on this expression.

<table>
<thead>
<tr>
<th>CD23 + cells</th>
<th>EBV-B cells</th>
<th>EBV-B cells + PL</th>
<th>EBV-B cells + ZPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>- IL-4</td>
<td>77.38 ± 7.80</td>
<td>62.18 ± 16.70</td>
<td>62.16 ± 12.00</td>
</tr>
<tr>
<td>+ IL-4</td>
<td>84.66 ± 6.66</td>
<td>84.41 ± 8.00</td>
<td>81.26 ±10.44</td>
</tr>
</tbody>
</table>

4.2.6 The effect of Prednisolone and ZPT on the mRNA for CD23 on monocytes and U937 cells.

In this chapter it has been demonstrated that both prednisolone and ZPT reduced CD23 expression on monocytes when induced either by IL-4 or IL-13. The mRNA for CD23 was analysed to see if the molecular basis of inhibition was similar for prednisolone and ZPT. Monocytes were cultured with IL-4 without or with prednisolone (10\(\mu\)g/ml) and ZPT and PL at 1mg/ml. Monocytes from two individuals were pooled just prior to RNA isolation. Initial studies showed that although the mRNA for CD23 could be detected by 8 hours the maximum signal was between 18 and 24 hours. The RNA was always isolated within the maximum signal time point. Fig 4.2.6a shows two representative autoradiograms of the effect of PL, ZPT and prednisolone on CD23 mRNA. The mRNA for \(\beta\) actin is shown as a control. The data of 9 pools were quantified by densitometry and the ratio of CD23 mRNA and actin mRNA was calculated. Both Prednisolone and ZPT significantly decreased the mRNA for CD23 after induction with IL-4 although ZPT was not as pronounced as prednisolone (Fig 4.2.6b). PL had no effect on the induction of mRNA for CD23 by IL-4.

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Figure 4.2.6a  Two representative autoradiograms of mRNA for CD23 in monocytes after induction with IL-4 only (A), IL-4 + PL (B), IL-4 + ZPT (C), IL-4 + Prednisolone (D). β actin mRNA is shown as a control for loading of RNA.
Figure 4.2.6b The ratio of mRNA for CD23/actin after culturing monocytes with IL-4 (n = 9). An arbitrary value of 1 was assigned to the CD23/actin ratio for monocytes cultured with IL-4 only. There was a significant reduction in IL-4 mRNA for CD23 after exposure to prednisolone and ZPT. PL had no influence on IL-4 induced mRNA for CD23.

As monocytes and U937 cells showed the same response to prednisolone in relationship to CD23, the effect of this drug on IL-4 and IL-13 induction of mRNA was considered. In fig 4.2.6c is shown the mRNA for CD23 when the cells were incubated with or without IL-4 or IL-13 and prednisolone. There is consistent reduction in the mRNA after culturing with prednisolone. This represents almost complete inhibition of the IL-4 and IL-13 induced mRNA for CD23.
4.2.7 The action of ZPT on newly synthesised CD23 molecules

As the mRNA for CD23 was not greatly decreased by ZPT it was thought that its action might be due to an effect on the translation of CD23 after induction of IL-4. Although the translated protein could be detected for the α chain of the HLA-DR molecules in monocytes, the translated CD23 could not be detected after several attempts with changes to the protocol (Fig 4.2.7a). Using U937 cells as a control, a band of approximately 90Kd was obtained but this was not consistent. The reason for the lack of detection of the CD23 molecule may be due to the fact that it degraded to its soluble form. The use of protease inhibitors such as TCLK and iodoacetamide might have ensured its detection.
Figure 4.2.7a  Autoradiograph showing incorporation $^{35}$S methionine into newly synthesised CD23 protein and the $\alpha$ chain of HLA-DR molecules. Lane A - Precleared lysate. Lane B - Immunoprecipitation with CD23. Lane C - The $\alpha$ chain of HLA-DR gave two bands at 28 and 14kd but there was no detection of the CD23 molecule.
4.3 DISCUSSION

Corticosteroids such as prednisolone, ZPT, cyclosporin and IFN-γ have all been used in the treatment of AE. Three of these substances were tested for their effect on IL-4 induced CD23 expression. Corticosteroids have been shown to inhibit CD23 expression on PBMCs, B cells and U937 cells (Fischer et al., 1990; Katira et al., 1993; Kaufman Paterson et al., 1994; Naray-Fejes-Toth et al., 1984; Naray-Fejes-Toth et al., 1985). The results in this chapter have confirmed this data showing that prednisolone inhibited IL-4 induced CD23 expression on monocytes and has been extended to show that the IL-13 induction was also reduced in monocytes and U937 cells. The inhibition by prednisolone was irrespective of whether the monocytes were part of a mixed cell culture (PMBCs), semi purified or a monocytic cell line. The data indicated that the IL-13 induction on monocytes was less responsive to prednisolone inhibition. However this was not borne out with U937 cells and further experiments would have to be performed at the molecular level to confirm this.

sCD23 production was also shown to be reduced and therefore this reduction was probably due to a decrease in translation of protein. Corticosteroid/receptor complexes can be shown to modulate certain genes by binding to glucocorticoid-responsive elements on DNA. Fischer et al suggested that the CD23 gene had putative glucocorticoid-responsive elements in its DNA sequence and thus the reduction in mRNA for CD23 seen in monocytes and U937 cells when cultured with prednisolone may be through this drug binding to these sequences (Fischer et al., 1990).

ZPT's action on CD23 in similarity to prednisolone was not restricted to the IL-4 pathway as it also inhibited IL-13 induction of CD23. However, unlike prednisolone both pathways were equally inhibited. The reduction of sCD23 from monocytes was only seen at the highest concentration of ZPT. sCD23 is normally formed from surface CD23 by proteolysis (Nakajima et al., 1987; Letellter et al., 1988; Letellier et al., 1989; Letellier et al., 1990) although some is produced intracellularly (Lee et al., 1989). In the case of ZPT, its inhibitory action on the induction of CD23 may be more pronounced than on the formation of sCD23. The reduction in mRNA for CD23 by ZPT was not as clearly seen as with prednisolone and thus the translation was investigated. However CD23 was not detected probably due to its degradation (Dr Sarfati pers.com.) and therefore other methods for investigating the protein translation will have to be attempted. ZPT showed different actions on
CD23 on monocytes compared with U937 cells. This discrepancy between monocytes and U937 cells has been shown before whereby certain cytokines have had opposing effects on IL-4 induced CD23 expression (IL-6: Willheim et al., 1991; IFN-α: Gessl et al., 1993; Hashimoto et al., 1995; IL-10: Spittler et al., 1995). This difference between monocytes and U937 cells may be due to the fact that the cell line represents an earlier stage of monocytic differentiation than blood monocytes. ZPT did not influence constitutively expressed CD23a or IL-4 induced CD23 expression (a and b) on B cells. This phenomena whereby an effect is seen on monocytes but not B cells has been demonstrated with 1,25(OH)₂ vitamin D₃ (Fargeas et al., 1990) and it would be interesting to see if ZPT contains any components similar to this molecule. However, 1,25(OH)₂ vitamin D₃ unlike the inhibitory factor in ZPT downregulated CD23 expression on U937 cells (Oberg et al., 1993). Prednisolone and ZPT showed similar inhibition of CD23 in monocyte preparations which were 75% CD14+ pure as when the cells were within a mixed cell population. This may indicate that both drugs are acting directly on monocytes; however the influence of the remaining T and B cells cannot be ruled out.

Cyclosporin, although at first thought only to be immunosuppressive for T cells has been also been found to decrease antigen presentation by monocytes (Palay et al., 1986) and inhibit and enhance IL-4 induced IgE production by PBMCs (Chang et al., 1993; Wheeler et al., 1995). It has also been shown to inhibit histamine release and PGD₂ production from human skin mast cells (Stellato et al., 1992). These results have made it a good candidate for treatment in AE. In the data presented here cyclosporin did not influence IL-4 induced CD23 expression except for a slight enhancement at one concentration. A study of immunological markers in the lesional skin of AE patients after treatment with cyclosporin showed that CD25+, CD14+ and IL-8+ cells were significantly reduced (van Joost et al., 1992). The success of cyclosporin in AE may be a combination of its effects on T cells by reducing cytokine production and decreasing antigen presentation.

Finally, IFN-γ has been shown either to decrease (Velde et al., 1990), enhance (Delespesse et al., 1989) or have no effect (Vercelli et al., 1988) on IL-4 induced CD23 expression on purified monocytes. In our laboratory, we obtained similar results to Vercelli et al., 1990 whereby IFN-γ (100U/ml) had no effect on CD23 on monocytes in a PBMC population (Dr Hawke unpublished data). This discrepancy may be due to different isolation procedures for monocytes and the influence of other
cell types in mixed cell population. This variation in results was not seen on B cells where IFN-γ inhibits IL-4 induced CD23 expression (Galizzi et al., 1988; Delespesse et al., 1989), CD23mRNA (Donoroy et al., 1990) and the inhibition is post-transcriptional (Lee et al., 1993). The role of IFN-γ in regulation of IL-4 induced CD23 expression and its action in the treatment of AE will have to be further elucidated.

The rationale for studying the effect of different drugs on IL-4 induced CD23 expression on monocytes was that if overexpression of CD23 was important in AE then these treatments would in some way reduce CD23 expression. There was a reduction of CD23 on Langerhans cells in AE patients treated with corticosteroids (Bieber et al., 1989) and on macrophages and Langerhans cells when treated with ZPT (Xiou et al., submitted for publication). Thus these results may indicate that downregulation of CD23 has a beneficial role in the resolving of AE. The changes in lesional skin after treatment with IFN-γ have not been performed and it would be interesting to discover if there is a change in CD23. Cyclosporin showed no effect on CD23 however it would be easy to speculate that its immunosuppressive action on T cells could lead to a reduction in IL-4 which would lead to a decrease in CD23. All these drugs will have multiple actions and of the four, ZPT has been the least studied. Thus its effect on a variety of other immunological systems would have to be tested as its effect on CD23 expression alone may not fully explain the total clearing of eczematous lesions seen in the clinical trials.
Chapter 5

The influence of ZPT and PL on cytokine production from patients with AE and normal subjects
5.1 INTRODUCTION

The study by Braathen et al demonstrated for the first time that the cellular infiltrate in skin of AE patients was mainly T cells and this was followed by the observation that these were of the CD4+ subset (Braathen et al., 1979; Zachary et al., 1985). The discovery that IgE production is regulated by IL-4 and inhibited by IFN-\(\gamma\) (Pene et al., 1988) has lead to an investigation into the profile of T cells infiltrating the skin and in the circulation of AE patients. IFN-\(\gamma\) has been shown to be decreased in vitro after mitogen stimulation of mononuclear cells from AE patients (Reinhold et al., 1989; Reinhold et al., 1990; Rousset et al., 1991; Jujo et al., 1992; Tang et al., 1993). The percentage of IFN-\(\gamma\)-producing cells in AE was found to be higher in unstimulated cultures (Tang & Kemp, 1994a) and the mRNA for IFN-\(\gamma\) was also increased compared to controls (Tang et al., 1994b). This group postulated that the reduced IFN-\(\gamma\) production was not due to a defect in transcription of mRNA but was post-transcriptional.

IL-4, in contrast to IFN-\(\gamma\) is increased on mitogen stimulation (Rousset et al., 1991; Jujo et al., 1992; Renz et al., 1992) and there is also an increase in mRNA for IL-4 receptor in AE compared to controls (Renz et al., 1992). Spontaneous expression of IL-4 mRNA has also been demonstrated (Tang & Kemp, 1994c). These findings have lead to the theory that AE is a ‘Th2 like disease’. Evidence for this has been shown were by the expansion of skin-infiltrating T cells from skin biopsies of patients with AE gave cells of the Th2 subtype (Reinhold et al., 1991) and allergen specific Th2 cells where derived from patch testing of AE patients with the allergen (Ramb-Lindhauser et al., 1991; van Reijsen et al., 1992). These T cells all produce high levels of IL-4 and low levels of IFN-\(\gamma\).

Along with the defects in IL-4/IFN-\(\gamma\) production, other cytokines have also been implicated in AE. TNF-\(\alpha\) has been shown to be decreased after PHA stimulation in AE compared to controls (Kapp et al., 1990; Takahasi et al., 1992). Another group has shown no difference in TNF-\(\alpha\) production compared to controls, however they found a significant correlation between TNF-\(\alpha\) production, IFN-\(\gamma\) production and IgE levels (Reinhold et al., 1989). Increased plasma TNF-\(\alpha\) concentration has also been found in AE (Sumimoto et al., 1992). Thus there is controversy over TNF-\(\alpha\) production and its role is still unclear. IL-10 production in the skin (Ohmen et al., 1995) and IL-6 (Toshitani et al., 1993) production by T cells have been showed to be
increased in AE whilst IL-2 (Kapp et al., 1991) and IL-1 by monocytes (Rasanen et al., 1987, Jakob et al., 1995) were decreased. Thus in AE there seems to be a fine balance in cytokine production.

Corticosteroids, one of the main treatments for AE, have been shown to inhibit the production of IL-4 from Th2 cells whilst cyclosporin also a treatment for AE is more effective at inhibiting IL-2 from Th1 cells (Schmidt et al., 1994). IL-5 was inhibited equally by both drugs (Mori et al., 1994). These treatments seem to target different populations of T cells and yet both are effective in AE. Thus it was decided to see if ZPT had an effect on cytokines which have been shown to be relevant in AE and if this cytokine production was linked to the inhibition of IL-4 induced CD23 expression.
5.2 RESULTS

5.2.1 Kinetics of cytokine production

Initial experiments were performed to establish the peak production of each cytokine to be measured. PBMCs from five normal individuals were cultured with concanavalin A (Con A) at a concentration of 10 μg/ml for a period of three days. Supernatants were taken at 24 hour intervals and tested for IL-4, IL-2, IFN-γ, IL-10, TNF-α and sCD23 production. Lymphocyte activation which was measured by tritiated thymidine incorporation showed that Con A stimulated cultures incorporated 68277 ± 10791.6 counts per minute (cpm). IL-2, IL-4 and TNF-α showed a peak in release after one day (Fig 5.2.1a, 5.2.1b). IFN-γ and IL-10 peaked after two days of incubation whilst sCD23 levels were at their maximum at three days (Fig 5.2.1b). In further experimentation, cytokines were measured at times of their maximum production.

Figure 5.2.1a  IL-2 and IL-4 production after three days stimulation of PBMCs with concanavalin A (5 individuals). Arrow indicates the day at which supernatant were taken for further experimentation.
Figure 5.2.1b  IFN-γ, IL-10, TNF-α and sCD23 production after three days stimulation of PBMCs with concanavalin A (5 individuals). Arrow indicates the day at which supernatant were taken for further experimentation.
5.2.2 The effect of ZPT on cytokine production in controls and AE patients

21 controls and 20 AE patients were studied for the effect of ZPT and PL on Con A induced cytokine production. The control group studied consisted of 11 males and 10 females with a mean age of 42.4 ± 10.4. The AE patients were diagnosed according to criteria described in the materials and methods. This group comprised of 7 males and 13 females with a mean age of 30.18 ± 8.3. The IgE levels in the control group ranged from 10 - 430 IU/ml (mean, 143 ± 25 IU/ml) whilst in the AE group from 420 - 10500 IU/ml (mean, 3929 ± 703 IU/ml).

5.2.2.1 Lymphocyte Activation

PBMCs were cultured at a concentration of 1 x 10^6 cells/ml with Con A (10\(\mu\)g/ml) with or without PL or ZPT. Tritiated ^[H] thymidine was added during the last 18 hours of culture. The Con A induced proliferation in the 21 controls decreased significantly from 38509 ± 2036 cpm to 32390 ± 2074 cpm when the cells were cultured with ZPT (p = 0.01) whilst with PL there was no significant change (37270 ± 2237). A similar result was seen with the 20 AE patients where Con A induced proliferation was 41533 ± 4490 cpm, ZPT 34907 ± 3704 cpm and PL 38316 ± 4079 cpm (Fig 5.2.2.1a). There was no difference in terms of their tritiated thymidine incorporation by Con A between AE and control subjects.

5.2.2.2 IL-2 production

The Con A induced IL-2 production in controls was 711 ± 182 pg/ml. This level was significantly decreased by PL (527 ± 93pg/ml) and ZPT (424.101 ± 95). However this decrease by the placebo and ZPT was not reflected in the AE patients where Con A induced IL-2 production was 882.07 ± 244 pg/ml, PL 723 ± 168 pg/ml and ZPT 742 ± 239 pg/ml (Fig 5.2.2.2a). There was no significant difference in the Con A induced IL-2 production by controls and AE patients. Cells cultured without Con A showed no IL-2 production with or without PL or ZPT.
Figure 5.2.1a (A) Proliferation in PBMCs from controls cultured with Con A without or with PL or ZPT. There was a significant reduction in proliferation when cells were cultured with ZPT.

(B) Proliferation in PBMCs from AE patients cultured with Con A without or with PL or ZPT. A similar reduction is seen as is shown in control subjects when cells were cultured with ZPT.
Figure 5.2.2a (A) Con A induced IL-2 production in controls. Both PL and ZPT showed a significant decrease on this production, $p = 0.01$ and $0.001$ respectively. (B) Con A induced IL-2 production in AE patients. There was no effect of PL or ZPT on this production.
5.2.2.3 IL-4 production

IL-4 production by Con A was $68 \pm 14.5 \text{ pg/ml}$ in controls and $68.1 \pm 10.1$ in AE patients i.e no difference between the groups. As seen in Figure 5.2.2.3a coincubation of ZPT with Con A showed a significant decrease in IL-4 production in both controls, $58.1 \pm 16.1 \text{ pg/ml}$ and AE patients, $58.2 \pm 10.1 \text{ pg/ml}$ ($p = 0.04$ and $p < 0.01$ respectively). There was no significant change in IL-4 levels when cells were cultured with PL.

5.2.2.4 IFN-γ production

Con A induced IFN-γ production was $818 \pm 159 \text{ IU/ml}$ in controls and $520 \pm 103 \text{ IU/ml}$ in patients with AE. Although the mean of IFN-γ production was less in the AE group compared to the controls this did not reach statistical significance. The PL or ZPT had no effect on IFN-γ production in either group studied (Fig 5.2.2.4a)

5.2.2.5 IL-10 production

PBMCs cultured without a stimulus produced detectable amounts of IL-10. In controls this production was $698 \pm 146 \text{ pg/ml}$ and in AE patients $895 \pm 234 \text{ pg/ml}$. This release of IL-10 was not affected by culturing with the PL. ZPT induced significantly higher levels of IL-10 in controls ($3452 \pm 413 \text{ pg/ml}$) and AE patients ($6435 \pm 506 \text{ pg/ml}$) (Fig 5.2.2.5a). When comparing controls to AE patients this production induced by ZPT was significantly higher in AE patients compared to controls ($p < 0.002$).

