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IMMUNE MECHANISMS IN ATOPIC ECZEMA AND THE IMPACT OF THERAPY

A thesis submitted to the Faculty of Medicine, London University for the degree

of

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by Dr Piu Banerjee MBBS, MRCP (Lond)

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The author is a graduate of St Bartholomew's Hospital Medical College, London.

The work described in this Thesis was performed in the Departments of Dermatology and Immunology of the Royal Free Hospital and School of Medicine, London.

Supervisors of research are Prof LW Poulter (Department of Immunology) and Dr MHA Rustin (Department of Dermatology).

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ABSTRACT

Lesional skin in atopic eczema (AE) exhibits a T cell dominated cellular infiltrate suggestive of a type IV hypersensitivity reaction. The T lymphocytes involved appear to be predominantly T helper type 2 cells. Raised IgE levels and positive prick test reactivity to aeroallergens are also commonly seen implicating involvement of allergic immediate type hypersensitivity. The presence of IgE receptors on antigen presenting cells may provide a link between these two mechanisms. In AE, the low affinity IgE receptor,FcεRII or CD23, is found in lesional skin to be largely expressed on Langerhans cells and dermal dendritic cells, that is those cells capable of antigen presentation. This study investigates the relevance of IgE receptors to the pathogenesis of AE in terms of cellular distribution and the regulation of their expression. Relationships between CD23 expression and clinical severity are revealed in the context of local immunological activity in the skin.

A cohort of patients with AE were recruited and treated with Chinese herbal therapy (CHT) to test the relationship between immunological parameters and disease severity. Firstly it was established that treatment with CHT in this patient population resulted in an improvement in clinical disease. The distribution of IgE receptors on immunocompetent cells in lesional skin in AE and the emergence of these receptors during differentiation of cells of the monocyte/macrophage series were investigated. The relationship between T cell cytokine release and the level of expression of IgE receptors within the skin was determined.

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Using a standard clinical scoring system, it was confirmed that CHT improved the clinical status of patients in terms of reducing erythema and surface damage and the area of skin involved. Sequential use of immunoperoxidase and alkaline phosphatase-anti-alkaline-phosphatase immunohistological methods were employed to reveal the distribution of CD23 on mature cells in lesional skin. The results showed that as clinical severity was reduced, the numbers of CD23+ Langerhans cells and the numbers of CD23+ macrophages also decreased. Treatment also lead to a downregulation of the level of CD23 expressed by antigen presenting cells.

To determine whether the changing proportions of CD23+ macrophage subsets in lesional skin resulted from dysfunction in monocyte differentiation, peripheral blood monocytes were isolated by adherence and cultured for 7 days. Immunocytochemical staining procedures were used to determine the relative proportions of emerging macrophage subsets and the distribution of CD23. Treatment did not affect the emergence of the RFD1+ inducer macrophage phenotype during the culture period however, there was a significant increase in the proportion of RFD7+ effector macrophages as compared to the situation before treatment. Interestingly, monocytes from patients with AE differentiated faster than those from normal controls but by day 7, the proportions of differentiated macrophages were similar. At day 0, therapy reduced the proportion of RFD1+ monocytes coexpressing CD23 however this difference was lost after 7 days of culture. Notably, throughout the 7 day culture period, larger proportions of monocytes from patients with AE were found to express CD23 compared with normal controls.

To investigate aspects of T cell control of CD23 expression, methods of In situ hybridisation were performed on lesional skin before and after clinical improvement to identify the presence of mRNA for Interleukin-2 and Interleukin-4, (Th1 and Th2 type cytokines respectively). Following treatment, there was a decrease in the level of expression of mRNA for IL-4 and no significant change in the level of IL-2 mRNA.

This study reveals an aberrant expression of CD23 on antigen presenting cells within lesional skin in AE and that treatment leading to clinical improvement results in a downregulation of CD23 expression. Therapy also leads to a decrease in Th2 activity as demonstrated by reduced IL-4 mRNA levels in the lesions, suggesting that this may also be relevant to the pathogenesis of AE. This research has thus revealed that CD23 expression by antigen presenting cells and local TH2 like T cell activity, is modulated by a course of therapy that improves clinical disease status.