The Con A induced IL-10 production was $5034 \pm 498 \text{ pg/ml}$ in controls and was lower than in AE patients ($6150 \pm 935$) although not statistically significant (Fig 5.2.2.5b). This level was reduced by PL in both controls ($3804 \pm 390 \text{ pg/ml}$) and AE patients ($4677 \pm 546 \text{ pg/ml}$) ($p = 0.01$). When PBMCs were cultured with Con A and ZPT higher levels of IL-10 were produced in controls, $7324 \pm 608 \text{ pg/ml}$ and AE patients $10610 \pm 800 \text{ pg/ml}$ ($p < 0.002$). Again there was a significant difference between IL-10 induced by ZPT by controls compared with the AE patients ($p = 0.002$).
**Figure 5.2.3a** (A) Con A induced IL-4 production in controls. There was a significant effect of ZPT in decreasing the production of IL-4 (p = 0.04). (B) Con A induced IL-4 production in AE patients. ZPT significantly inhibited IL-4 production (p < 0.01).
Figure 5.2.2.4a (A) Con A induced IFN-γ in controls. PL and ZPT had no effect on this production.

(B) Con A induced IFN-γ in controls. PL and ZPT had no effect on this production.
Figure 5.2.2.5a (A) IL-10 production by unstimulated PBMCs from controls. ZPT significantly increased this IL-10 production (p < 0.001) whilst the PL had no effect.

(B) IL-10 production by unstimulated PBMCs from AE patients. PL had no effect whilst ZPT increased the production (p < 0.001).
Figure 5.2.2.5b (A) Con A induced IL-10 production by PBMCs from controls. ZPT significantly increased this IL-10 production (p < 0.001) whilst PL decreased this production (p = 0.03).

(B) Con A induced IL-10 production by PBMCs from AE patients. PL had no effect whilst ZPT increased the production (p < 0.001).
5.2.2.6 TNF-α production

Unstimulated PBMCs from controls released 112.1 ± 26 pg/ml of TNF-α whilst AE patients released 65.43 ± 112 pg/ml (Fig 5.2.2.6a). There was a significant increase in TNF-α production when cells were cultured with ZPT in controls, 799 ± 132.5 pg/ml and AE patients, 648 ± 112 pg/ml (p < 0.001). PL had no effect on TNF α production from control cells however there was a slight increase in production from AE cells (p = 0.01).

Although, the Con A induced TNF-α production was higher in controls (762 ± 144 pg/ml) than AE patients (472 ± 119 pg/ml), this was not statistically different (Fig 5.2.2.6b). However, ZPT increased Con A induced TNF-α production in controls (1990 ± 408 pg/ml) and AE patients (1341 ± 217 pg/ml) (p < 0.002).

5.2.2.7 sCD23 production

Similar amounts of sCD23 were found in unstimulated PBMCs from controls (5.14 ± 3.46 IU/ml) and AE patients (5.95 ± 4.76 IU/ml). Increased levels of sCD23 was found in the supernatants after stimulation with Con A. However cells from patients with AE produced higher levels of sCD23 (89.28 ± 8) compared to controls cells (63.68 ± 7.3) (p < 0.03). This production was unaffected by PL but ZPT decreased the levels in controls to 38.54 ± 8.9 IU/ml and AE patients to 48.7 ± 5.79 IU/ml (p < 0.001) (Fig 5.2.2.7a). Thus ZPT is able to suppress the production of sCD23.
Figure 5.2.2.6a (A) TNF-α production from unstimulated cells from controls. ZPT significantly enhanced TNF-α production compared with cells alone or cells with PL. (B) TNF-α production from unstimulated cells from AE patients. PL slightly increased TNF-α release however ZPT had a greater effect.
Figure 5.2.2.6b (A) TNF-α production from Con A stimulated cells from controls. ZPT significantly enhanced TNF-α production. (B) TNF-α production from Con A stimulated cells from AE patients. ZPT significantly enhanced TNF-α production.
Figure 5.2.2.7a (A) sCD23 production after Con A stimulation in controls. ZPT significantly inhibited sCD23 release whilst PL had no effect.

(B) sCD23 production after Con A stimulation in AE patients. ZPT significantly inhibited sCD23 release whilst the PL had no effect.
5.2.3 Time course induction of IL-10 and TNF-\(\alpha\) by ZPT

The kinetics of cytokine induction by ZPT was investigated. PBMCs from five normal individuals were cultured with or without ZPT or placebo extract (1 mg/ml) and supernatants taken at 1, 2, 4, 8, 12 and 18 hours to measure the IL-10 and TNF-\(\alpha\) concentrations.

5.2.3.1 IL-10

ZPT induced IL-10 production after 8 hours of culture whilst there was no significant production by PL or cells cultured alone. This production increased in a time dependant manner (Fig 5.2.3.1a).

![Figure 5.2.3.1a](image)

*Figure 5.2.3.1a* Time course induction of IL-10 by ZPT. Cells only (○), cells + PL (■) and cells + ZPT (●). IL-10 production began after 8 hours culture with ZPT.

5.2.3.2 TNF-\(\alpha\)

TNF-\(\alpha\) production appeared 1 hour after culturing with ZPT and peaked at 18 hours (Fig 5.2.3.2a). However there was plateau of production between 4 and 8 hours with an increase again at 18 hours. As a mixed population of cells was used this could
indicate that different populations of cells were being induced to produce TNF-α at different times. There was no significant release with cells cultured alone or with PL.

**Figure 5.2.3.2a** Time course induction of TNF-α by ZPT. Cells only (○), cells + PL (■) and cells + ZPT (●). TNF-α production started after 1 hour culturing with ZPT.

### 5.2.4 Induction of IL-10 and TNF-α from semi-purified monocytes by ZPT

As IL-10 and TNF-α are produced by a variety of cells including monocytes (de Waal Malefyt et al., 1991) the ability of ZPT to induce these cytokines in monocytes was investigated. Monocytes (2 x 10⁶/ml) were obtained as previously described and cultured for eighteen hours with different concentrations of ZPT and PL (1mg/ml). Monocytes cultured alone released 654 ± 247 pg/ml of IL-10. This production was increased in a dose dependent fashion by ZPT reaching 5411 ± 2169 pg/ml at a concentration of 1mg/ml. There was a slight decrease in IL-10 production when the monocytes were cultured with PL (Fig 5.2.4a).
Figure 5.2.4a  Induction of IL-10 from semi-purified monocytes by different concentrations of
ZPT (●). PL (1mg/ml) extract is shown as control (○).

TNF-α release from monocytes was 62 ± 30 pg/ml and this increased in a dose
dependant manner peaking at 1580 ± 221 pg/ml when the cells were cultured with
ZPT (Fig. 5.2.4b). There was no change in TNF-α production when the cells were
cultured with PL.
Figure 5.2.4b  Induction of TNF α from semi-purified monocytes by different concentrations of ZPT (●). Placebo (1mg/ml) extract is shown as control (○).

5.2.5  Relationship between ZPT and LPS

Human PBMCs and isolated monocytes produce TNF α (Morrison & Ryan 1987) and IL-10 (de Waal Malefyt et al., 1991) when activated with lipopolysacchride (LPS). LPS is found in the bacterial coat of gram negative bacteria and the antibiotic polymyxin B has been shown to bind to LPS and inhibit its action (Morrison & Jacob 1976). The possibility that ZPT was contaminated with bacteria during the extraction and therefore contained LPS was investigated.

5.2.5.1  PBMCs

LPS was mixed with polymyxin B (20µg/ml) and then added to PBMCs (n = 3) as a control to see the effect on TNF α and IL-10 production. Polymyxin B abrogated TNF α and IL-10 production by LPS at all concentrations except the highest concentration (100ng) where 93% of the cytokine production was inhibited (Fig 5.2.5.1a).
PBMCs (n = 6) were cultured with ZPT or PL (1mg/ml) with or without polymyxin B (20μg/ml) to check if ZPT contained LPS. Polymyxin B had no significant effect on TNF-α and IL-10 production by ZPT (Table 5.2.5.1a). At these levels of cytokine production polymyxin B abolished the action of LPS. (Fig 5.16). PL or cells alone did not produce either cytokine. Thus from the results, ZPT is not contaminated with LPS and therefore components in this herbal mixture must be responsible for the cytokine induction.

Table 5.2.5.1a Effect of polymyxin B on cytokine production induced by ZPT. There was no significant change in cytokine production thus excluding bacterial contamination as a cause of IL-10 and TNF-α production.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>- Polymyxin B (pg/ml)</th>
<th>+ Polymyxin B (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>2152 ± 816</td>
<td>1950 ± 701</td>
</tr>
<tr>
<td>IL-10</td>
<td>1725 ± 469</td>
<td>1654 ± 408</td>
</tr>
</tbody>
</table>
U937 cells

U937 cell lines from various laboratories have shown different responses to LPS in their cytokine production. Activation with PMA or GM-CSF has been necessary for the LPS induction of TNF-α (Cannistra et al., 1987) on some sublines whilst other responded to LPS alone (Ucla et al., 1990). The U937 subline cultured within the laboratory showed a small production of TNF-α 144.5 ± 124.2 pg/ml (n=4) when stimulated with LPS at 100ng/ml for 18 hours. The LPS induced production was totally abolished with polymyxin B. There was no constitutive cytokine production or IL-10 production when the cells were cultured with LPS. ZPT or PL did not induce TNF-α or IL-10 from U937 cells when cultured overnight. When the cell line was stimulated with PMA (50ng/ml) overnight there was stimulation of TNF-α production of 2750 ± 141 pg/ml but no IL-10 production. LPS at 100ng/ml did not influence this production. However there was an increase to 3500 ± 191 pg/ml when the cells were cultured with PMA and ZPT (p < 0.003). This increase was not affected by also culturing with polymyxin B, 3450 ± 263pg/ml. Again there was no IL-10 production by these cells.

5.2.6 Relationship between inhibitory effects of ZPT on CD23 expression and cytokine production

As both IL-10 (Spittler et al., 1995) and TNF α (Gessl et al.,1993, Hashimoto et al.,1995) have been shown to down regulate IL-4 induced CD23 on purified monocytes, the inhibition of CD23 by ZPT was investigated to see if this was due to the release of these two cytokines.

5.2.6.1 The effect of an anti IL-10 antibody on ZPT inhibition

PBMCs (n=6) were cultured with IL-4 and ZPT or PL as previously described except with the addition of a mouse monoclonal against IL-10 at different concentrations. As seen in Fig 5.2.6.1a the antibody against IL-10 had no effect on the ZPT inhibition of IL-4 induced CD23 expression on monocytes. The antibody at a concentration of 10μg/ml reduced the IL-10 production by ZPT from 6075 ± 765 pg/ml to 2880 ± 1327 pg/ml. There is a possibility that this level of IL-10 present could still have biological activity thus a dose response curve with ZPT and anti IL-10 may need to done to confirm this data.
5.2.6.2  The effect of an anti TNF-α antibody on ZPT inhibition

A mouse monoclonal anti TNF-α antibody was added to the culture system to see if TNF-α was responsible for the inhibition produced by ZPT. The antibody had little effect on CD23 expression on monocytes whether the cells were cultured with IL-4 alone or in the presence of IL-4 with the placebo extract. However adding the antibody to cells cultured with IL-4 and ZPT, partially reversed the inhibition seen with ZPT (Fig 5.2.6.2a). The TNF-α release by ZPT was 1971 ± 699 pg/ml and this was reduced to 688 ± 255 pg/ml with the anti TNF antibody at its highest concentration (10ug/ml). The IL-10 production by ZPT was unaffected.
5.2.6.2a (A) The effect of an anti TNF-α antibody on IL-4 induction of CD23 on monocytes. IL-4 only (□), IL-4 + PL (○) and IL-4 + ZPT (●).

(B) The effect of different concentrations of an anti TNF-α antibody on the inhibition of CD23 by ZPT. IL-4 (□), IL-4 + PL (○) and IL-4 + ZPT (●). The antibody reduced the inhibition caused by ZPT from a maximum of 70% to less than 40%.

5.2.7 The effect of TNF-α and IL-10 on IL-4 induced CD23 expression on monocytes

When PBMCs were cultured with IL-4 and different concentrations of TNF-α (n = 6) or IL-10 (n = 4), both cytokines showed no effect on IL-4 induced CD23 expression (Table 5.2.7a). This is in contrast to the data with the anti TNF α antibody which indicated that TNF-α could partially be responsible for the inhibitory effect of ZPT.
Table 5.2.7a  Effect of different concentrations of TNF-α and IL-10 on IL-4 induced CD23 expression on monocytes. There was no significant effect of either cytokine.

<table>
<thead>
<tr>
<th>Concentration ng/ml</th>
<th>IL-4 induced CD14+CD23+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
</tr>
<tr>
<td>0</td>
<td>45.20 ± 7.7</td>
</tr>
<tr>
<td>0.1</td>
<td>49.10 ± 7.0</td>
</tr>
<tr>
<td>1.0</td>
<td>49.85 ± 7.7</td>
</tr>
<tr>
<td>10.0</td>
<td>43.34 ± 6.2</td>
</tr>
</tbody>
</table>
5.3 DISCUSSION

An imbalance of cytokine production has been thought to be a major feature in AE with a predominance of Th2 cytokines. In the data presented there was no significant difference between the Con A cytokine production between AE patients and controls. Many authors have shown an increase in IL-4 (Rousset et al., 1991; Jujo et al., 1992; Renz et al., 1992) and a concomitant decrease in IFN-γ (Reinhold et al., 1988; Reinhold et al., 1990; Rousset et al., 1991; Jujo et al., 1992; Tang et al., 1993). However in relation to IL-4 and IFN-γ the results obtained were more in line with those of Takahashi et al., 1992 and Simon et al., 1995 whereby although the trend for decreased IFN-γ and increase IL-4 was seen in AE the result did not reach statistical significance. The patients in this study had a range of disease from moderate to severe and a clear distinction between cytokine production in patients with AE and control subjects was not found. With respect to IL-4 and IFN-γ, a clear cut result may have been obtained if a homogenous population of very severely affected patients was used. There was an increased production of sCD23 from AE patients compared to controls when activated with mitogen as has been previously described (Kim et al., 1987; Kicza et al., 1989).

The purpose of this study was to investigate the effect of ZPT on cytokine production. These data have shown that culturing PBMCs with Con A and ZPT decreased both IL-4 and sCD23 and enhanced IL-10 and TNF-α production. There was no modulation of Con A activation of the other cytokines either by ZPT or PL. If the ability of ZPT to decrease IL-4 production is mirrored in vivo then the imbalance between IFN-γ and IL-4 seen in AE would be redressed leading to switch from Th2 to a Th1 or Th0 profile. Although a change in IL-4 levels may be favourable in AE, IL-13 has been shown to have similar effects to IL-4 (Zurawski & de Vries 1994). Thus a concomitant reduction in IL-13 by ZPT would also be necessary. Studies of mRNA of cytokine production in biopsies of lesional skin of AE patients before and after treatment with ZPT would give a clearer indication of the change in the T cell infiltrate. The reduction in sCD23 seen may be a secondary phenomenon due to the changes in IL-4 production.

The other two major changes of cytokine production which were induced by ZPT were on IL-10 and TNF-α production. This production was irrespective of mitogen activation and there was a time dependant release of both cytokines. As PBMCs
were a mixed cell population, purified monocytes (80% CD14+) were investigated and it was demonstrated that they released both cytokines in a dose dependant fashion in response to ZPT but not PL. The obvious explanation for the induction of these two cytokines was that in the extraction process, ZPT had become contaminated with LPS. Polymyxin B an antibiotic is known to bind to LPS (Morison & Jacob 1976). When ZPT was mixed with this antibiotic there was only a slight decrease in IL-10 and TNF-α. The concentration of polymyxin B (20μg/ml) used abolished 93% of the release of both cytokines by LPS at 100ng/ml and 100% at lower concentrations of LPS. ZPT induced the similar levels of IL-10 and TNF-α as 20ng/ml of LPS. ZPT but not LPS superinduces TNF-α production when U937 cells were activated with PMA. PL was produced in the same manner as ZPT and did not induce either cytokine, however this does not totally rule out the presence of LPS and a test such as the Limulus amebocyte lysate assay may need to be performed on both extracts.

In a previous chapter it has been shown that ZPT inhibited IL-4 and IL-13 induced CD23 expression. Results given in this chapter suggest that TNF-α but not IL-10 might be responsible for this inhibition. TNF α has been shown to downregulate mRNA for CD23 and increase sCD23 production from monocytes. (Hashimoto et al., 1995). The results presented for sCD23 release from monocytes (Fig 4.2.3.1b) showed that there was an inhibition of sCD23 but only at the highest concentration of ZPT. Also the mRNA for CD23 in monocytes was slightly decreased by ZPT (Fig 4.2.6b). However, in the experiments done by Hashimoto et al. their maximum sCD23 enhancement was after three days of culturing with IL-4 and TNF-α whilst the supernatants in the results presented were taken after eighteen hours (Hashimoto et al., 1995). Likewise for mRNA for CD23, the maximum inhibition was seen after 24 hours. Thus further experimentation with ZPT at the time points described above would confirm if the induction of TNF-α is responsible for inhibiting CD23 expression. In this same study they showed that TNF-α had no effect on IL-4 induced CD23 expression on monocyte depleted PBMCs. This could explain why ZPT had no effect on EBV transformed B cells or purified B cells (Fig 4.2.5a & Table 4.2.5.1a).

IL-10 (Spittler et al., 1995) and TNF-α (Gessl et al.,1993; Hashimoto et al.,1995) have been shown to down regulate IL-4 induced CD23 on purified monocytes however when either cytokine was used with PBMCs there was no inhibition of IL-4 induced CD23 expression. Even when both cytokines were used at their highest
concentration (10ng/ml) in the same culture system there was no effect on IL-4 induced CD23 expression. This must imply that another factor is being induced by ZPT which is synergising with TNF-α to reduce CD23 expression. IL-6 would be a probable candidate as it has been shown to reduce IL-4 induction of CD23 on purified monocytes (Willheim et al., 1991). This possibility would have to be investigated on PBMCs and monocytes. The Con A proliferative response of PBMCs from AE patients and controls showed a small but significant reduction when ZPT but not PL was added to the culture system. This reduction could be due to the release of IL-10 as it has been shown to inhibit PHA-induced T cell proliferation (Spittler et al., 1995). IL-10 has been shown to inhibit IFN-γ production (Fiorentino et al., 1989) but there was no change in Con-A induced IFN-γ production when cells were also cultured with ZPT. Perhaps, suboptimal doses of Con A might have shown a difference in IFN-γ production. The missing data in the induction of these two cytokines by ZPT is the upregulating of their mRNA in monocytes. This would obviously have to be pursued.

These results may point to the targetting of monocytes in the treatment of AE. The combination of release of IL-10, TNF-α and maybe other cytokines at the site of inflammation by components in ZPT may be important in the improvement seen when patients are treated with this therapy. To obtain a better understanding of this treatment it will be important to study the immunological changes that occur when patients are being treated.
Chapter 6

Association of immunological changes with clinical efficacy in patients with AE treated with ZPT
6.1 Introduction

The aetiology of AE is not known, treatment is empirical and based on topical steroids which abates the immunopathology of the skin. In more severe cases systemic steroids, cyclophosphamide, azathioprine and phototherapy have been used (Morrison & Schulz 1978; Morrison et al., 1978).

In recent years newer approaches have been used to target specific defects. One of the main features of AE is a raised IgE level; IFNγ has been shown to suppress IL-4 induced IgE production in vitro (Pene et al., 1988). Thus immune intervention with IFNγ has shown encouraging results (Boguniewicz et al., 1990; Hanifin et al., 1993; Reinhold et al.; 1993, Pung et al., 1993). The infiltration of T cells is thought to play a central role in the disease with most groups demonstrating a preponderance of Th2 like cells (Reinhold et al., 1991; Ramb-Lindhauser et al., 1991; Van der Heijden et al., 1991; Sowden et al., 1992, ). However paradoxically Grewe et al., have recently shown a Th1 type response with increased levels of mRNA for IFNγ in the skin of AE patients which decreases following treatment (Grewe et al., 1994).

Cyclosporin is mainly immunosuppressive for T cells by blocking the calcium dependant pathways leading to the induction of various cytokine genes including IL-2, IL-4 and IFNγ (Rao 1994). This drug has been used successfully in the treatment of AE (Sowden et al., 1991; Joost et al., 1994; Granuland et al., 1995) although due to the various side effects long term treatment is not recommended.

Despite these therapeutic approaches, there are still patients who are unresponsive to all these forms of treatments. Two double blind placebo controlled trials and a long term study have demonstrated the efficacy and safety of a traditional Chinese herbal therapy (ZPT) in such patients (Sheehan et al., 1992a, Sheehan et al., 1992b, Sheehan et al., 1994). This is a new treatment which may have novel pharmacological actions or include a range of therapeutic chemicals which intervene in several points in the inflammatory process.

A number of factors contribute to the damage seen in the skin of AE patients and some of the serological and cellular changes in the peripheral blood can correlate with disease severity. Recently a soluble form of the intercellular adhesion molecule (ICAM) has been found to be raised in the serum of patients with AE and could reflect the increased trafficking of cells into the skin. The vascular adhesion molecule (VCAM) binds basophils, eosinophils and monocytes and is induced by IL-4 and IL-13 (Schleimer et al., 1992; Meerschaert & Furie 1995; Bocher et al., 1995). A
soluble form of VCAM is also found in the serum (Wellicome et al., 1993) and thus the participation of these adhesion molecules was investigated. sCD23, surface CD23 on cells, IgE and IgE complexes are all raised in AE and may be involved in antigen processing and mediator release in the skin. The rationale behind this study was to investigate these immunological changes when patients undergo a specific Chinese herbal therapy treatment to give an insight into the mechanism of actions of the herbs and therefore the disease process. This chapter covers the findings of this study.
6.2 RESULTS

6.2.1 Treatment
The original decoction was made from finely ground herbs which were packaged in sealed porous sachets (Sheehan et al., 1992a; Sheehan et al., 1992b; Sheehan et al., 1994). The new palatable form was made from a water extract of the herbal mixture which was freeze dried to granules.

6.2.2 Patients
Forty eight patients with AE took part in an open study which assessed a new palatable form of freeze dried granules of ZPT compared to its original decoction prepared from the ground herbs. Eighteen of these patients were randomly chosen to take part in this study. This patient group consisted of 11 males and 7 females with a mean age of 33.4 ± 13 (mean ± SD). Blood samples were taken at 0 and 8 weeks after treatment from all patients. The skin was scored at each visit using a system described in the materials and methods. All the patients had moderate to severe disease which had been recalcitrant to previous treatment.

6.2.3 Controls
The control group consisted of 13 males and 6 female healthy volunteers with no personal or family history of atopy and a mean age ± SD was 34.84 ± 12.25. The Wilcoxon ranked signed test was used to compare the various parameters before and after treatment. The unpaired Student's t-test was used to analyse differences between controls and patients. Graphs show the data from individuals as well as the mean ± SE.

6.2.4 Dermatological and Immunological changes after treatment with ZPT

6.2.4.1 Erythema and Surface Damage
There was a significant improvement in surface damage (A) and erythema (B) in the 18 patients who took part in this study (Fig 6.2.4.1a). 10 out of eighteen patients were given the original decoction of traditional Chinese herbal therapy while 8 received a more palatable formulation of freeze dried granules. The groups showed
no difference in clinical outcome whether receiving either formulation. This has been confirmed by the result of the larger open clinical trial (Banjee & Rustin 1994). Thus data for the eighteen patients was analysed as one group.

![Figure 6.2.4.1a Erythema (A) and surface damage (B) in eighteen patients before and after treatment with ZPT. There was a significant reduction in both parameters (p < 0.001).](image)

### 6.2.4.2 Total IgE

All the patients had elevated levels of serum IgE, $5640 \pm 1036$ IU/ml (mean ±SEM) at the beginning of the study. After eight weeks of treatment there was no significant difference in the total IgE level, $7104.72 \pm 1813$ IU/ml (Fig 6.2.4.2a). Seven of the patients showed a decrease whilst the rest remained unchanged or increased.
6.4.2.3 IgE complexes

There was a significant difference in the levels of IgE containing complexes between controls and patients (p = 0.001) (Fig 6.4.2.3a). Complexes in patients showed a fall from 64.11 ± 8.22 IU/ml to 47.86 ± 6.74 IU/ml after 8 weeks of treatment (p = 0.045).

Figure 6.2.4.2a Serum IgE in controls and patients before and after treatment. There was no change in the IgE levels in patients.
6.2.4.4 Monocytes expressing CD23
6.2.4.4.1 In vivo expression
The percentage of circulating monocytes coexpressing CD23 and CD14 was 4.55 ± 1.10 before treatment and 3.51 ± 2.38 after treatment. There was no difference between controls and AE patients and the values were not affected by treatment (Fig 6.2.4.4.1a). There was no correlation between patients who showed a fall in the percentage of circulating monocytes expressing CD23 and clinical improvement.
Figure 6.2.4.4.1a CD23 expression on monocytes of controls and patients before and after treatment. There was no difference in the expression of CD23 on monocytes of controls or patients and there was no effect of treatment.

6.2.4.4.2 IL-4 induction of CD23 on monocytes

PBMCs were cultured overnight with IL-4 (200U/ml) and then analysed for monocytes coexpressing CD23 and CD14. In Fig 6.2.4.4.2a it is seen that the IL-4 induced % CD14+CD23+ cells was significantly reduced from 51.63 ± 3.29 to 39.73 ± 4.23 after 8 weeks of herbal treatment (p < 0.01).
Figure 6.2.4.4.2a  IL-4 induced CD23 expression on monocytes of controls and patients. The inducibility of CD23 by IL-4 was reduced after eight weeks of treatment (p<0.01).

6.2.4.4.3  The effect of ZPT on CD23 expression before and after in vivo treatment

PBMCs were cultured with IL-4 and ZPT and placebo extracts at 1mg/ml and analysed for CD23 expression on monocytes at time 0 and 8 after weeks treatment with ZPT. There was a significant inhibition by ZPT at both time points (p = 0.002) but this was not affected by in vivo treatment with ZPT (Fig 6.2.4.4.3c).
Figure 6.2.4.3c In vitro inhibition of ZPT (■) and PL (□) on IL-4 induced CD23 expression on monocytes before and after 8 weeks of treatment with ZPT. There was no significant difference between the inhibition before and after treatment.

6.2.4.5 sCD23

sCD23 levels in the serum of patients was kindly measured by Ms A Katira in Prof. J Gordon’s laboratory. Although the soluble CD23 levels in serum were elevated in two-thirds of the atopic eczema patients, no change was detected following treatment (Fig 6.2.4.5a).
Figure 6.2.4.5a  sCD23 levels in the serum of patients before and after eight weeks of treatment with ZPT. The levels were unaffected by treatment.

6.2.4.6 Soluble Interleukin 2 receptor (sIL-2R)

It has previously been shown that sIL-2R correlates with disease activity and decreased on treatment with corticosteroids (Colver et al., 1989). It was confirmed that patients with atopic eczema have a raised sIL-2R level compared with controls (p < 0.002) (Fig 6.2.4.6a). Patients undergoing treatment showed significantly decreased levels from 4070.59 ± 467.68 pg/ml to 3277.78 ± 431 pg/ml (p < 0.05). However, these values were still above those of the controls (2310.53 ± 226.32 pg/ml).
Figure 6.2.4.6a  Soluble interleukin 2 receptor (sIL-2R) in controls and patients after treatment with ZPT. Post treatment levels are decreased compared to pre treatment levels in AE patients (p < 0.05). There is a clear statistical difference between AE patients and controls (p < 0.002).

6.2.4.7  Soluble vascular adhesion molecule (sVCAM)

sVCAM is raised in a variety of diseases including rheumatoid arthritis (Wellicome et al., 1993). The potential part that it might play in atopic eczema and the effect on treatment was examined. sVCAM was significantly raised compared to controls (p < 0.001) and significantly diminished from 1011.11 ± 82ng/ml to 856.94 ± 79.98 ng/ml after 8 weeks of treatment (p < 0.05) (Fig 6.2.4.7a). Again the post treatment values were still slightly higher than the controls (653.10 ± 29 ng/ml).
Figure 6.2.4.7a Soluble vascular cellular adhesion molecule (sVCAM) in the serum of AE patients and controls. AE patients have raised levels of sVCAM compared to control individuals ($p < 0.001$). After treatment with the Chinese herbal mixture there is a significant fall in this level ($p < 0.05$).

6.2.4.8 Soluble Intercellular adhesion molecule (sICAM)

The serum levels in atopic patients (296 ± 62 ng/ml) showed no difference from the normal levels established by the manufacturers of the ELISA kit used (210.6 ng/ml) and there was no change after treatment, 250.2 ± 20.1 ng/ml (Fig 6.2.4.8a).
Figure 6.2.4.8a Soluble intercellular cellular adhesion molecule (sICAM) values in the serum of patients before and after treatment with ZPT. There was no significant change and the levels in the serum did not differ from levels found in controls.
6.3 DISCUSSION

In data presented previously ZPT has been shown to inhibit CD23 expression on monocytes and induce IL-10 and TNF-\(\alpha\). This current study now reveals immunological changes in patients undergoing treatment with this preparation. The parameters that were significantly altered by the in vivo treatment were levels of IgE complexes, sIL-2 receptor, sVCAM and the inducibility of CD23.

There was a significant decrease in IgE complexes after treatment. In this research the nature of these complexes was not analysed but it would not be difficult to speculate that these were IgE complexed to anti IgE antibody which have previously been described (Nawata et al., 1985; Quinti et al., 1986; Marone et al., 1989). Czech et al., 1995 showed no change in the IgE complexes after treatment of AE patients. However this group analysed these complexes after 2-3 weeks of treatment and suggested that this might have been too short a duration to observe a change in the IgG anti IgE antibodies as the half life of IgG antibodies is about 3 weeks. This decrease in complexes detected could lead to less complexed IgE binding to mast cells, B cells, eosinophils and monocytes through their IgE receptors and therefore a diminution in mediator production which is known to exacerbate skin damage.

Patients being treated with ZPT did not show any reduction in total IgE in spite of a significant reduction in the extent of their eczema as judged by scoring of erythema and surface damage. Other studies in patients with AE undergoing treatment with corticosteroids, azathioprine (Johansson & Juhlin 1970), cyclosporin A (Munro et al., 1991) and even IFN-\(\gamma\) (Weindel et al., 1994) also failed to show an immediate fall in total serum IgE levels in spite of a significant reduction in the extent of their eczema as judged by scoring of erythema and surface damage. Other studies in patients with AE undergoing treatment with corticosteroids, azathioprine (Johansson & Juhlin 1970), cyclosporin A (Munro et al., 1991) and even IFN-\(\gamma\) (Weindel et al., 1994) also failed to show an immediate fall in total serum IgE levels in spite of a significant reduction in the extent of their eczema as judged by scoring of erythema and surface damage.

Another prominent feature in AE is the upregulation of the CD23 on PBMCs and Langerhans cells in the skin of patients with AE (Melewicz et al 1981; Nakamura et al., 1981; Buckley et al., 1992). In particular, the levels of CD23 on monocytes have correlated with superoxide production (Polla et al., 1992) and induction of nitric oxide (Mossalayi et al., 1994). In this study the results have shown two different effects of treatment on CD23 expression on peripheral blood monocytes. Firstly, the percentage of circulating monocytes expressing CD23 following treatment was unchanged, although the expression of CD23 on monocytes in this group of patients was much lower than previously described (Nakamura et al., 1991). Secondly, the level of IL-4 induced CD23 expression on monocytes in vitro was significantly reduced after treatment. This indicates that treatment is able to block IL-4
induction of CD23. This is in accordance with the finding that ZPT but not PL, can inhibit IL-4 induced CD23 expression in vitro. The in vivo treatment with ZPT did not effect its in vitro inhibition which may indicate that this treatment is inducing factors like cytokines which influence the immune system rather than altering the monocytes per se. Although CD23 on blood monocytes and sCD23 in the serum were not affected, biopsy data in other studies with this treatment have shown that CD23 is downregulated on antigen presenting cells in the skin of treated patients (Xiou et al submitted for publication). This might suggest that this therapy is directed at the site of inflammation where the cells are activated rather than acting as a general immunosuppressant.

Activated T cells express the IL-2 R, of which the α chain can be shed and maintain its affinity for IL-2 (Rubin et al., 1986). This soluble form (sIL-2R) can be detected in the serum of AE patients. The level of sIL-2R correlates with disease activity and is reduced after treatment with corticosteroids (Colver et al., 1989, Wuthrich et al., 1990). The results presented do show a small but significant decrease in sIL-2R in the serum of patients on treatment. Shed IL-2R is a reflection of surface expression thus a reduction in this marker may mirror a decrease in activated T cells either as a primary or secondary phenomenon.

For the first time it has been shown that sVCAM levels are significantly raised in patients with AE compared with controls. There is also a reduction in sVCAM after 8 weeks of treatment. As VCAM is an adhesion molecule which preferentially binds basophils and eosinophils and is induced by IL-4, it may have a potential role in allergic reactions (Schleimer et al.,1992). The relationship between the expression of VCAM and the soluble form is not known but the decrease in sVCAM in the serum could be a reflection of downregulation of adhesion molecules on the endothelial cells and therefore a reduction in cells entering the skin. sICAM has recently been implicated as a marker for AE with levels decreasing on treatment with topical steroids (Kojima et al., 1994; Wuthrich et al., 1995). In this study no difference was seen between sICAM levels in controls and patients and there was no change following treatment. Wuthrich et al., 1995 has suggested that ICAM may be a new clinical marker for AE, however our results do not support that conclusion. These data suggest that sVCAM may be more relevant as a diagnostic marker. To justify the relevance of our observations it will be necessary to show that VCAM and IL-2R are downregulated in the skin biopsies of AE patients after treatment.
This study has confirmed the efficacy of ZPT in patients with AE who have been unresponsive to standard Western Medicine. The treatment consists of a mixture of ten herbs of which many of the components may be unnecessary. The final aim must be the isolation of a compound or compounds which are responsible for the in vivo efficacy.
Chapter 7

Isolation and purification of factor(s) which inhibit CD23 expression on monocytes
7.1 INTRODUCTION

Two compounds used in treatment of atopic diseases, theophylline and disodium chromoglycate originated from plants. Theophylline, from the plant *Theo sinensis* belongs to the family of xanthines and is known for its ability to inhibit the PDE action (Butcher & Sutherland 1962). Its property as a bronchodilator has lead to its use in asthma. Patients with AE have high levels of PDE (Grewe et al., 1982; Chan et al., 1982; Sawai et al., 1995) but surprisingly some patients with AE and asthma only see an improvement in their asthma when treated with theophylline. (Giustina et al., 1984). However unpublished observations from Hanifin & Tofte suggest that other PDE inhibitors might be efficacious in AE (Hanifin & Chan 1995).

Disodium cromoglycate (DSCG) a member of the chromone family was developed to improve on the benzopyrone bronchodilator khellin which was isolated from *Amni visnaga* (Shapiro & Konig 1985). However DSCG is devoid of the bronchodialatory effects but prevents mast cells from releasing their mediators. This property may be due to its ability to reduce intracellular calcium in sensitized mast cells (White et al., 1984). DSCG is used in the prophylactic treatment of asthma but has been shown by one group to be effective in young children with AE when applied to the skin (Kimata & Igarashi, 1990).