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ABBREVIATIONS

AE	atopic eczema
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
APC	antigen presenting cells
BCIP	5-bromo-4-chloro-3 indolyl phosphate
BSA	bovine serum albumin
CD	cluster of differentiation
CHT	Chinese herbal therapy
CLA	cutaneous lymphocyte associated antigen
DAB	3,3 diaminobenzidine tetrahydrochloride
Der p	Dermatophagoides pteronyssinus allergen
DNA	deoxyribonucleic acid
ELAM-1	endothelial leucocyte adhesion molecule-1
FcεRI	high affinity IgE receptor
FcεRII	low affinity IgE receptor
FITC	fluorescein isothiocyanate
HCL	hydrochloric acid
HDM	house dust mite
HLA	human leucocyte antigen
ICAM-1	intercellular adhesion molecule-1
IFN	Interferon
Ig	immunoglobulin
IL	interleukin
LC	Langerhans cells

LFA-1	lymphocyte function associated antigen-1
$MgCl_2$	magnesium chloride
MoAb	monoclonal antibody
mRNA	messenger ribonucleic acid
Mw	molecular weight
NaCl	sodium chloride
NBT	nitro blue tetrazolium
PB	peripheral blood
PBM	peripheral blood monocytes
PBS	phosphate buffered saline
PBSM	phosphate buffered saline containing magnesium chloride
RAST	radioallergosorbent test
RNA	ribonucleic acid
RNAse	ribonuclease
ROD	relative optical density
SSC	saline sodium citrate
TBS	Tris-buffered saline
Th1	T helper type 1 lymphocytes
Th2	T helper type 2 lymphocytes
TNF	tumour necrosis factor
TNMT	trinitro monotetrazolium
Tris	Tris(hydroxymethyl) methylamine
TRITC	tetramethylrhodamine isothiocyanate

CHAPTER 1

MAIN INTRODUCTION

1. MAIN INTRODUCTION

1.1 DEFINITION AND CLINICAL FEATURES OF ATOPIC ECZEMA

Atopic eczema (AE) is an inflammatory skin disease presenting with severe pruritis and classical features of dryness and erythema of the skin [Hanifin and Rajka 1980]. Lesions commence as vesicles or papules and secondary to scratching or itching, excoriations and lichenification develop. It has a chronic and fluctuating course with an age of onset usually between 2 and 6 months although the disease can start at any age [Hanifin and Rajka 1980]. During the infantile phase, the face is often involved and as the child begins to crawl, the extensor surfaces are commonly affected. The erythematous papules may often become secondarily infected leading to exudation and crusting.

As the name implies, AE is a disease associated with dysfunction of immunological mechanisms. The term atopy describes a state of hyperreactivity to environmental allergens where the immunological mechanisms of affected individuals "over respond", to a point where clinical signs or symptoms may be manifest. Atopy was originally described by Coca and Cooke in 1923. The immunological mechanisms involved are principally those associated with the production of IgE class antibodies and the binding of these antibodies to mast cells and basophils with the subsequent degranulation of these cells causing the release of pharmacologically active factors that may promote the signs and symptoms of disease. The commonest environmental agents that can promote

these reactions are house dust mite (the commonest allergens being Der p I and Der p II, enzymes found in faecal pellets), pollens, animal dander and some fungi [Thompson and Stewart 1993].

The clinical manifestations of atopic reactions range from hayfever (allergic rhinitis) to life threatening systemic anaphylactic reactions that some people experience after being sensitised to allergens such as antibiotics, most commonly penicillin; or in food allergies such as in allergy to peanuts. As well as these severe acute responses, atopic reactions are also associated with chronic clinical conditions. The commonest of these are allergic asthma and atopic eczema. Both these conditions may continue for years. In allergic asthma, reversible episodes of bronchospasm are induced in the lungs by exposure to environmental allergens. Interestingly, asthma may develop in subjects who show no evidence of atopy [Poulter and Burke 1996], and such observations led to research that has identified further underlying immunoregulatory dysfunction promoting a chronic inflammatory reaction in the bronchioles of asthmatics [Poulter *et al.* 1994]. It is now thought that a similar underlying problem may be associated with AE (see below).