ZPT is a standardized aqueous extract consisting of plant materials from ten individual herbs. Separation of this mixture by thin layer chromatography reveals that it consists of many constituents (Fig 7.1a). The individual herbs have been shown to have a variety of activities; these include anti-bacterial, anti fungal, PAF inhibition and non steroid anti-inflammatory actions (Chang & Butt 1987). Particular compounds in this mixture have had a long history of research, this includes glycyrrhetinic acid found in *Glycyrrhiza uralensis* which is a potent inhibitor of 11β hydroxysteroid dehydrogenase (Kumagai et al.,1966). This enzyme is responsible for the conversion of cortisol to cortisone. Recently, glycyrrhetinic acid has been found to have an effect on the immune system by enhancing the IFN-γ production from human lymphocytes (Shinda et al., 1986) and IL-2 production by mouse spleen cells (Zhang et al.,1993; Zhang et al.,1995). Another compound found in ZPT, paeoniflorin (*P.lactiflorin*) has been shown to inhibit binding of steroids to their receptors and may positively or negatively influence steroid levels through these receptors (Tamaya et al., 1986).
Many acidic polysaccharides have also been isolated from the individual herbs in ZPT. These polysaccharides consist mainly of four monosaccharides L-arabinose, D-galactose, L-rhamnose and D-galacturonic acid, varying in the content of each one. Two acidic and one neutral polysaccharide have been isolated from *Paeonia lactiflora* (Tomoda et al., 1993; Tomoda et al., 1994). Glycyrrhizans UA and UB, saposhnikovan A and C, rehmannan SA and SB have all been isolated from *Glycyrrhiza uralensis*, *Ledebouriella seseloides* and *Rehmanniae glutinosa* respectively (Tomoda et al., 1990; Shimizu et al., 1989a; Shimizu et al., 1989b; Tomoda et al., 1994). All these polysaccharides and glycosides increase carbon clearance in mice and it has been suggested that they have potentiating activity on the reticuloendothelial system. Along with the aforementioned compounds, alkaloids, glycosides and tannins have been isolated from the ten herbs of which the mechanisms of actions have not yet been defined (Huang 1993).

AE patients treated with ZPT showed no change in renal or hepatic function tests after long term treatment (Sheehan et al., 1994) but there are still fears concerning the toxicity of Chinese herbs (Chan et al., 1993). Thus there is a need to isolate the active component(s). Due the role of CD23 in AE it was decided to isolate the component(s) which inhibited CD23 expression.
7.2 RESULTS

7.2.1 The effect of Glycyrrhetic acid and paeoniflorin on IL-4 induced CD23 expression on monocytes

As glycyrrhetic acid and paeoniflorin are two of the known components in ZPT, their effect on the induction of CD23 on monocytes was tested to establish if these were the active components responsible for the inhibitory effect of ZPT (n=6). As seen in Fig 7.2.1a both compound showed little effect on CD23 expression on monocytes.

Figure 7.2.1a The effect of glycyrrhetic acid (O) and paeoniflorin (Δ) on IL-4 induction of CD23 on monocytes. Neither compound had any effect on CD23 expression.

Therefore it can be concluded that glycyrrhetic acid and paeoniflorin have no inhibitory effect on IL-4 induced CD23 expression and are unlikely to be the active components of ZPT.

7.2.2 The effect of the separate herbs in ZPT on CD23 expression

As ZPT consists of ten separate herbs the individual herbs were examined for their contribution to the inhibitory effect on IL-4 induced CD23 expression. Water extracts
of the ten herbs were made at Phytopharm and then were dissolved in RPMI at concentrations equivalent to that in the whole mixture as described in the materials and methods. Fig 7.2.2a shows the mean of five experiments where 4 out of the 10 herbs, *Dictamnus dasycarpus*, *Glycyrrhiza uralensis*, *Paeonia lactiflora* and *Schizonepeta tenuifolia* were shown to have the largest inhibitory effect.

All four of the highest herbal extracts seem to have the similar inhibitory effect as the original mixture (ZPT), so they were diluted out to see if a difference could be seen at lower concentrations. The starting concentration of the four herbal extracts was the same as was found in ZPT and doubling dilutions were made. All four extracts showed dose dependant response curves (Fig 7.2.2b).

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**Figure 7.2.2a** The inhibitory effects of the ten separate herbs of ZPT on IL-4 induced CD23 expression on monocytes. The ten individual herbs were identified by their botanical names.
Figure 7.2.2b  Dose response curves of four herbal extracts that are responsible for CD23 inhibition in ZPT. Error bars have been removed for ease of viewing the diagram.

7.2.3  Separation of the active molecule(s) from ZPT which inhibit CD23 expression

Using a Biorad Protein estimation kit with BSA as a standard it was determined that ZPT and PL contained 980 ng/ml and 1250ng/ml of protein respectively per 1 mg of dried extract. It is unlikely that this proteinacious material present had biological activity since the extraction process included boiling. Thus the presence of other types of molecules was investigated.

The isolation of active components from ZPT (performed in collaboration with Dr Alan Jaques and Prof Tom Rademacher)

7.2.3.1  Isolation of components from ZPT by Paper Chromatography

ZPT was spotted onto cellulose paper and descending paper chromatography
eluting with butanol:ethanol: water (4:1:1) was performed. Paper segments were eluted with methanol and then eluted with water. The samples were taken to dryness and then redissolved in the original volume. The different fractions were analysed for their ability to inhibit IL-4 induced CD23 on monocytes. Two major peaks were identified in the water eluted fractions (W) which were inhibitory (Fig 7.2.3.1a). The first peak is at the origin of the paper chromatography (W1) and the second runs 11-13 cms from the origin (W2). The results represent three independent experiments on IL-4 induced CD23 expression. The paper chromatography was repeated three times and similar profiles were obtained each time. However it was not clear where the peak of the inhibitory activity was in this profile and further purification prior to paper chromatography was necessary.

The methanol elution of the paper chromatography contained two peaks AL1 and AL2 that eluted near the solvent front as shown in Fig 7.2.3.1b. Interestingly, in the fractions from the methanol elution there were two peaks which enhanced IL-4 induced CD23 expression on monocytes.

Figure 7.2.3.1a Inhibitory effect of fractions from ZPT after water elution from paper chromatography. There were two inhibitory peaks (W1 & W2).
7.2.3.2 Hexane Extraction

As a starting point to purification, the effect of solvent extraction using hexane was investigated. Both the solvent fraction and an aqueous fraction were tested for their inhibitory ability (n = 6). As seen in Fig 7.2.3.2a hexane extraction contained enhancing constituents and the inhibitory material was found in the aqueous phase.
Figure 7.2.3.2a Solvent extraction of ZPT with hexane. The fraction from hexane enhanced IL-4 induced CD23 expression whilst the water fraction was inhibitory.

7.2.3.3 Butanol extraction

As the original paper chromatography (Fig 7.2.3.1a) was performed with alcohol mixture, a butanol extraction was done after a hexane extraction to see if the inhibitory activity could be separated by alcohol. A scheme of the extraction procedure is shown in Fig 7.2.3.3a. ZPT and all ten individual herbs were extracted in parallel to identify any similar properties between the individual herbs and ZPT. The extracts analysed were normalised to the ZPT activity. Each set of extractions was tested on PBMCs from five different individuals.

\[
\text{Ratio compared to control} = \frac{\% \text{ Inhibition of CD14+CD23+ cells by extract}}{\% \text{ Inhibition of CD14+CD23+ cells by ZPT}} \quad (\text{PSE222/9240})
\]
Figure 7.2.3.3a Hexane and butanol extraction scheme of ZPT and the individual herbs.
In Fig 7.2.3.3b it is seen that the inhibitory activity in ZPT largely remained within the aqueous phase throughout the extraction. This indicates that the inhibitory factor(s) is extremely hydrophilic. Again a slight enhancing factor was obtained in the hexane fraction. The four herbal extracts (*Dictamnus dasycarpus*, *Glycyrrhiza uralensis*, *Paeonia lactiflora*, *Schizonepeta tenuifoli*) that had shown inhibitory activity in Fig 7.2.2a exhibited the same profile as ZPT i.e the inhibitory activity remained within the aqueous phase (Fig 7.2.3.3b). The other six herbs also showed this to a lesser extent (Fig 7.2.3.3c, 7.2.3.3d). Two of the individual herbal extracts *Caulis Clematis armandi* and *Rehmanniae glutinosa* had the majority of the enhancing activity.

**Figure 7.2.3.3b** Inhibitory and enhancing of profile of ZPT and the four major inhibitory herbs in ZPT after hexane and butanol extraction. The inhibitory factor(s) remained within the aqueous phase.
Figure 7.2.3.3c Inhibitory and enhancing profiles of the four of the herbs in ZPT with medium activity after hexane and butanol extraction. The enhancing(s) factor was contained in Caulis Clematis armandi.

Figure 7.2.3.3d Inhibitory and enhancing profiles of the two remaining herbs in ZPT with low activity after hexane and butanol extraction.
7.2.3.4 Large scale Extraction of ZPT

To obtain an indication of the mass of active material present in ZPT, a large scale solvent extraction of ZPT followed by further purification by paper chromatography was performed. Modifications were made to the protocol, in the alcohol extraction, the alcohol was changed from butanol to hexanol as the latter is immiscible with water. In the original paper chromatography 2 cm segments were eluted with methanol and then water, in this procedure 5 cm segments were eluted. The weight of each extract was taken throughout the experiment. In Fig 7.2.3.4a it is clearly seen that the inhibitory constituent(s) remain within the first 5 cm segment from paper chromatography (n = 3). There is only a slight decrease in activity in this fraction when the fractions were adjusted back to their original volume. This final weight of fraction accounted for 25% of the original weight. By weight this fraction would have approximately 2.5 x the inhibitory capacity of the original starting material. No inhibitory or enhancing factor(s) were seen in the methanol eluted fractions.

Figure 7.2.3.4a Large scale purification of the inhibitory constituent(s) in ZPT using solvent extraction and paper chromatography. (■) shows the extraction process when the extracts are dissolved back to the original volume. (□) shows the data adjusted to a standard weight of 1 mg/ml.
7.2.4 Ion Exchange and Reverse Phase Columns

To investigate further a suitable protocol for purifying the inhibitory factor(s), a range of ion exchange columns were used. ZPT was extracted through solvents as previously described and then analysed on a variety of columns. In Figure 7.2.4a (n = 5) it is seen that the inhibitory factor was eluted from a strong anion exchange column (AG1) and cation exchange column (AG50) indicating that the inhibitory factor was apparently neutral. However the activity was retained on AG3 and AG3 acetate which are weaker anion exchange columns than AG1. A Sep-Pak® C18 column was used to investigate the separation that could be achieved by a reverse phase HPLC column. As most of the activity was eluted with water, the result with this column confirms the hydrophilicity of the inhibitory molecule. Subsequent elution with 10% methanol and pure methanol did not elute a significant amount of activity (Fig 7.2.4a).

![Figure 7.2.4a](image-url) Effects of ion exchange and reverse phase columns. The activity was eluted from strong anion and cation exchange columns. However it was retained on weaker anion columns (AG3 and AG3 acetate). On a Sep-Pak® column the activity was eluted with water.
7.2.5 Effects of Charcoal

When mixed with charcoal the activity was absorbed onto charcoal and could not be eluted either with water or pyridine buffer (Fig 7.2.5a).

![Figure 7.2.5a](image)

**Figure 7.2.5a** Elution of the activity of ZPT from a suspension of charcoal. The activity remained bound to charcoal even after elution with water and pyridine buffer.

7.2.6 Purification of ZPT for Mass Spectroscopy

The procedure for the purification was according to the scheme seen in Fig 7.2.6a. 1.211g of ZPT was extracted through solvents as previously described. The extract was then placed through a mixed bed column of AG1/AG 50. 0.592g was obtained from this procedure (yield 49%). After passing through the Sep-pak ® C-18 columns the weight was 0.473g (yield 39%) and the final extract gave 0.329g (yield 27%) after passing through the final mixed bed column. After methanol precipitation 0.137g of the final extract gave 0.103g from the supernatant and 10mg of precipitate. This would give a total yield from ZPT for the supernatant of 20% and precipitate of 1.97%.
Figure 7.2.6a  Scheme showing the purification of ZPT using a variety of columns
As seen in Fig 7.2.6b when the extract was dissolved according to its original volume the inhibitory activity remained constant throughout the procedure (n = 3) i.e no increase in total activity. If the data was interpreted according to a fixed weight of 1mg/ml the final product was 4x as potent as the original ZPT.

![Graph](image)

Figure 7.2.6b  Inhibitory activity of ZPT after partial purification through a series of columns (■) shows the extraction process when the extracts are dissolved back to the original volume. (E) shows the data adjusted to a standard weight of 1mg/ml.

After methanol precipitation the final column eluant (E), the supernatant (S) and the precipitate (P) were compared to ZPT in terms of inhibitory activity. The fractions were diluted to the original volume and then further diluted to obtain a dilution curve for each sample. As seen in Figure 7.2.6c ZPT, E, S and P gave similar dose response curves. The concentrations of ZPT, E, S and P at the mid range point of inhibition (25% inhibition) were 120µg/ml, 30µg/ml, 15µg/ml and 3µg/ml respectively. This indicates that the P is 40x more active than ZPT on a weight/volume basis. As S required double the concentration of E to obtain the same inhibition, it can be speculated that half the inhibitory activity has been removed by methanol precipitation (P). The mass spectroscopy data will give a clearer indication of the purity of P.
Figure 7.2.6c Dilution curves of the column eluant (E) supernatant (S) and precipitate (P) after methanol precipitation. These three fractions are compared to ZPT.

7.2.6.1 Electrospray Mass Spectroscopy Results

The negative ion electrospray mass spectrum (fig 7.2.6.1a) of the methanol precipitated fraction yielded an apparent molecular ion of molecular electron (m/z) 779 which could undergo loss of 162 Da to m/z 617 possibility corresponding to loss of a hexose, or deoxygenation by the loss of 16 Da to m/z763. The peak at m/z 617 could either undergo deoxygenation to m/z 601 or loss of hexose to form the base peak at m/z 455. The base peak at m/z 455 could also undergo deoxygenation to m/z 439. The only other significant peak was at m/z 195 which could be a loss of 260 Da from m/z 455. There was evidence of a minor component also present in the mass spectrum of apparent molecular weight of m/z 665 which could indicate a loss of hexose to m/z 503 and then further loss of a hexose to m/z 341. The interpretation of the mass spectrum of the methanol precipitate is summarized in Fig 7.2.6.1b.
Figure 7.2.6.1a  Negative ion electrospray mass spectrum of the methanol precipitated fraction of ZPT. Courtesy of Mr Nigel Haverington, GSG, UK.
Figure 7.2.6.1b  Schematic of the negative ion electrospray mass spectrum of the methanol precipitated fraction of ZPT.
7.3 CONCLUSION

The isolation of component(s) which are responsible for the efficacy of ZPT in AE is essential so that a specific therapeutic compound drug be administrated with minimal side effects. ZPT has been shown to have many effects i.e inhibition of CD23 expression and cytokine production in vitro and reduction of activation markers in vivo. This could indicate that ZPT has one active compound like a corticosteroid which interacts in many pathways or multiple active compound(s) which simultaneously attack a number of inflammatory processes in the skin. As the possibility of one or more active compound(s) exist the activity which inhibited IL-4 induced CD23 expression was investigated.

ZPT consists of ten herbs of which an aqueous extract is made, 4 out of the 10 herbal extracts when dissolved at a concentration equivalent to that of the whole mixture showed similar levels of inhibitory activities on CD23. These were extracts from Dictamnus dasycarpus, Glycyrrhiza uralensis, Paeonia lactiflora and Schizonepeta tenuifolia. The inhibition by these four extracts was dose dependant and might indicate that they have the same or similar inhibitory factor(s) but present at different concentrations. Further analysis of these four extracts would have to be done to verify this possibility.

The extraction and subsequent paper chromatographic analysis showed that the major inhibitory component(s) remained at the origin of the paper. This would be characteristic of a hydrophilic molecule as highly hydrophobic molecules, such as peptides will be carried down the paper. This was confirmed by the fact that the major inhibitory factor(s) was neither hexane or alcohol soluble. The hexane and alcohol extraction of the individual herbal extracts showed that activity of four major inhibitory herbs also showed the same characteristics as ZPT i.e hexane and alcohol insoluble. The properties of ZPT were further elucidated by ionic exchange columns which showed that the activity was apparently of a neutral charge, although the activity was reduced after passing through a weakly anionic column (AG3 and AG3 acetate). AG1 is a strong anionic exchange column which binds inorganic and organic anions whereby phenolate, nitrate and citrate ions are more readily exchanged for the chloride ion bound to the resin. However AG3 columns bind mineral acids better and may indicate that the inhibitory factor may be weakly acidic. Preliminary data shows that the inhibitory factor is also pH stable as it is not affected by overnight treatment with strong acid or base. The negative ion mass spectrum of the precipitated
product revealed that this product consisted of one major compound and evidence of a minor component. Both had the ability to lose mass corresponding to loss of hexose residues and therefore is some form of carbohydrate but the exact structure has to be further investigated. Due to the lectin binding ability of CD23 it could be argued that the carbohydrates from this active component could be blocking the anti CD23 antibody in the assay and therefore experiments using monoclonals which bind to different parts of the CD23 molecule will have to be done. The fact that the active component affects the mRNA for CD23 may rule out a simple blocking of the antibody against CD23. ZPT has also been shown to induce IL-10 and TNF-α in vitro the effect of the precipitated product on these cytokines is currently under investigation.

Most of the enhancing property seem to reside in the herbal extracts *Caulis Clematis armandi* and *Rehmannia glutinosa*. These herbs are known to contain aristolochic acid and the saponin akebin (Haung 1993). However their effects on CD23 expression have not been investigated. Although the compound(s) might not be of therapeutic value in AE, it would be of scientific benefit to further investigate this enhancing compound. Glycyrrhetinic acid and paeoniflorin, the major components of *Glycyrrhiza uralensis* and *Paeonia lactiflora* respectively had no inhibitory effect on IL-4 induced CD23 expression but glycyrrhetinic acid slightly enhanced this expression. Glycyrrhetinic acid has been shown to enhance IFN-γ production from human lymphocytes (Shinda et al., 1986) and in some culture systems this cytokine has been shown to increase IL-4 induced CD23 expression on monocytes (Delespesse et al., 1989). Thus this molecule may by indirectly affecting CD23 expression by the release of IFN-γ; this would have to be investigated.

The isolation and purification of a component which inhibits CD23 expression has revealed a molecule(s) which is consistent with it being a carbohydrate. The potency is 80x more than ZPT and is 1.9% by weight of the original mixture. Patients with AE take 30g of ZPT per day of which 15% is estimated to be absorbed, if this purified component is effective in an animal model then 500mg would need to be taken daily which is comparable with other therapeutic agents. Further elucidation of the structure will have to be done to identify its therapeutic value in AE.
Chapter 8

Final Discussion and Conclusions
8.1 DISCUSSION

The findings of this thesis can be summarised as follows:

a) ZPT and Prednisolone inhibited IL-4 and IL-13 induced CD23 expression on monocytes from controls and AE patients in vitro. The inhibition by the two agents was dose dependant and not due to cell death.

b) Constitutive and IL4/IL13 induced CD23 expression on the human monoblastic/monocytic leukaemia cell line U937 was inhibited by prednisolone but not ZPT. sCD23 production was also similarly inhibited by prednisolone but not ZPT.

c) The mRNA for CD23 in monocytes was decreased by prednisolone and to a lesser extent by ZPT. Prednisolone also decreased the mRNA for CD23 in U937 cells.

d) ZPT induced IL-10 and TNF-α production from unstimulated and Con A stimulated PBMCs from controls and AE patients. Con A induced IL-4 production was decreased but IL-2 and IFN-γ production was not affected by ZPT. Cytokine production was not affected by PL.

e) AE patients treated with ZPT showed a decrease in IL-2 receptor, sVCAM and IgE complexes in the serum but no change in total IgE, sICAM and sCD23. The inducibility of CD23 on monocytes by IL-4 from patients was decreased by in vivo treatment with ZPT.

f) Isolation of components from ZPT using hexane, hexanol and paper chromatography revealed a peak near the origin which inhibits CD23 expression. Methanol extraction from paper chromatography also showed that ZPT contained a factor(s) which enhanced CD23 expression. Using a series of anionic exchange columns the inhibitory factor was found have a neutral charge and highly hydrophilic. Methanol precipitation of the column eluant followed by mass spectroscopy revealed a molecule with carbohydrate moieties which inhibited CD23 expression.
In the light of the pathogenesis of AE, the relationship between these results and how treatment with ZPT or other agents can lead to improvement of the disease will be discussed further.

AE is a complex disease with various defects including an overexpression of CD23. One of the theories of the initiation of the disease was proposed by Bruijnzeel-Koomen et al who identified that Langerhans cells isolated from the skin of patients with AE had IgE bound to the surface and proposed that this IgE could take part in allergen presentation in the skin leading to a delayed type hypersensitivity reaction which involved the activation of Th2 cells (Bruijnzeel-Koomen et al., 1986). These cells produce cytokines such as IL-4 and IL-13 which enhanced IgE and CD23 levels on a variety of cells. The Langerhans cell seems to be pivotal in the immunopathology of AE with its ability to bind IgE through IgE receptors FceR1 and CD23 (Bieber et al., 1992; Wang et al., 1992; Grabbe et al., 1993; Torresani et al., 1991). However, there is also evidence that monocytes express at least two of these receptors FceR1 and CD23 (Maurer et al., 1994; Melewicz et al., 1981; Nakamura et al., 1991) and thus this cell may also contribute to the disease.

Downregulation of CD23 is seen on Langerhans cells and macrophages in skin biopsies from patients taken before and after treatment with prednisolone and ZPT (Bieber et al., 1989; Xiou et al., submitted for publication). In the results presented here, two out of the three agents used in treatment of AE, prednisolone and ZPT inhibited CD23 surface expression and mRNA for CD23 in monocytes in vitro. Thus the importance of this downregulation may be related to the resolving of the disease. The mechanism by which this occurs is unclear but in vitro evidence suggests that the increased binding of IgE to monocytes in AE could lead to a cascade of events resulting in tissue damage in the skin.

Many studies have shown that CD23 expression on monocytes correlated with elevated IgE levels (Melewicz et al., 1980; Melewicz et al., 1981; Nakamura et al., 1991, Polla et al., 1992). In parasitic diseases and allergic disorders, CD23 bearing monocytes are involved in phagocytosis of IgE coated particles (Spiegelberg 1984), IgE-dependent cytotoxicity and the release of mediators (Capron et al., 1983; Joseph et al., 1983). Ferreri et al. have demonstrated that using aggregated IgG, IgA and IgE to stimulate monocytes from normal individuals resulted in the release of PGE₂, LTC4 and LTB₄ (Ferreri et al., 1986). However, this group could show no difference
in mediator release between AE patients and non-atopics and they postulated that as monocytes of patients with AE are exposed to higher concentrations of IgE in the circulation, this may shift their dose response curve and increase their ability to release mediators (Ferreri et al., 1988). More recently the monocytes of AE patients have been found to have an increase in production of superoxide (Polla et al., 1992) and this correlated with CD23 expression. However this group did not show that ligation of CD23 induced superoxide production but only speculated that this could happen in AE. Macrophages when incubated with IgE dimers can release superoxide and IL-1 (Dessaint et al., 1982) and this may indicate that this process is able to occur.

IL-1β and TNF α production from monocytes of asthmatic patients but not control individuals have been shown to be induced by stimulation with IgE complexes (Borish et al., 1991). Although this has not been demonstrated in monocytes from AE patients it is not difficult to speculate that the same production would occur as these patients have increased Fcε receptors. IL-6 and thromboxane (TxB₂) are also produced on stimulation of monocytes with IgE complexes (Paul-Eugene et al., 1992). This group also demonstrated that IgE complexes as well as an anti-CD23 antibody induced TNF-α, IL-6, superoxide and TxB₂ on CD23+ monocytes but not CD23- monocytes (Mossalayi et al. 1994). Monocytes from normal individuals were pretreated with IL-4 to obtain CD23+ monocytes that would mimic the monocytes of patients with AE in vivo. This IgE dependent monocyte activation was also linked with the nitric oxide pathway and it has been suggested that ligation of CD23 induces nitric oxide which could influence TNF α production (Dugas et al., 1995).

The consequences of mediator release from monocytes could lead to tissue damage and the recruitment of different cell types into the skin of patients with AE. Mitogen stimulation of PBMCs showed an increase in PGE₂ production from patients with AE compared to controls (Jacob et al., 1990; Chan et al., 1993). A more convincing argument PGE₂ having a role in AE is that it is elevated in lesional skin compared to uninvolved skin in patients (Fogh et al., 1989). IL-2, IFN-γ and IL-12 production from human T cells and IL-1 production from human monocytes were all inhibited by the addition of PGE₂ (Chouaib et al., 1984; Hasler et al 1983; van der Pouw Kraan et al., 1995). Betz et al reported that in the murine model PGE₂ inhibited Th1 cytokines but not Th2 cytokines and this has been confirmed in the human system (Betz & Fox 1991; Snijdewint et al., 1993). The overproduction of PGE₂ in the skin
of AE patients could lead to the preferential proliferation of Th2 cells, a phenomenon that is seen from T cells isolated from skin biopsies from patients (Reinhold et al., 1991; Ramb-Lindhauser et al., 1991; van Reijsen et al., 1992). Chan et al. has linked the increased PGE₂ production by monocytes to the increased levels PDE, an enzyme which hydrolyses cAMP (Chan et al., 1993). The group also suggests that a defective monocyte function may be responsible for the propagation of the disease. In contrast to the deleterious effects of PGE₂, it has also been shown to inhibit IL-4 induced IgE production (Pene et al., 1988). However, Phipps et al., argue that in human diseases elevated IgE is associated with high levels of PGE₂ and the inhibition of IFN-γ by PGE₂ would more likely lead to an increased IgE production in vivo (Phipps et al., 1991).

Poll et al. showed there was general activation of the respiratory burst enzymes as superoxide production was increased in AE irrespective of the activation used i.e. opsonized zymosan or PMA. Toxic oxygen metabolites such as superoxides are generated when polymorphs are activated and can take part in tissue damage in some inflammatory conditions (Fantone & Ward 1982). Another toxic molecule which can be generated is nitric oxide. It is formed from the oxidation of guanidino nitrogen of L-arginine by the enzyme nitric oxide synthase (NOS) which exist in many forms. One of the isoforms of this enzyme inducible NOS (iNOS) is not found in resting cells but can be induced by TNF-α, IL-1β and IFN-γ and is thought to be involved in inflammatory responses. This enzyme can be upregulated in murine macrophages and monocytes but evidence is only now emerging for its existence in human monocytes (Dugas et al., 1995). In the murine model it has been shown that nitric oxide inhibits the proliferation of Th1 cells and its cytokines whilst having no effect on Th2 cells (Taylor-Robinson et al., 1994). This evidence and the observation that iNOS is found in the epithelial cells in the lung of asthmatic patients but not in normal individuals (Hamid et al., 1993) has lead Barnes & Liew to postulate that nitric oxide production in the airways of the lungs suppress Th1 cells leading to propagation of Th2 cells (Barnes & Liew 1995). The iNOS production by epithelial cells or monocytes in the skin of patients with AE has not been demonstrated but the link between the nitric oxide pathway and CD23 could indicate that in chronic inflammation in the skin nitric oxide mediated toxic effects could cause damage to the skin and a proliferation of Th2 cells.

The in vitro model of CD23 expression used showed that IL-4 not only increases
CD23 expression but also sCD23 production from monocytes (Vercelli et al., 1988; te Velde et al., 1990). Patients with AE have increased levels of sCD23 in their serum compared to controls (Kim et al., 1989; Wuthrich et al., 1992; Kagi et al., 1992). sCD23 actions include the increase in spontaneous IgE production from allergic individuals (Sarfati et al., 1984a; Sarfati et al., 1984b) and synergy with low levels of IL-4 to produce IgE (Pene et al., 1988; Chretien et al., 1990). It has also been shown to increase the production of various cytokines such as IL-6, IL-1α, TNF-α, and PGE₂ from mononuclear cells and monocytes (Armant et al., 1994; Herbelin et al., 1994). This release of cytokines and increased production of IgE would further aggravate the inflammatory response in AE.

In the treatment of AE agents which modulate CD23 would not only lead to a decrease in binding of IgE to monocytes and macrophages but a subsequent decrease in mediator release which skews the immune response towards a Th2 profile. Corticosteroids have been shown to downregulate CD23 expression and sCD23 production on B cells (Katira et al., 1993; Kaufman Paterson et al., 1994) and also monocytes by results presented in this thesis. ZPT decreased CD23 expression on monocytes but was unable to downregulate CD23 expression on B cells, U937 cells and sCD23 production from monocytes. The conclusions from this data might be that ZPT is acting specifically on monocytes or that the regulatory pathways for CD23 expression are different in monocytes and B cells. Isolations of B cells from tonsils and peripheral blood would have to be investigated to give a clearer understanding of the effect of ZPT. In relation to mediator release PGE₂, superoxide and nitric oxide have also been shown to be inhibited by corticosteroids (Goode et al., 1991; Maridonneau-Parini et al., 1989; Radomski et al. 1990). Preliminary data from our laboratory has shown that ZPT inhibited PGE₂ production from PBMCs from patients with AE (Chow, 1993 BSc project). However purified monocytes from patients with AE will have to be investigated to justify this result. Superoxide production by the activation of monocytes by PMA was not affected by ZPT in normal individuals however the superoxide and nitric oxide production by the monocytes of AE patients cultured with ZPT was not investigated. It has been suggested by Greenspan & Aruoma that plant derived metabolites with antioxidant activity would be useful in AIDS where oxidative stress induced cell damage and death is seen (Greenspan & Aruoma, 1994). It is possible that ZPT could contain some of these anti-oxidant metabolites and this possibility has to be further investigated.
The data presented has only shown a role for CD23 on monocytes and obviously its expression on B cells, Langerhans cells and eosinophils will also be important in the disease. Without the investigation of prednisolone and ZPT on these cells it would be difficult to speculate if CD23 is playing a major role in the immunopathology of AE.

The finding of high affinity IgE receptors on monocytes, Langerhans cells and eosinophils must cast suspicion of IgE binding only to CD23 when the higher affinity receptor exists on the same cell (Bieber et al., 1992; Wang et al., 1992; Maurer et al., 1994, Gounni et al., 1994). Reischl et al. have examined carefully the regulation of FcεR1 on normal monocytes and concluded that normal individuals and AE patients had equivalent levels of FcεR1 on their surface which was subject to a recycling mechanism (Reischl et al., 1995). They concluded that the difference seen in AE and healthy individual was that the FcεR1 was being retained on the surface due to the binding of IgE. Thus increased levels of FcεR1 seen in AE are being detected due the increased levels of IgE seen in the disease. This group also showed that IL-4 had no effect on FcεR1 regulation. It has been proposed that the FcεR1 is important in allergen presentation on monocytes as monocyte enriched-PBMCs were capable of presenting birch pollen more efficiently to birch pollen specific T cell clones when allergen specific IgE antibody was used (Maurer et al., 1995). PGE₂ production has also been shown through activation of FcεR1 by IgE complexes. Thus the contribution of the different IgE receptors to the inflammatory disease process is unknown but with high levels of IgE in patients with AE, they may all have a role.

AE is characterized as a 'Th2 like disease' due to the higher levels of IL-4 found on stimulation of PBMCs and T cells isolated from the skin of affected individuals. This over production of IL-4, central to the upregulation of IgE and CD23 can have effects on other molecules and modulate different cell types. IL-4 is well known for its ability to cause resting B cells to enter the S phase of the cell cycle and cause isotype switching in activated B cells (Howard et al., 1982). It augments mitogen and antigen driven T cells and its presence shifts the profile of human PBMCs to Th0 or Th2 (Ferandez-Botran et al., 1986; Romangnani 1993) and stimulates mouse mast cells (Mossmann et al., 1986). IL-4 has been shown to upregulate class II MHC expression on B cells and monocytes (DeFrance et al 1987; Gerard et al., 1990) and this can increase the ability of monocytes to stimulate T cells. Furthermore,
it modulates the production of other cytokines by reduction of mRNA and the release of IL-1, IL-6, TNF-α, IL-8, IL-10 and IL-12 (Hart et al., 1989; Essner et al., 1989; Standiford et al., 1990; Gauchat et al., 1990; de Waal Malefyt et al., 1991). By downregulating IL-12 from monocytes, IL-4 skews the immune response towards a Th2 profile. IL-4 has also been shown to suppress PGE_2 production from monocytes (Hart et al. 1989). This is in contrast to the situation in AE where PGE_2 levels and IL-4 levels are elevated. IL-4 as well as up regulating the receptor for IgE, downregulates Fcγ receptors on monocytes therefore downregulating IgG responses (te Velde et al., 1990). In comparison to this in vitro data, administration of IL-4 to humans showed no decrease in cytokine production by monocytes but exogenous IL-4 no longer inhibited cytokine production in vitro (Wong et al., 1992). However CD14, Fcγ receptors and PGE_2 production were reduced whilst CD23 expression was increased. Thus the effects of IL-4 that operate in vivo may be different from those seen in vitro.

In addition to its effects on mononuclear cells, IL-4 induced VCAM on endothelial cells which preferentially bind eosinophils and basophils (Schleimer et al., 1992; Moser et al., 1992). IL-13 has similar effects as IL-4 although it seems to be more prolonged in production but has no effect on T cells (de Waal Malefyt et al., 1993; Zurawski & de Vries 1994; Cosentino et al., 1995; Bocher et al., 1995). Evidence has been provided using a mutant IL-4 protein to show that IL-4 and IL-13 share a common component in their receptors (Aversa et al., 1993). Increased mRNA for IL-4 has been found in skin biopsies of acute lesions however mRNA for IL-13 in the skin of affected patients has not been investigated (Hamid et al., 1994). Allergen challenge in patients with asthma showed an increase in the mRNA for IL-13 in broncholavage cells (Huang et al., 1995) and thus a similar result may be obtained with patch testing of skin of AE patients with allergen. The other Th2 cytokine implicated in AE is IL-5. It is known to promote the differentiation and survival of eosinophils (Weller 1992) and eosinophil derived proteins may be another cause of tissue damage in AE (Leiferman 1989).

In AE the switching from a Th2 to Th1 or Th0 profile could lead to a resolving of the disease. Corticosteroids have been shown to inhibit IL-4 and IL-5 from T cell clones whilst cyclosporin was more effective at the inhibition of IL-2 (Schmidt et al., 1994). However with PBMC preparations corticosteroids and cyclosporin were equally effective in the inhibition of IL-4, IL-5 and IL-2 production (Snijdwint et
In data presented IL-4 was significantly inhibited by ZPT but these results were not as impressive as those seen with cyclosporin and corticosteroids. If the in vitro result is mimicked in vivo then corticosteroids, cyclosporin, IFN-γ and ZPT would all downregulate IL-4 and thus lead to improvement in the disease. ZPT was shown to downregulate IL-4 from PBMCs and increase IL-10 and TNF-α production from PBMCs and in particular monocytes. The actions of ZPT demonstrated here are in vitro and may not be reflected in vivo. However it can be speculated on how changes in the three cytokines illustrated can be beneficial in AE. A decrease in IL-4 and hopefully IL-13 would be advantageous in restoring the balance of cytokines produced as mentioned above.

IL-10 is produced by human Th0, Th1, Th2 cells and monocytes and has been shown to have many immunosuppressive properties (de Waal Malefyt et al., 1991; Howard & O’Garra, 1992). It has been shown to inhibit IL-4 induced IgE and IgG4 production in PBMCs and this inhibition is mediated through monocytes (Punnonen et al., 1993). Bober et al. extended this work by showing that IL-10 inhibited allergen driven proliferation and reduced the number of IL-4 and allergen stimulated IgE B cells in atopic PBMCs (Bober et al., 1994), thus a reduction in IgE would be favourable in AE. This cytokine has also been demonstrated to suppress antigen activation of all subsets of CD4+ clones (de Waal Malefyt et al., 1991) and this may be through the downregulation of class II on monocytes (de Waal Malefyt et al., 1991). IL-10 inhibited the production of IFN-γ, IL1-α, IL-6, G-CSF and TNF-α, by monocytes (Fiorentino et al., 1989; de Waal Malefyt et al., 1991; Vieira et al., 1991) and the transcription of these cytokines in PBMCs (Wang et al., 1994). In activation of monocytes, IL-10 was produced after 24-48 hours and switched off other cytokines as well as its own production and this has lead to the notion that IL-10 is involved in the switching of ongoing immune responses (de Waal Malefyt et al., 1991).