Although the association with allergen driven IgE responses and granulocyte release of mediators still holds, it is clear that atopic reactivity to allergens is not the sole cause of eczema. Atopic status (allergic type reactivity to environmental allergen) can be demonstrated *in vivo* by "prick testing". Here a solution of allergen is placed on the skin and a prick is made through this solution into the skin thus introducing the allergen intradermally. Atopic subjects

will exhibit a classic acute wheal and flare reaction that is read 15 minutes after testing. Using a panel of common environmental allergens, it is known that up to 30% of the general population exhibit positive allergic responses. However the vast majority of these people do not have eczema. Furthermore, these artificially induced immediate type hypersensitivity reactions develop and resolve within minutes whereas AE is recognised as a chronic inflammatory condition. Such anomalies make it important to look more closely at the pathogenic mechanisms at play in this disease.

1.2 AETIOLOGY AND PATHOGENESIS OF ATOPIC ECZEMA

There are many reports of immunological abnormalities associated with AE. 80% of patients with AE have raised IgE levels in the peripheral blood and this is considered to be related to immediate allergic, or type I hypersensitivity [Johansson 1969]. Indeed an immediate type hypersensitivity response can be demonstrated in most patients with AE and they develop a wheal and flare response 15-20 minutes after pricking the skin with allergens (see above). "Prick" test positivity is only manifest to allergens that the subject has encountered and become sensitised to. The immune reactivity is caused by a specific IgE response to the allergen. The allergen specific IgE binds via Fc receptors to mast cells and other basophils and following further exposure to the allergen, (as occurs when prick testing is performed), there is cross-linking of the bound IgE, leading to degranulation of the mast cells and the release of vasoactive mediators. It is recognised however, that the clinical manifestations of AE can occur in subjects with serum IgE levels within the normal range.

Furthermore levels of IgE may fluctuate with time and may not necessarily correlate with disease severity [Jones *et al.* 1975].

The clinical severity of the disease including the area of skin affected also varies with time although there appear to be no consistent precipitating factors other than infection. Patients with AE are recognised as being more susceptible to superficial bacterial skin infections [Brook *et al.* 1996, White and Noble 1986]. This may simply result from bacterial colonisation within damaged skin, or may be a manifestation of an impaired immune defence system [Rogge and Hanifin 1976].

There are further features of AE that suggest that the immune system in these patients is abnormal. Attacks of herpes simplex may lead to a more widespread eruption, eczema herpeticum (Kaposi's varicelliform eruption), suggesting an increased susceptibility to the spreading of cutaneous Herpes in AE [Goodyear *et al.* 1996, Rasanen *et al.* 1987]. This indicates a failure of defence mechanisms to control the virus. There is undoubtedly depressed cell-mediated immunity in AE [McGeady *et al.* 1975] with diminished tuberculin reactivity [Uehara 1977]. This can be seen by the poor handling of viral, fungal and bacterial infections of the skin in AE patients. However the incidence of viral warts does not appear to be increased compared with non atopic individuals [Williams *et al.* 1993]. Sensitivity to contact allergens such as Dinitrochlorobenzene (DNCB) is also diminished in patients with extensive AE but this reaction returns to normal with remission of the disease [Uehara and Sawai 1989].

Regarding specific components of the immune defence system, cytotoxic responses of natural killer cells and cytotoxic T cells have been reported to be lowered in AE [Leung *et al.* 1983]. This may be the cause of the increased incidence of disseminated Herpes simplex infections mentioned above. Chemotaxis is impaired with slower migration of monocytes and neutrophils [Rogge and Hanifin 1976] perhaps contributing to the frequent superficial Staphylococcal infections [White and Noble 1986]. Indeed, Staphlococcus aureus can be isolated from the involved skin in more than 90% of patients with AE [Leyden *et al.* 1974].