ICAM expression and PGE2 production have also been shown to be downregulated by IL-10 (Willems et al., 1994, Spittler et al., 1995; Niiro et al., 1994). The reduction in allergen driven proliferation, ICAM (Kojima et al., 1994, Grewa et al., 1994, Wuthrich et al., 1995) and PGE2 levels (Jakob et al., 1989, Chan et al., 1993) which have all been implicated in AE could lead to a improvement of the disease if a component of ZPT is able to induce IL-10 production in the skin. Interestingly, IL-10 inhibited IgE mediated nitric oxide synthase induction by human keratinocytes and
the authors suggest that IL-10 may be an important anti-inflammatory agent in the skin (Becherel et al., 1995). Furthermore, PDE hyperactivity has been implicated in AE and the inhibition of PDE have been shown to augment IL-10 production in monocytes (Kambayashi et al., 1995) and thus PDE activity in AE may lead to reduced IL-10 by monocytes. A controlled trial of IL-10 in normal individuals has demonstrated an inhibitory effect on T cells and cytokine production (Chernoff et al., 1995). The argument against IL-10 being beneficial in AE has been the data recently presented by Ohmen et al that has shown an overexpression of mRNA of IL-10, IFN-γ but no IL-4 in the skin of AE patients compared to contact dermatitis patients (Ohmen et al., 1995). The authors concluded that the down regulation of IL-10 was important in AE. The upregulation of IFN-γ and no detectable IL-4 reported in this study is contrary to what has previously been reported. However Grewe et al., also showed an upregulation of mRNA IFN-γ which decreased after treatment in AE (Grewe et al. 1994a). Both these groups extracted RNA from skin biopsies and then performed PCR which was in contrast to other groups who used in situ hybridization technique. The differences in results obtained maybe due techniques used however the possibility cannot be ruled out that IL-10 and IFN-γ may have an adverse role to play in the immunopathology of AE. The finding of IFN-γ in the skin has lead to the proposal that the initiation phase of AE is Th2 dependent but the chronic phase is Th1 dependent (Grewe et al., 1994b).

It is difficult to conceive how a proinflammatory cytokine like TNF-α could be effective in AE (Vassalli 1992) but it has been argued that depending on the environment of this cytokine it may act in a positive manner in resolving some diseases (Jacob 1992). The production of TNF α from monocytes is suppressed by IL-4 and IL-13 (Hart et al., 1989; Essner et al., 1989 Cosentino et al., 1995) and thus the reduced levels of TNF-α reported by some authors maybe due to the increase of both these other cytokines (Kapp et al., 1990; Takahasi et al., 1992). TNF-α is produced by monocytes when CD23 is ligated and this cytokine can then down regulate CD23. Thus in a normal immune response further TNF-α production should be reduced through this pathway but in AE the overproduction of IL-4 or IL-13 may lead to a downregulation of the mRNA for proinflammatory cytokines like IL-1 and TNF α and therefore CD23 is not decreased. IL-1 production from monocytes in AE has been shown to be decreased however this has not been shown for TNF-α and thus would have to be investigated to verify this conclusion (Rasanen
et al., 1987; Jakob et al., 1995). Although TNF α has been shown to decrease CD23 expression, it has an upregulating effect on sCD23 and IgE production (Hashimoto et al., 1995; Gauchat et al., 1992) and therefore this cytokine could have positive and negative influences in AE (Gauchat et al., 1992). Furthermore mast cells constitutively express mRNA and produce TNF-α on crosslinking of their FcεR1 receptor and thus the contribution of this source is unknown in the skin of AE patients (Okayama et al., 1995). IL-10 reduces the production of TNF-α and thus a combinatorial effect of these two cytokines if released in vivo could lead to improvement of AE.

The in vitro findings can only be justified in relationship to disease by a similar finding in vivo. The study of skin biopsies and serological factors from patients with AE before and after treatment may give an indication of the relevant contribution of certain cells and surface molecules in the disease process. Many studies have shown that sCD23, sIL-2R, IgE, IgE complexes and ECP in the serum of patients with AE are elevated along with an increased T cell infiltration and CD23 expression in the skin. Table 8.1 shows a comparison of ZPT with other treatment in respect to serological changes. ZPT correlated well with other treatment in AE in demonstrating that the IL-2R decreased with treatment (Colver et al., 1989; Wuthrich et al., 1990; Kagi et al., 1992) indicating that activated T cells are important in the pathology of the disease. Unfortunately, ECP which is a good indicator of disease activity was not measured and investigations are in hand to address this (Czech et al., 1991). Other factors such as IgE levels and sCD23 production although elevated did not change with treatment in agreement with other workers in the field (Johansson & Juhlin 1970; Munro et al., 1991; Weindel Nelson et al., 1994; Wuthrich et al., 1992; Kagi et al., 1992). Although CD23 expression on circulating monocytes remained unchanged, CD23 expression on RFD1+, RFD7+ and CD1+ cells (Dendritic like, macrophages and Langerhans cells respectively) showed a significant reduction whilst cells expressing the high affinity IgE receptor were unaffected in patients before and after treatment with ZPT (Xiou et al submitted for publication). The results indicate that CD23 expression on peripheral monocytes although found to be elevated is not a good gauge of improvement in the skin and investigation of CD23 in the skin or on other cells such as B or T cells in the periphery may be more informative. A decrease in sICAM on treatment was not verified by results presented here however sVCAM was shown to be elevated and decreased following treatment (Koide et al., 1994; Wuthrich et al., 1995; Kowalzick et al, 1995a). This increase in sVCAM in AE has not
been demonstrated before but elevation has been shown in asthma during attacks (Koizumi et al., 1995). The reason why a concomitant reduction in ICAM is not seen is unclear as in an inflammatory process both adhesion molecules are likely to be present on endothelial cells. Allergen specific lymphocytes from allergic patients have been shown to induce both VCAM and ICAM on endothelial cells (Delneste et al., 1995). However IL-4 and IL-13 have been shown to selectively increase VCAM without affecting ICAM and E-selectin on human umbilical vein endothelial cells and thus if ZPT is selectively inhibiting IL-4 and IL-13 processes then this could explain the result obtained (Schleimer et al., 1992; Bochner et al., 1995).

Table 8.1 Comparison of serological changes when patients with AE are treated with different agents. NC = no change, NT = not tested, ↓ = decrease. A decrease in activation of T cells and production of ECP from eosinophils is consistent with an improvement in AE.

<table>
<thead>
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<th>Corticosteroids</th>
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<th>Cyclosporin</th>
<th>UVA/UVB</th>
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<td>sCD23</td>
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<td>ECP</td>
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<td>sIL2R</td>
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<td>sCD14</td>
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<td>sICAM</td>
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<td>sVCAM</td>
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The mechanism by which ZPT downregulates IL-2R, IgE complexes and VCAM from the in vitro data presented is unknown. The reduction in IL-4 could lead to a decrease in VCAM however if ZPT also induced TNF α in vivo, this cytokine also upregulates VCAM and therefore it is difficult to reconcile these two events (Thornhill & Haskard 1990). IL-10 is known not to have any effect on VLA-4 expression on monocytes which is the counterpart for VCAM, however it has been shown to decrease VCAM and ICAM expression on IL-1 activated endothelial cells (de Waal Malefyt et al., 1991b; Krakauer, 1995). Clearly, factors that reduce emigration...
of cells from the circulation to the skin will alleviate the severity of eczema. It may also be necessary to target ligands on T cells such as CLA which allows specific homing of these cells into the skin (Rossiter et al., 1994; Santamaria et al., 1995). Any decrease of endothelial and cell adhesion molecules coupled with reduction of mediators from cells triggered by immune complexes will enhance the healing process in the skin.

The results from this project have indicated that ZPT has one or more active components which might be worthwhile in the treatment of AE. The mechanism by which ZPT acts has not fully been explained but it has the ability to inhibit CD23 expression in a similar fashion to prednisolone at the mRNA level and downregulate IL-2 receptor without changing serum IgE levels. However, unlike prednisolone it is able to induce the production of IL-10 and TNF-α in vitro which may account for some of its activity. One of the aims of this project was to isolate therapeutic molecules for the treatment of AE. A chemical which is thought to be responsible for the inhibition of CD23 has been isolated from ZPT. In the final analysis, AE manifests many immunological defects and other forms of treatment such as corticosteroids and cyclosporin have been shown to intervene in many pathways in the inflammatory process and therefore a molecule which only downregulates CD23 may not be as effective as ZPT as a whole. Finally the future extensions of this project should be:

a) Determining if ZPT induces the mRNA for IL-10 and TNF-α
b) Extending the work of ZPT effect on PGE₂ production by monocytes and if the downregulation of CD23 results in the reduction of mediators such as PGE₂ and nitric oxide.
c) Elucidation of the structure and the family of compounds to which the molecule that inhibits CD23 belongs.
d) Comparison of the isolated compound(s) with ZPT to determine if it has the same abilities i.e induction of cytokines, inhibition of histamine release from mast cells and decreasing delayed hypersensitivity in the guinea pigs.

If all these aims can be achieved with positive results then hopefully the isolated molecule(s) may have many immunoregulatory properties and thus could be useful in treatment of patients with AE where the basic treatments have failed.
Chapter 9

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2 Latchman YE, Brostoff J: The effect of prednisolone on IL-4 and IL-13 induced CD23 expression on U937 cells and peripheral blood monocytes (Abstract) Immunology 1994; 83 Suppl 1:62


Papers submitted
1 Latchman YE, Banerjee P, Rustin M, Brostoff J: The influence of Traditional Chinese Herbal therapy (Zemaphyte\textsuperscript{TM}) on cytokine production in vitro: The role of IL-10 in atopic eczema J Allergy and Clin Immunol (submitted)
The Efficacy of Traditional Chinese Herbal Therapy in Atopic Eczema

Abstract

A traditional Chinese herbal therapy (Zemaphyte®) for the treatment of atopic eczema (AE) is currently being assessed. This review attempts to highlight its success in patients who are recalcitrant to Western forms of treatment and the rationale behind its use. The herbal preparation is a mixture of 10 herbs with some known pharmacological agents and actions. The concept of such a complex mixture in clinical treatment is anathema to Western medicine but acceptable in traditional Chinese medicine. As this formation has been shown to be effective in two double-blind crossover trials, investigative work on components from the mixture must be established in order to find the active constituent(s) and describe their mode of action. This research will also lead to a greater understanding of the complex immunopathology of AE.

Introduction

Atopic eczema (AE) is a chronic relapsing skin disorder with a genetic predisposition. It has been estimated that 10% of the Western population suffers from this disease with an increasing prevalence in children. The aetiology of AE is unknown; however, there are numerous cellular and humoral defects including a raised total IgE and multiple positive specific IgE antibodies to a wide range of allergens. Although many of the defects are indicative of a type 1 hypersensitivity, there is infiltration of the skin with T cells which is typical of a delayed-type hypersensitivity reaction [1]. It has been demonstrated that lymphocytes from patients with AE produce higher levels of interleukin 4 (IL-4) [2, 3], and clones isolated from allergic individuals in response to allergen generate IL-4 [4]. These findings have led to the theory that in AE there is a preferential infiltration of Th2 cells into the skin [5]. Evidence for this has been shown by Reinhold et al. [6] in the skin and in the circulation of patients with AE [7]. The high-affinity receptor (FcεR1) and the low-affinity IgE receptor (CD23) [8−10] have now been identified on Langerhans cells in the skin, and there is an increased expression of CD23 on the circulating monocytes of AE patients [11, 12] and in the skin of affected individuals [13]. Thus, the proposed activation of Th2 cells is the binding of aero-allergens percutaneously absorbed onto IgE bound through its receptors on cutaneous antigen-presenting cells.

Although the finer details of the immunological basis of this disease are being unravelled, the general treatment of AE is to reduce the inflammation in the skin by topical steroids and, in severer cases, systemic steroids. As interferon γ is known to reduce IL-4-induced IgE production [14], interferon γ was assessed as a therapeutic agent [15]. There was clinical improvement in patients treated but this was followed by a rapid relapse. Cyclosporin, an effective im-
munosuppressant, has also been administered to patients with AE [16]; this has also been successful but fears about its toxic effects to the hepatic and renal system have reduced its use in children. However, even with these effective treatments which have significant side-effects there is still a proportion of patients who are recalcitrant to all forms of Western treatment. To fill this therapeutic void traditional Chinese herbal therapy (TCHT) has recently been successfully used to treat the severest forms of eczema.

**What Diseases Are Treated by TCHT?**

There is a variety of herbal treatments for a collection of diseases in China. These include rheumatoid arthritis, asthma and psoriasis. At present the only TCHT studied in a Western setting, with rigorous double-blind placebo-controlled trials [19, 20], has been a formulation of 10 herbs for AE. Thus only the pharmacological aspects and treatment with this mixture (trade name: Zemaphyte®) will be discussed further.

**History and Theory of TCHT**

Traditional Chinese medicine predates its current interest in the West by over 2,000 years. Its pharmacopoeias contain a variety of medicines from plant, animal and mineral origin [17]. Traditional Chinese medicine seeks to treat the whole person not just the disease; thus the emotional and environmental aspects of the patients are considered. The essential theme behind this approach resides in the two forces Yin and Yang. Yin and Yang literally translated represent two banks of a river, one is the shade and the other is the sun. Yin can also portray the female unresisting nature and Yang the masculine controlling side of the universe. These aspects of Yin and Yang may appear antagonistic; however, in Chinese philosophy they are complementary to each other. In healthy individuals the Yin and Yang are in perfect balance, thus illness only occurs when there is an inequality between them. The objective of the traditional Chinese medicine practitioner is to detect the imbalance and to treat the patient to achieve harmony. There is an exchange between the environment and the body: food, drink, air entering and waste leaving, and this interchange occurs through the Zang Fu (Chinese organ systems). Transport within the body is carried out by the blood vessels and other parts of connective tissues (Jing Luo, Xue Mai) to various organs [18]. All these factors are taken into account, and it is likely that a traditional Chinese doctor will also examine the tongue, iris and pulse of the individual. The treatment of a patient may be mixed including herbal remedies, massage and acupuncture. This concept leads to individual cures for each patient embracing physiological and psychological aspects. In China TCHT and conventional medicine are practised together; however, in the West practitioners find the TCHT difficult to comprehend. The astonishing results achieved in atopic eczema have led most Western doctors to believe that the Chinese have perfected the science or art of choosing specific traditional Chinese herbs to treat specific disease rather than as they do to redress the balance between the Yin and Yang.

**Clinical Evidence for the Efficacy of Zemaphyte**

The first double-blind placebo-controlled trial of this preparation for AE was carried out in children at the Hospital for Sick Children, London [19]. Chinese practitioners work out a personalised prescription for each patient. Thus the initial challenge for this trial was to formulate a standard preparation for all patients, a concept alien to Chinese herbal practitioners. However with the help from Dr. Luo, a Chinese doctor with vast experience in the area of AE, 10 individual herbs were recommended. The constituents of these herbs were ground and placed in porous paper sachets. These ‘teabags’ were boiled, and approximately 100 ml of the decoction was drunk by each patient. Placebo sachets with similar smell, taste and appearance were used. The structure of the trial was that of a double-blind placebo-controlled study with a wash-out period between treatments. Forty-seven children entered the study and 37 completed it. There was a significant decrease in erythema and surface damage with the active but not the placebo formula. In the later adult study [20], there was an even more significant improvement in erythema and surface damage (fig. 1). The median decrease in erythema was 91.4% compared to 10.6% with placebo. Surface damage scores were decreased by 85.7% (active) and 17.3% (placebo). Many of the patients reported that they were less itchy and had a greater ability to sleep.

In both studies renal function, full blood count and a liver function test were all measured and were all found to be normal. However, patients have been reported with hepatotoxicity [21] and recurrent facial herpes [22] who used traditional Chinese herbs unrelated to this treatment. With these incidents in mind research on the active components of TCHT is desirable.
Treatments phase 1  Treatment phase 2

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**Fig. 1.** Sequential total body scores (geometric means) for erythema (a) and surface damage (b). Patients were assessed monthly. The body was divided into 20 equal parts, and the percentage area of each zone affected was assessed. A standardised scoring system for degree of erythema and surface damage (0-3) was made. The sum of the severity scores multiplied by the area scores gave a total body score, the maximum being 180. The scores for each patient at the end of each 8-week study period were plotted. Kindly reproduced by the permission of *The Lancet*, from Sheehan et al. [20].

### Pharmacology

Many herbs have been investigated, and agents with pharmacological action have been identified. The preparation we have used consists of a standardized preparation containing plant materials widespread in China which have been identified by their botanical names as: *Ledebouriella seseloides*, *Potentilla chinensis*, *Clematis armandii*, *Rehmannia glutinosa*, *Paeonia lactiflora*, *Lophatherum gracile*, *Dictamnus dasycarpus*, *Tribulus terrestris*, *Glycyrrhiza glabrae*, *Schizonepeta tenuifolia*. This is ‘fingerprinted’ by thin-layer chromatography and compared to reference samples by the supplier. Table 1 summarises the principal known pharmacological activities of the herbs in the eczema formulation [23]. This table should be regarded as a guide not as an exhaustive inventory of biological activities. Some tests are relatively simple to carry out, and, for example, the large number of compounds shown to have antibacterial and non-steroid anti-inflammatory activities may reflect the relative ease of testing. Particular compounds in this mixture have a long history of research; this includes glycyrrhetinic acid which is a potent inhibitor of 11β-hydroxysteroid dehydrogenase which in the body is responsible for conversion of cortisol to cortisone [24]. This compound is found in *G. glabrae*. Taylor et al. [submitted for publication] have shown that after administration of the recommended doses of the herbs there was no significant change in absolute levels or ratio of cortisone/cortisol levels in the urine. However this does not address the possibility that organ-specific changes in the handling of corticosteroids occur which are not reflected in the urine. Recently, glycyrrhetinic acid has been found to have an effect on the immune system by enhancing the proliferation induced by concanavalin A and increasing the production of IL-2 [25]. This may indicate that glycyrrhetinic acid may target chemical pathways in cells and increase or decrease the production of immunological mediators.

Sheehan and Atherton [19] also compared the effects of liquorice (*G. glabrae*) which was present in both the active and placebo preparations in their first trial. This must indicate that there are other active components in TCHT. Paeoniflorin found in *P. lactiflora* has been shown to inhibit binding of steroids to their receptors and may positively or negatively influence steroid levels through their receptors [26]. Gas chromatography and mass spectroscopy of a different formulation of Chinese herbs has shown that it contains a large quantity of paeonol [27], and this component which is present in the tree peony may also be worthy of further investigation. From the current knowledge at our disposal the following types of action of compounds in the eczema preparation have been proposed by Whittle [28] and other:

1. endogenous cortisol/cortisone release by adrenocortical stimulation;
2. potentiation of the action of endogenous corticosteroids in specific tissues including the skin and lungs, by inhibition of metabolising enzymes;
3. corticosteroid-like activity of plant surrogates;
4. interference in the generation of inflammatory mediators from the arachidonic acid/prostaglandin/leukotriene cascade;
5. an antipruritic action not necessarily mediated via corticosteroids (vasoconstrictor effect);
Table 1. Summary of the principal known pharmacological activities of the 10 herbs used in the mixture used for the treatment of AE

<table>
<thead>
<tr>
<th>Herb</th>
<th>Anti-bacterial</th>
<th>Anti-fungal</th>
<th>Anti-viral</th>
<th>Anti-histaminic</th>
<th>PAF inhibitor</th>
<th>Steroid-like</th>
<th>Non-steroid anti-inflammatory</th>
<th>Immunosuppressant</th>
<th>Smooth muscle relaxant</th>
<th>Anti-ulcer</th>
</tr>
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<tbody>
<tr>
<td>Clematis armandii</td>
<td>+</td>
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<tr>
<td>Dictamnus dasycarpus</td>
<td>+</td>
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<tr>
<td>Glycyrrhiza glabrae</td>
<td>+</td>
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<tr>
<td>Ledebouriella saseloides</td>
<td>+</td>
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<td>Lophatherum gracile</td>
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<td>Rehmannia glutinosa</td>
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<tr>
<td>Paeonia lactiflora</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Potentilla chinensis</td>
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<tr>
<td>Tribulus terrestris</td>
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<tr>
<td>Schizonepeta tenuifolia</td>
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</table>

PAF = Platelet-activating factor.

(6) antibacterial activity;
(7) anti-allergic activity (interference in pathways leading to an allergic response).

In terms of the pharmacological properties the clinical efficacy may well be the result of a multiple, simultaneous attack on a number of inflammatory processes in the skin involving mediators which differ in origin and time course. The limited success of Western monotherapy in the treatment of severe AE with agents such as azathioprine and corticosteroids only emphasises this point.

Possible Mechanisms of Action

The efficacy of the herbs has been shown at the clinical level but in the absence of a suitable animal model for AE an array of immunological systems has to be examined. In our quest to find the mechanism of action we have investigated its effect on CD23. CD23 is the low-affinity IgE receptor (FcεRII) and has been implicated as having a central role in AE with the result being chronic inflammation. It exists in two forms: type A is constitutively expressed on B cells, and type B can be induced by IL-4 on a variety of cells including monocytes. CD23 is expressed on the surface of monocytes and this has been correlated to superoxide production. We postulated that any drug that may block or inhibit IgE binding on cutaneous antigen-presenting cells would arrest the efferent cellular response in AE. To test this hypothesis we have used a model system to study the effect of an aqueous extract of the herbs on IL-4-induced expression of CD23 on normal monocytes. These data are at present being submitted for publication but clearly show that the aqueous extract inhibits the IgE receptor (CD23) while the placebo extract used in the double-blind trial had no effect. This is being corroborated by preliminary data from co-workers who show that CD23 in skin biopsies from treated patients is also reduced. These data are in accordance with those of Bieber et al. [10] who have demonstrated a similar finding with the reduction of IgE+ Langerhans cells in AE patients treated with corticosteroids. At present other groups are investigating effects on histamine release from mast cells, platelet-activating factor inhibition and the cellular changes that occur on treatment. Various parameters have to be considered as there are no validated laboratory models for eczema. The key to the mechanism of action will be in the isolation of active components from the mixture. Not only will this bring to light new and exciting drugs but it may help greatly in our understanding of AE.

Conclusion

Two double-blind placebo-controlled trials have demonstrated that Zemaphyte is successful in the treatment of AE. As the patients in these trials were all recalcitrant to Western forms of medicine, it can be speculated that TCHT is certainly as effective as corticosteroids, azathioprine and cyclosporin in the management of this particular disease and with fewer side-effects. In our model system of IL-4-induced CD23 expression we have shown a reduction in CD23 expression on the surface of monocytes. This evidence and preliminary data on CD23 expression in the skin of treated patients have led us to surmise that one mecha-
nism of action is to target antigen-presenting cells in the skin. A reduction in molecules such as CD23 would lead to a decrease in the 'delayed-type hypersensitivity reaction' seen in AE. This hypothesis would certainly account for the success in the majority of patients with sensitivities to aero-allergens and high IgE levels. Our preparation is a mixture of ten herbs, all containing distinct pharmacological agents, therefore, the success of this treatment may be in its ability to intercept and reduce many immunological pathways. In our search to find the active component(s) in this mixture, the fact that AE is a complex and multifactorial disease leads us to speculate that polypharmacy could be a desirable treatment for AE.

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Efficacy of traditional Chinese herbal therapy in vitro.
A model system for atopic eczema: inhibition of CD23 expression on blood monocytes

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Summary
Recently, there has been growing interest in the use of traditional Chinese herbal therapy (TCHT) decoctions for the treatment of atopic eczema (AE). The mode of action of this treatment is still unknown, and in order to investigate this we have analysed the effect of an extract of these herbs (TCHTE) on interleukin 4 (IL-4)-induced CD23 expression on peripheral blood monocytes from non-atopic subjects. We found that TCHTE inhibited CD23 expression up to 60% (P < 0.001), whereas the placebo extract had no significant effect on CD23 expression. This inhibition was dose-dependent, and TCHTE was effective at a concentration of 250 μg/ml (P = 0.001). If TCHTE or placebo was added after IL-4, the action of TCHTE could still be seen at 12 h. This inhibition was not due to cell death, as peripheral blood mononuclear cells (PBMCs) cultured with TCHTE or placebo at a concentration used in these experiments had a similar viability to control cultures. Down-regulation of the low affinity receptors for IgE on antigen-presenting cells in patients with AE may contribute to the benefit observed following treatment with TCHT.

Traditional Chinese herbal therapy (TCHT) has been shown to have beneficial effects in recalcitrant atopic eczema (AE). Many anecdotal cases have testified to this, and the efficacy has recently been confirmed by two double-blind placebo-controlled cross-over studies carried out in children and adults. These results have stimulated investigation into the mode of action of TCHT. There are numerous cellular and humoral defects in AE, including raised total IgE and multiple positive specific IgE antibodies to a wide range of allergens. Although these markers reflect a type 1 hypersensitivity, the skin in AE resembles a delayed-type hypersensitivity reaction, indicating T-cell involvement. The initiation of such a reaction has been the proposed binding of percutaneously absorbed Aeroallergens to IgE bound to the surface of cutaneous antigen-presenting cells.

CD23, the low affinity IgE receptor (FceRII), has been implicated in the pathogenesis of AE. It was initially found on B cells and several lymphoblastoid B-cell lines, and is now known to exist in two forms. Type A is constitutively expressed on B cells, and type B can be induced by interleukin 4 (IL-4) on a variety of cells, including monocytes. Monocytes from patients with AE have been shown to express elevated levels of CD23 on their surface and this has been correlated with superoxide production. There is also increased expression of CD23 in the skin of affected individuals. As it has been demonstrated that lymphocytes from patients with AE produce higher levels of IL-4 and any drug which might block or down-regulate CD23 cell surface expression may be effective in decreasing the afferent pathway of the cell-mediated reaction in AE. With this in mind, it was decided to use a model system to study the effect of TCHT on the expression of CD23 on monocytes under the influence of IL-4.

Methods
Subjects
Samples were obtained from 20 normal individuals, and included some which were supplied by the South Thames Blood Transfusion Service (12 females, eight males: mean age 29 ± 8.9). None of these subjects had a history of atopic diseases.
Reagents

A CD23-specific monoclonal IgG2b (H107) was obtained from the Nichirei Corporation (Tokyo, Japan). Phycoerythrin (PE)-conjugated anti-Leu M3 (CD14) and Simultest control (FITC + PE) were purchased from Becton Dickinson (Mountain View, CA, U.S.A.). The secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab)2 anti-mouse immunoglobulin (Dako Ltd, High Wycombe, U.K.). Recombinant IL-4 was obtained from Genzyme (Boston, MA, U.S.A.). The TCHT extract (TCHTE) and placebo extract were a kind gift from Dr B. Whittle, Phytopharm Ltd (Brough, U.K.), and were produced from the same herbs that were administered in the clinical studies.2,3 TCHT consisted of a preparation standardized by botanical identification and thin layer chromatography, containing plants which are widespread in China, and which have been identified by their botanical names as: Ledebouriella seseloides (4), Potentilla chinensis (6), Clematis armandii (3), Rehmannia glutinosa (6), Paeonia lactiflora (4), Lophatherum gracile (4), Dictamnus dasycarpus (6), Tribulus terrestris (4), Glycyrrhiza uralensis (2), and Schizonepeta tenuifolia (2). The figures in parentheses refer to the relative weight of each herb in the mixture. The placebo was composed of Homulus lupulus, Hordeum distichon, Hordeum distichon ustum, baker’s bran, sucrose, Salvia spp, Thymus vulgaris, Rosmarinus officinalis, Mentha piperita and clove oil. TCHT and placebo were supplied as water extracts taken to dryness. The yield from the extraction of TCHT was 5-75 kg of dried powder from 18-25 kg of raw herbs (31-3%). The extracts were then redissolved in RPMI 1640 (Gibco, Grand Island, NY, U.S.A.), and filtered before use.

Peripheral blood mononuclear cell (PBMC) separation and culture

Heparinized venous blood or buffy coat cells from the South Thames Blood Transfusion Service were diluted 1:1 with RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) and layered on to Lymphoprep (Nycomed, Oslo, Norway) within 2 h of collection. The gradients were centrifuged at 800g for 25 min at room temperature. The mononuclear cells were separated from the interface and washed twice in RPMI 1640. The cells were then resuspended at a concentration of 5 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum (Northumbria Biologicals, U.K.), 2 mmol/l L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco, Grand Island, NY, U.S.A.). Two hundred microlitres (1 x 10^6 cells) were cultured with various concentrations of IL-4, either alone, or with TCHTE or placebo at a final concentration of 1 mg/ml at 37°C, in 5% CO2 for 18 h. Cells from the same individual were always incubated with both preparations, i.e. TCHTE and placebo.

Double-staining FACS analysis

After culturing with IL-4, the cells were washed twice in phosphate-buffered saline with 1% bovine serum albumin (PBS–1% BSA), and then incubated with H107 at a concentration of 50 μg/ml and human gammaglobulin at 20 mg/ml (Sigma, Poole, U.K.) to saturate the IgG receptors. The cells were washed twice with PBS–1% BSA and then stained with FITC-conjugated rabbit anti-mouse immunoglobulin. After washing, the unreactive sites were blocked with 2% normal mouse serum in PBS–1% BSA. The figures in parentheses refer to the relative weight of each herb in the mixture. The placebo was composed of Homulus lupulus, Hordeum distichon, Hordeum distichon ustum, baker’s bran, sucrose, Salvia spp, Thymus vulgaris, Rosmarinus officinalis, Mentha piperita and clove oil. TCHT and placebo were supplied as water extracts taken to dryness. The yield from the extraction of TCHT was 5-75 kg of dried powder from 18-25 kg of raw herbs (31-3%). The extracts were then redissolved in RPMI 1640 (Gibco, Grand Island, NY, U.S.A.), and filtered before use.

The percentage of CD14^CD23^ cells in the monocyte gate was always greater than 80%. The results were expressed as percentage positive cells (CD14^CD23^) in the presence of IL-4 minus the double-stained cells in the absence of IL-4. All assays were carried out in duplicate, using double staining for CD14 and CD23.

Time course of addition of TCHTE and placebo

PBMCs were cultured as above, except that TCHTE or placebo extracts were added at 0, 0.5, 1, 2, 3, 4, 8, 12, 18 and 24 h after the addition of IL-4 at a concentration of 200 U/ml.

Assessing cell death

PBMCs were cultured in triplicate with TCHTE or placebo for a period of 3 days. The cell counts were assessed using acridine-orange/ethidium-bromide (Sigma, Poole, U.K.) under a fluorescence microscope, using a standard technique.15 The viability of the cells
was also determined using propidium iodide. Twenty microlitres of propidium iodide (50 μg/ml) were added to 200 μl of PBMCs (1 × 10⁶), and incubated for 10 min. The cells were washed twice with PBS, and resuspended in 200 μl of PBS. The samples were analysed by FACScan within an hour of the incubation.¹⁷

Assessing superoxide production by monocytes cultured with TCHTE or placebo

PBMCs were cultured, as previously described, with IL-4 (200 U/ml) and TCHTE or placebo (1 mg/ml). After culture, the cells were induced to release superoxide by incubating with phorbol 12-myristate-13-acetate (PMA) (Sigma), and this was measured by the reduction of nitroblue tetrazolium (NBT) (Sigma) to formazan, according to the method of Rook et al.¹⁸ Briefly, the cells were washed twice with PBS–1% BSA, and then 100 μl of RPMI 1640 (warmed at 37°C) was added. NBT and PMA were added to give a final concentration of 1 mg/ml and 10 μg/ml, respectively, in a volume of 200 μl. The cells were incubated for 30 min at 37°C, in 5% CO₂, and then centrifuged for 10 min. The supernatant was removed, and the cells were washed four times with methanol to remove the unreduced NBT. The wells were left to dry, and the formazan precipitate was then redissolved in 120 μl of 2 mol/l potassium hydroxide and 140 μl of DMSO. The optical density of the wells was read at 630 nm in an ELISA reader (Dynatech MR500, Dynatech, West Sussex, U.K.). Known amounts of NBT were reduced to construct a standard curve, beginning from 10 nmol/l of NBT per well. Thus, the amount of unknown NBT reduced by the monocytes could be determined. Negative wells without cells, but undergoing the same procedure, were used as blanks for the optical density readings. All assays were performed in triplicate.

Statistical analysis

The paired Student’s t-test was used to analyse the effect of TCHTE and placebo on IL-4-induced CD23 expression on monocytes.

Results

Dose-response of IL-4 induction of surface CD23

PMBCs were incubated with three concentrations of IL-4, and the surface CD23 on monocytes measured

Figure 1. The dose–response curve of IL-4-induced CD23 expression on monocytes after 18 h (n = 25). A concentration of 200 U/ml of IL-4 produced approximately 50% expression, and was considered optimum for the subsequent inhibition studies (mean ± SE).

Time course of inhibition of TCHTE and placebo on CD23 expression

PMBCs from three different individuals were cultured with IL-4 (200 U/ml) and TCHTE or placebo for periods of 18, 42 and 66 h. The maximum inhibition of CD23 expression was found to be at 18 h (Fig. 2). All subsequent experiments were carried out at this time point.

The effect of TCHTE on CD23 expression using varying concentrations of IL-4

The inhibitory effect of TCHTE on IL-4-induced CD23 expression was examined using three concentrations of IL-4 (200, 100 and 50 U/ml). In Figure 3, the mean inhibition of expression ± SE of the 20 individuals shows that TCHTE significantly inhibits (P < 0.001) CD23 expression on monocytes irrespective of the concentration of IL-4 used. The mean fluorescence intensity of CD23 also reflected the inhibition by
The effect of varying the concentrations of TCHTE and placebo on CD23 expression

We then considered if TCHTE could inhibit at a lower concentration. Using concentrations of 1.0, 0.5, 0.25 and 0.125 mg/ml of TCHTE or placebo extracts co-incubated with 200 U/ml of IL-4, TCHTE inhibited in a dose-dependent fashion. The mean inhibition ± SE of the 12 individuals studied was significant \( P = 0.001 \) down to a concentration of 0.25 mg/ml (Fig. 4).

Time course of addition of TCHTE and placebo

TCHTE inhibited CD23 expression on monocytes when added up to 12 h after IL-4 addition (Fig. 5). Again, placebo had little effect on CD23 expression on monocytes. There was an increase in CD23 expression between 12 and 18 h as the cells were cultured for a further 24 h.
Figure 5. The ability of traditional Chinese herbal therapy extract (TCHTE) or placebo extract to inhibit IL-4-induced CD23 expression on monocytes at intervals after the addition of IL-4. IL-4 alone ( ), IL-4 + placebo ( ), and IL-4 + TCHTE ( ). Inhibition was still present even when the TCHTE was added 12 h after the addition of IL-4. The mean of duplicate samples is shown at each time point.

Cell death

To examine whether the inhibition of IL-4-induced CD23 expression by TCHTE was due to cell death, PMBCs were cultured with varying concentrations of TCHTE or placebo extract for 3 days. The results, using acridine-orange/ethidium-bromide, are expressed as the mean of four experiments (Fig. 6). There was no difference in cell death between control and TCHTE/placebo cultures up to 2.5 mg/ml. However, TCHTE became toxic when used at 5 mg/ml whereas this did not occur with the placebo extract until a concentration of 10 mg/ml was reached. In all our experiments the dose of TCHTE used never exceeded 1 mg/ml, at which the viability was 97%.

The viability was also confirmed using propidium iodide. In three separate experiments the percentage viability ± SD was 95.6 ± 2 in control cultures; with TCHTE it was 90.1 ± 2.6 and with placebo 96 ± 3, at a concentration of 1 mg/ml.

Superoxide production

The possibility that TCHTE or placebo was affecting cell metabolism but not killing cells was considered. The reduction of NBT by superoxide produced by monocytes is a clear measure of cell viability. As can be seen from Table 1, there was no variation in NBT reduction by PMA-stimulated monocytes whether in the presence of TCHTE or placebo. This clearly shows that there was no impairment of superoxide production at the dose of TCHTE previously shown to reduce the expression of CD23. This indicates that the alteration of CD23 expression was neither due to cell death nor to a toxic effect on cell metabolism.

<table>
<thead>
<tr>
<th>mmol/l NBT reduced per well</th>
<th>% inhibition of CD14 CD23</th>
</tr>
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<tbody>
<tr>
<td>-IL-4</td>
<td>+IL-4 (200 U/ml)</td>
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<tr>
<td>Cells only</td>
<td>2.11 ± 0.8</td>
</tr>
<tr>
<td>TCHTE</td>
<td>2.09 ± 0.9</td>
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<tr>
<td>Placebo</td>
<td>2.08 ± 0.8</td>
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Table 1. Nitroblue tetrazolium (NBT) reduction by phorbol 12-myristate-13-acetate-stimulated monocytes cultured in the absence or presence of IL-4 with traditional Chinese herbal therapy extract (TCHTE) or placebo (n = 5). There was no difference in the superoxide production and reduction in NBT between the control cultures and cells cultured with TCHTE or placebo, although there was a significant inhibition of CD23 in the parallel cultures of the same individuals (mean ± SD). The results are expressed as mmol/l of NBT reduced per well.
Discussion

A formulation of traditional Chinese herbs has been found to be an effective treatment in patients with recalcitrant AE. In this study, we have shown that an extract of TCHTE significantly inhibits the expression of CD23 induced by IL-4 on normal monocytes. An extract of a placebo previously used in two double-blind clinical trials had little effect on this IL-4-driven CD23 expression. The expression of CD14 on monocytes was not affected by TCHTE or the placebo extract. This presumably indicates that the effect of TCHTE may be specific for CD23, and does not cause a general decrease in cell-surface markers on monocytes. One potential criticism of this study may be the high concentration of TCHTE used, in relation to the concentration achieved in vivo. However, adult patients receive 200 ml of this extract per day, containing 38 g of soluble material, and 1 mg/ml is a rough estimate of the circulating dosage in vivo after oral administration, allowing for 15% absorption.