Evidence of immunosuppression may appear paradoxical when chronic inflammatory lesions persist. However many of the bacteria known to colonise the AE lesions secrete exotoxins which act as superantigens and stimulate macrophages to release pro-inflammatory cytokines, as well as activating large numbers of T cells [Leung *et al.* 1995]. The T cells will secrete cytokines that may induce tissue inflammation [Campbell and Kemp 1997]. Thus immune dysfunction may work in a variety of ways and the development of the disease may depend on a multitude of factors that can build one on the other over time.

It has been suggested that dairy products aggravate the disease [Agata *et al.* 1993, Chandra *et al.* 1989] but studies in children have produced conflicting evidence as to whether egg and milk exclusion, for example, leads to clinical improvement of the disease [Neild *et al.* 1986, Atherton *et al.* 1978, Webber *et al.* 1989]. However one double-blind controlled crossover trial suggested that

dietary manipulation involving egg and milk avoidance is of benefit in reducing disease activity in some children with AE [Atherton *et al.* 1978]. Another study has shown that in families with a history of atopic disease, milk and egg avoidance in mothers breastfeeding infants reduced the subsequent incidence of AE in those children [Chandra *et al.* 1989]. Much work has investigated the role of house dust mites (HDM) in the development of AE associated with allergic reactivity to HDM related allergens Studies have shown that HDM are present in higher quantities in the mattresses of patients with moderate to severe AE [Beck and Korsgaard 1989, Colloff 1992], thus indicating that levels of exposure to HDM may be related to disease severity. However, there is conflicting evidence as to the benefit of eradication of the mites [Colloff *et al.* 1989, Tan *et al.* 1996]. A recent double blind placebo controlled study showed that the use of vacuuming and gortex covered mattresses led to a marked reduction in dust with an associated clinical improvement of the eczema [Tan *et al.* 1996].

In addition to these clinical findings, prick test reactivity to house dust mite antigen is usually positive in AE patients; there are often raised titres of IgE complexes in the serum [Stone *et al.* 1973, Jones *et al.* 1975] and HDM antigen specific T lymphocytes are present in lesional skin [Van der Heijden *et al.* 1991]. Despite the overwhelming evidence of the involvement of HDM in the immunopathogenesis of AE, the relative importance of this as a precipitating factor remains unclear.

There is increasing evidence that AE has a genetic component. Twin studies have shown for example that the incidence of AE in both individuals is significantly greater in monozygotic pairs, than in dizygotic twins where one subject may develop AE while the other twin does not [Shultz Larsen *et al.* 1986]. The allergen-specific IgE-mediated hypersensitivity that is associated with atopy has been shown to be transferred from an atopic bone marrow donor to a non-atopic recipient [Agosti *et al.* 1988]. This was shown by the occurrence of positive prick tests to aerollergens in previously negative subjects who were recipients of allogenic bone marrow transplantation from atopic donors. One study of seven atopic families revealed an autosomal dominant inheritance linked to a DNA marker on chromosome 11q [Cookson *et al.* 1989]. However a further study of 95 families with AE refuted such an association [Coleman *et al.* 1993]. Linkage studies have shown an association between atopy and the beta unit of the Fc epsilon RI which has been localised to human chromosome 11q 12-13 [Cox *et al.* 1998]. There have been further genetic linkages of AE with a mast cell chymase variant [Mao *et al.* 1998]. Finally atopy has been associated with a mutation in the alpha subunit of the interleukin 4 receptor [Hershey *et al.* 1997].

Another contributing factor to the development of atopic diseases including eczema may be exposure to aeroallergens and dietary allergens in the neonatal period [Holt *et al.* 1997]. In those genetically predisposed to AE, the immature immune system of the young child appears to preferentially produce T lymphocytes that secrete a cytokine profile that is associated with T helper type 2 (Th2) lymphocytes. Should this occur, the immune system appears to

maintain this tendency permanently thus possibly promoting atopic reactivity as this T cell subset secretes IL-4 and IL-5 [Yabuhara *et al.* 1997].

The Th2 cytokines interleukin 4 and 5 promote IgE production and eosinophilia. These features are both characteristic of atopy. During normal infancy, exposure to environmental allergens is thought to result in those T cells that respond to these allergens undergoing deletion or anergy and the subsequent development of immunological tolerance [Prescott *et al.* 1997]. This process may not occur in the atopic subject.