Many studies have shown that CD23 expression on monocytes correlates with elevated IgE levels, but its role in AE has yet to be elucidated. In parasitic diseases and allergic disorders CD23-bearing monocytes are involved in phagocytosis of IgE-coated particles. IgE-dependent cytotoxicity, and the release of mediators. There is also a requirement for an association with a complement receptor. Ferreri et al. have demonstrated that, using aggregated IgG, IgA and IgE, the monocytes from patients with AE will release leukotriene C4 (LTC4). However, this group could show no difference in mediator release between AE patients and non-atopics, and they postulated that as monocytes of patients with AE are exposed to higher concentrations of IgE in the circulation, this may increase their ability to release mediators. More recently, the monocytes of AE patients have been found to have an increase in production of superoxide, and this correlates with CD23 expression. Macrophages, when incubated with IgE dimers, can release superoxide and interleukin 1 (IL-1), and this may point to a role for CD23 expression in parasitic diseases, and an overexpression in allergic disorders due to high levels of IgE. Langerhans cells of AE patients bind IgE, and normal Langerhans cells can be induced to express CD23. However, as the high-affinity IgE receptor has also been demonstrated on Langerhans cells, CD23 may only be a small component in the IgE-binding capacity of these cells. TCHTE down-regulates CD23 expression, and this will lead to reduced binding of IgE to monocytes and macrophages, and a subsequent decrease in mediator release and superoxide production. This would be one way of decreasing the inflammatory response in AE. Many of the herbs in this mixture of TCHTE have been demonstrated to have anti-inflammatory properties, which may synergize with other factors to produce clinical benefit.

We have used an artificial system of CD23 induction in which the levels of CD23 expression induced by IL-4 (200 U/ml) are greater than those seen on the monocytes of patients with AE. Our results indicate that TCHTE is more effective at lower levels of CD23 expression, and this may be comparable with the situation in vivo. The time-course study showed that TCHTE could be effective after the addition of IL-4, and this may be realistic in view of the in vivo situation. The data presented do not rule out the possibility that TCHTE might interfere with the action of, or bind to, IL-4. Hence, other systems using IL-4 as an inducer will have to be employed to assess whether TCHTE has any effect on its biological activity. So far, we have only shown that CD23 expression can be inhibited, and preliminary data suggest that extracts from only four of the plants have this ability to inhibit CD23. However, the overall efficacy of the decoction is likely to be multifactorial, and further studies are required to analyse its precise mechanism of action. With our present data we cannot tell whether TCHTE is specifically targeting the activation of monocytes or the expression of CD23. Studies on purified populations of monocytes and B cells need to be performed to answer this question. However, TCHTE may be affecting CD23 induced by IL-4 (type B) rather than the form constitutively expressed on B cells (type A).

These subtypes may have different functions, and similar experiments with Langerhans cells may provide a clearer indication of which form of CD23 is being inhibited. In the absence of a suitable animal model for AE, the effect of TCHTE needs to be examined in a variety of immunological systems, as its effect on CD23 expression may not fully explain the clearing of eczematous lesions seen in the clinical trials. The eventual aim must be to isolate the active components from TCHTE.

Acknowledgments

We would like to thank Miss Clare Melikian for performing the viability studies. Special thanks to Dr Brian
Whittle and Phytopharm for the supply of TCHT (Zemaphyte™) and placebo herbs.

References

Association of Immunological Changes with Clinical Efficacy in Atopic Eczema Patients Treated with Traditional Chinese Herbal Therapy (Zemaphyte®)

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Abstract
The efficacy of the Chinese herbal therapy (Zemaphyte®) has been well established as a treatment for atopic eczema (AE) in clinical trials. The purpose of this study was to probe the immunological changes that occurred when patients were treated with the herbs for a period of 8 weeks. This treatment decreased serum IgE complexes (p<0.05) but did not affect total serum IgE or CD23 expression on peripheral blood monocytes. Peripheral blood mononuclear cells from patients before and after treatment were cultured overnight with interleukin 4 and the ability of this cytokine to induce CD23 on monocytes from treated patients was found to be significantly diminished (p<0.01). Soluble interleukin 2 receptor and soluble vascular cell adhesion molecule were both raised in the serum of AE patients compared to control individuals. Both these parameters were decreased following treatment (p<0.05). All these changes coincided with improvement in erythema and surface damage scores. There was no alteration in soluble intracellular adhesion molecule or soluble CD23. The results of these investigations would suggest that this herbal treatment has the ability to target various immunological parameters which may be involved in the pathogenesis of AE.

Introduction
Atopic eczema (AE) is a chronic skin disorder with numerous cellular and humoral defects and is often associated with other allergic diseases such as hay fever and asthma. As the aetiology of the disease is not known, treatment is empirical and based on topical steroids which reduce the inflammation of the skin. In severer cases, systemic steroids, cyclophosphamide, azathioprine and phototherapy have been used [1, 2].

In recent years newer approaches have been used to target specific defects. One of the main features of AE is a raised IgE level: interferon γ has been shown to suppress interleukin 4 (IL-4)-induced IgE production in vitro [3]. Thus immune intervention with interferon γ has shown encouraging results [4–7]. The infiltration of T cells is thought to play a central role in the disease with most groups demonstrating a preponderance of Th2-like cells [8–11]. However, Grewe et al. [12] have recently shown a Th1-type response with increased levels of mRNA for interferon γ in
the skin of AE patients which decreases on treatment. Cyclosporin is mainly immunosuppressive for T cells by blocking the calcium-dependent pathways leading to the induction of various cytokine genes including IL-2, IL-4 and interferon γ [13]. This drug has been used successfully in the treatment for AE [14–16] although due to the various side-effects long-term treatment is not recommended. Despite these therapeutic approaches, there are still patients who are recalcitrant to all these forms of treatments. Two double-blind placebo-controlled trials and a long-term study have demonstrated the efficacy and safety of a traditional Chinese herbal therapy (Zemaphyte®) in such patients [17–19].

The immunopathology of AE is complex with no single immunological intervention so far leading to significant long-term benefit. The rationale behind this study was to investigate the immunological changes when patients undergo a specific Chinese herbal therapy treatment to give an insight into the mechanism of actions of the herbs and therefore the disease process.

Materials and Methods

Treatment

The traditional Chinese herbal treatment consisted of a preparation standardized by botanical identification and thin-layer chromatography containing plant material widespread in China which has been identified by the botanical names as: Ledebouriella seseloides, Potentilla chinensis, Clematis armandii, Rehmannia glutinosa, Paeonia lactiflora, Lophatherum gracile, Dictamnus dasycarpus, Tribulus terrestris, Glycyrrhiza uralensis and Schizonepeta tenuifolia. The original decoction was made from finely ground herbs which were packaged in sealed porous sachets [17–19]. The new palatable form was made from a water extract of the herbal mixture which was freeze-dried to granules.

Patients

Forty-eight patients with AE took part in an open study which assessed a new palatable form of freeze-dried granules of traditional Chinese herbal therapy compared to its original decoction prepared from the grounds herbs. The patients were randomized to prepare a decoction from 4 sachets or take 4 packets of freeze-dried granules and undergo a specific Chinese herbal therapy treatment to give an insight into the mechanism of actions of the herbs and therefore the disease process. The control group consisted of 13 males and 6 females healthy volunteers with no personal or family history of atopy and mean age (± SD) of 34.84±12.25 years.

Isolation of IgE Complexes

IgE complexes were isolated by a standard method described elsewhere [20]. The serum was mixed with polyethylene glycol 6000 (BDH Chemical Ltd, Poole, UK) and left overnight at 4°C. Complexes were centrifuged to a pellet at 2,000 g, washed twice and resuspended in Veronal-buffered saline. The immune complexes were then assayed for IgE content.

Measurement of IgE and IgE Complexes

Maxisorb ELISA plates (Nunc Immuno plate II) were coated with rabbit antihuman IgE (Dako Ltd, High Wycombe, UK) at a concentration of 10 μg/ml. After overnight incubation at 4°C, the plate was washed 3 times with phosphate-buffered saline (PBS) + 0.05% Tween 20 and blocked with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., USA) + PBS + Tween 20, for 1 h. Serum samples were diluted 1 in 100 in bovine serum albumin + PBS + Tween 20 and placed on the plate for 2 h. The final stage involved a peroxidase-labelled rabbit antihuman IgE. The samples were detected with tetramethyl benzidine as a substrate and read at 450 nm on a Dynatech ELISA reader (Dynatech, Biltinghurst, UK). A standard curve was constructed from the NIH International IgE reference sample (NBSB, Potters Bar, UK) starting with a concentration of 100 IU/l.

Peripheral Blood Mononuclear Cells: Separation and Culture

Heparinized venous blood 1:1 diluted with RPMI 1640 (Gibco, Grand Island, N.Y., USA) was layered onto Lymphoprep (Nycomed, Oslo, Norway). The gradients were centrifuged at 800 g for 25 min at room temperature. The mononuclear cells were separated from the interface and washed twice in RPMI 1640. The cells were resuspended at a concentration of 4×10^5 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum (Northumbria Biologicals, Carrlington, UK), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco). The cells were immediately stained for CD23 expression on monococytes or were cultured overnight with IL-4 (200 U/ml; Genzyme, Boston, Mass., USA) at 37°C in 5% CO_2 for 18 h.

Double-Staining FACS Analysis

After culturing with IL-4, the cells were washed twice in PBS with 1% bovine serum albumin. The cells were incubated with anti-CD23 monoclonal (Becton Dickinson, Mountain View, Calif., USA) and human gammaglobulin at 20 mg/ml (Sigma, Poole, UK) to saturate the IgG receptors. The cells were washed twice with bovine serum albumin + PBS and then stained with a fluorescein-isothiocyanate-conjugated rabbit F(ab)2, antimouse immunoglobulin (Dako). The cells were washed and the unreactive sites were blocked with 2% normal mouse serum in PBS. The final step after further washings was an incubation with phyco-erythrin-conjugated anti-Leu M3 (CD14; Becton Dickinson). All incubations were carried out at 4°C for 35 min. The cells were finally resuspended in PBS + 1% formaldehyde for fixation and analysed by a FACScan (Becton Dickinson) using the forward and side scatter to optimize for monococytes. The results were expressed as percentage positive cells (% CD14 +).
Soluble Interleukin 2 Receptor
Soluble interleukin 2 receptor (sIL-2R) was detected in serum using a Biosource Cytoscreen® sIL-2R kit (Biosource International, Camarillo, Calif., USA). The assay was conducted following the manufacturer's instruction and the minimum detectable dose of sIL-2R was 32 pg/ml.

Soluble Vascular Cellular Adhesion Molecule
Soluble vascular cellular adhesion molecule (sVCAM) was measured in the serum using a commercially available kit (Parameter, R&D Systems, Abingdon, UK). The minimum detection level was 4 ng/ml.

Statistical analysis
The Wilcoxon signed-rank test was used to compare the various parameters before and after treatment. The paired Student's t test was used to analyse differences between controls and patients.

Results
Erythema and Surface Damage
There was a significant improvement in erythema (fig. 1a) and surface damage (fig. 1b) in the 18 patients who took part in this study. Ten out of 18 patients were given the original decoction of traditional Chinese herbal therapy while 8 received a more palatable formulation of freeze-dried granules. The groups showed no difference in clinical outcome whether receiving either formulation. This has been confirmed by the result of the larger open clinical trial [21]. Thus, data for the 18 patients were analysed as one group. In essence there was an approximately 50% improvement in both surface damage and erythema in the study group.

Calculation of Results
All the results are presented as means ± SEM.

IgE and IgE complexes
All the patients had elevated levels of IgE, 5,640 ±1,036 IU/ml at the beginning of the study. After 8 weeks of treatment there was no significant difference in the IgE level, 7,104.7 ±1,813 IU/ml. Seven of the patients showed a decrease whilst the rest remained unchanged or increased. There was a significant difference in the levels of IgE-containing complexes between controls and patients (fig. 2). Complexes in patients manifested a fall from 64.1 ±18.2 to 47.8 ±16.7 IU/ml after 8 weeks of treatment (p = 0.045).

Monocytes Expressing CD23 and Soluble CD23
The percentage of circulating monocytes co-expressing CD23 and CD14 was 4.55 ±1.10 before treatment and 3.51 ±2.38 after treatment and there was no difference between control and AE patients.
Peripheral blood mononuclear cells were cultured overnight with IL-4 (200 U/ml) and then analysed for monocytes co-expressing CD23 and CD14. In figure 3 it is seen that the IL-4-induced percentage of CD14+23+ cells was significantly reduced from 51.63±3.29 to 39.73±4.23 after 8 weeks of herbal treatment (p<0.01).

Although the soluble CD23 levels in serum were elevated in two thirds of the AE patients, no change was detected following treatment (data not shown).

Soluble Interleukin 2 Receptor

It has previously been shown that sIL-2R correlates with disease activity and improves on treatment with corticosteroids [22]. It was confirmed that patients with AE have a raised sIL-2R level compared with controls (p<0.001; fig. 4). Patients undergoing treatment showed a significantly decreased level from 4,070.59±467.68 to 3,277.78±431 pg/ml (p<0.05). However, these values were still above those of the controls (2,310.53±226.32 pg/ml).

Soluble Vascular Cellular Adhesion Molecule

sVCAM is raised in a variety of diseases including rheumatoid arthritis [23]. The potential part that it might play in AE and the effect on treatment was examined. sVCAM was significantly raised compared to controls (p<0.001) and significantly diminished from 1,011.11±82 to 856.94±79.98 ng/ml after treatment of 8 weeks (p<0.05; fig. 5). Again the posttreatment values were still slightly higher than those of the controls (653.10±29 ng/ml).

Soluble intracellular adhesion molecule was also measured. The serum levels in atopic patients showed no difference from controls and no change after treatment (data not shown).

Discussion

The clinical efficacy of a formulation of Chinese herbs in treating AE has been proven in two double-blind placebo-controlled studies [17, 18]. This current study now reveals immunological changes in patients undergoing treatment with this preparation. Those that showed significant chang-
es were IgE complexes, inducibility of CD23, sIL-2R and sVCAM.

There was a significant decrease in IgE complexes after treatment. In this research the nature of these complexes was not analysed but it would not be difficult to speculate that these were IgE complexed to anti-IgE which have previously been described [24]. This decrease could lead to less complexed IgE binding to mast cells, B cells, eosinophils and monocytes through their IgE receptors and therefore a diminution in mediator production which can exacerbate skin damage. Patients being treated with Zemaphyte did not show any reduction in total IgE in spite of a significant reduction in the extent of their eczema as judged by scoring of erythema and surface damage. Other studies in patients with AE undergoing treatment with corticosteroids, azathioprine [25] and even interferon γ [26] also fail to show an immediate fall in total serum IgE levels in spite of clinical improvement.

Another prominent feature in AE is that various studies have shown the up-regulation of the low-affinity IgE receptor (CD23) on peripheral blood mononuclear cells and Langerhans cells in the skin of patients with AE [27–29]. In particular, the levels of CD23 on monocytes have correlated with superoxide production [30] and induction of nitric oxide [31]. These results have shown two different effects of treatment on CD23 expression on peripheral blood monocytes. Firstly, the percentage of monocytes expressing CD23 following treatment was unchanged, although in this study the expression on monocytes was much lower than previously described [28]. Secondly, the level of IL-4-induced CD23 expression on monocytes in vitro was significantly reduced after treatment. This indicates that treatment is able to block IL-4 induction of CD23. This is in accordance with the finding that Zemaphyte, but not a placebo extract, can inhibit IL-4-induced CD23 expression in vitro [32]. Although CD23 on blood monocytes and soluble CD23 in the serum were not affected, biopsy data in other studies with this treatment have shown that CD23 is down-regulated on antigen-presenting cells in the skin of treated patients [submitted for publication]. This might suggest that this therapy is directed at the site of inflammation rather than acting as a general immunosuppressant.

Activated T cells express the IL-2R, of which the α-chain can be shed and maintain its affinity for IL-2 [33].
This soluble form (sIL-2R) can be detected in the serum of AE patients. The level of sIL-2R correlates with disease activity and is reduced after treatment with corticosteroids [22, 34]. Our results do show a small but significant decrease in sIL-2R in the serum of patients on treatment. Shed IL-2R is a reflection of surface expression; thus, a reduction in this marker may mirror a decrease in activated T cells either as a primary or secondary phenomenon. For the first time it has been shown that sVCAM levels are significantly raised in patients with AE compared with controls. There is also a reduction in sVCAM after 8 weeks of treatment. As VCAM is an adhesion molecule which preferentially binds basophils and eosinophils and is induced by IL-4, it may have a potential role in allergic reactions [35]. The relationship between the expression of VCAM and the soluble form is not known but the decrease in sVCAM in the serum could be a reflection of down-regulation of adhesion molecules on the endothelial cells and therefore a reduction in cells entering the skin. Soluble intercellular adhesion molecule has recently been implicated as a marker for AE with levels decreasing on treatment with topical steroids [36, 37]. In this study no difference was seen between soluble intercellular adhesion molecule levels in controls and patients and there was no change following treatment. Wuthrich et al. [37] have suggested that intercellular adhesion molecule may be a new clinical marker for AE; however, our results do not support that conclusion. The data suggest that sVCAM may be more relevant as a diagnostic marker. To justify the relevance of our observations it will be necessary to show that VCAM and IL-2R are down-regulated in the skin biopsies of AE patients after treatment. Clearly, factors that will reduce emigration of cells from the circulation to the skin will alleviate the severity of eczema. It may also be necessary to target ligands on T cells such as the cutaneous lymphocyte antigen which allows specific homing of these cells into the skin [38, 39]. Any decrease in endothelial and cell adhesion molecules coupled with reduction of mediators from cells triggered by immune complexes will enhance the healing process in the skin.

This study has confirmed the efficacy of Zemaphyte in patients with AE who have been unresponsive to standard Western medicine. AE is a complex disease with various immunological abnormalities; the success of this treatment in AE may be in its ability of a compound or series of compounds to target a variety of immunological parameters particularly involved in adhesion molecule expression, IL-4, IgE and CD23 pathways.

References


Immunology of Herbal Treatment for Eczema

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