Thus the aetiology of AE is almost certainly multifactorial with genetic, immunologic and environmental factors interacting to produce the disease phenotype. With the emergence of new technology and epidemiological studies, elucidation of the cellular and molecular interactions in AE and therefore the exact pathogenesis may become an achievable goal.

1.3 HISTOLOGY OF LESIONAL SKIN

The histological features of lesional skin in AE change with time [McKee 1996]. In the acute lesion, there is marked intercellular oedema (spongiosis) which may result in accumulation of enough fluid to result in intraepidermal vesicle formation. There is also some epithelial thickening (acanthosis), vascular dilatation in the dermis, and parakeratosis. In chronic lesions there is less spongiosis with more marked epidermal proliferation with acanthosis and elongation of the rete ridges leading to psoriasiform hyperplasia . There is also

prominent hyperkeratosis as well as some parakeratosis. Although these features may be used to distinguish acute from chronic lesions, most characteristics are common to both. However it is recognised that the appearance of the lesions is not uniform and may vary, not only between individuals, but also within individual patients when separate sites are investigated.

In both acute and chronic lesions there is a dermal perivascular cellular infiltrate consisting mainly of mononuclear cells [Zachary *et al.* 1985a]. The majority of these are T lymphocytes [Braathen 1979]. There are also macrophages, Langerhans cells and mast cells present; with only occasional neutrophils, eosinophils, and basophils [Mihm 1976]. The preponderence of T lymphocytes and antigen presenting cells in the dermis is suggestive of a cell-mediated immune response within the lesion. Strikingly, the dermal infiltrate contains very few plasma cells which suggests that raised IgE titres commonly seen in the circulation are not a consequence of B cell activity in the skin.

1.4 IMMUNOPATHOLOGY

The immunopathology of AE presents us with an apparent paradox. On the one hand raised IgE levels, circulating IgE-allergen complexes and positive prick tests are all indicative of an antibody mediated response. On the other hand, the inflammatory infiltrate within lesional skin exhibits a T cell dominated cellular infiltrate suggestive of a chronic cell mediated response [Zachary *et al.* 1985a].

One is drawn to the conclusion that both types of immune response occur in AE and both may therefore have an impact on the pathogenesis.

Such a situation is not unique to AE. There are other conditions where both humoral and cell mediated responses are involved. In rheumatoid arthritis there are circulating levels of autoantibodies while the synovial tissue within the joints is predominantly infiltrated with macrophages and T lymphocytes, a T cell mediated response.

In asthma, there is evidence of type IV mediated hypersensitivity with a T cell infiltrate in the the bronchial wall [Poulter *et al.* 1994], yet allergic IgE responses may promote acute bronchoconstriction following exposure to aeroallergens. Indeed it has been suggested that it is a failure to downregulate the cell mediated response within the bronchial walls rather than the antibody response to aeroallergens that promotes the chronicity of the disease [Poulter *et al.* 1994].

There is little doubt that raised titres of circulating IgE occur in the majority of cases of AE and are responsible for the atopic status of the patient. Of the five classes of immunoglobulin found in the circulation, the IgE isotype is normally present at the lowest titre contributing less than 0.1% of total immunoglobulin [Ward *et al.* 1996] Normally expressed as International Units (IU), the normal range is quoted as 0-120 IU/ml [Ward *et al.* 1996]. In patients with AE, this figure may rise to >2000 IU/ml. Such changes are not unique to AE but reflect atopic status in general and are seen in cases of asthma and rhinitis [Jones *et*

al. 1975]. As IgE is a cytophilic antibody, such high titres inevitably cause attachment of the immunoglobulin to granulocytes via Fc receptors.

1.4.1 T Cells

Although the increase in serum IgE represents an abnormal antibody response, the dermal perivascular infiltrate in the lesions of AE is predominantly mononuclear with T lymphocytes, Langerhans cells and macrophages. The major cell type in lesional skin is the T lymphocyte, the majority of which are T helper lymphocytes expressing CD4, with a helper:suppressor ratio of 7:1 [Lever *et al.* 1987]. Many of these lymphocytes have undergone activation and express CD25 (Interleukin-2 receptor) and HLA DR (a class II major histocompatability complex molecule). It is now recognised that T lymphocytes may be functionally divided into Th1 and Th2 subsets [Del Prete *et al.* 1994], the balance of which may be functionally important in AE, (see below).

1.4.2 Langerhans Cells

There are increased numbers of Langerhans cells (LC) in the dermis of AE lesional skin [Zachary *et al.* 1985a]. These cells are identified under electron microscopy by the presence of Birbeck granules, or at the light microscopic level by expression of the antigen detected by MoAb CD1 [Ray and Schmitt 1988].

Langerhans cells are large dendritic cells normally found in the epidermis [Mihm 1976]. These cells differentiate from monocytes [Hanau *et al.* 1987] and are recognised as part of the "dendritic cell" family. As LC constitutively express surface class II MHC antigens (HLA-DR,DP,DQ) [Ruco *et al.* 1989], they are considered to be the antigen presenting cells of the skin. During inflammatory reactions, numbers of these cells migrate to the dermis where they are seen interdigitating with the accumulations of T cells [Bos *et al.* 1986, Alegre *et al.* 1986].

Although dendritic cells of the dermis have been called "indeterminate cells", these are now thought to be Langerhans cells involved in local immune reactivity [Zachary *et al.* 1985b, Alegre *et al.* 1986]. In lesions of AE, a proportion of these LC have been shown to have IgE bound to their surface [Bieber *et al.* 1989a]; a situation not seen in clinically normal skin, even in patients who exhibit raised serum IgE [Barker *et al.* 1988]. It would seem likely therefore that LC activity contributes to the pathogenesis of AE.

1.4.3 Eosinophils

Eosinophils are recognised as being associated with allergy and Type I hypersensitivity. Although there are normally only small numbers of eosinophils present in AE lesions, deposits of eosinophil derived major basic protein have been identified, probably as a result of eosinophil degranulation [Leiferman *et al.* 1985]. In other allergic diseases such as asthma, eosinophils are thought to play a major role [Busse and Sedgwick 1992]. Indeed circulating levels of

eosinophilic cationic protein have been found to correlate with disease severity [Czech *et al.* 1992, Halmerbauer *et al.* 1997].

Several studies have demonstrated that raised levels of serum eosinophilic cationic protein are present in patients with eczema [Miyasato *et al.* 1996, Halmerbauer *et al.* 1997]. This must be released from eosinophil granules and suggests that there is widespread activation of the eosinophil pool occurring in AE [Czech *et al.* 1992, Uehara *et al.* 1990]. It is not uncommon for a systemic eosinophilia to be present in patients with AE. Again however this phenomenon appears to be an atopy related phenomenon as it may also be seen in subjects with rhinitis and asthma [Griffin *et al.* 1991, Borres *et al.* 1995]. This eosinophilia is also highly variable as many patients with AE have normal levels of these cells in the circulation [Uehara *et al.* 1990]. It remains unclear what may regulate or influence the eosinophil count but the cytokine Interleukin-5 (released by a Th2 type T cell) may be a contributing factor, as this is recognised as chemotactic for eosinophils and can also promote eosinophil activation [Adachi and Alam 1998] (see below).

1.4.4 Cytokines

As stated above, T lymphocytes dominate in the lesions of AE. Within this population are high numbers of allergen specific T lymphocytes [Van der Heijden *et al.* 1991], clearly suggesting that the dermal T cell infiltrate represents a recall reaction induced locally by immune reactivity to aeroallergens. Activation of T cells within a cell-mediated response is in part

under the regulation of cytokines [Xu *et al.* 1996] and T cells contribute by being cytokine producers [Romagnani 1992a]. Thus knowledge of changes in levels of cytokines and information about their synthesis associated with the inflammatory reactions in AE may offer clues as to the mechanisms underlying the immunopathology.

Analysis of the cytokines produced by the housedust mite, Dermatophagoides pteronyssinus (Der p.), specific T cells in the cellular infiltrate has revealed that there are increased proportions of Interleukin-4 (IL-4) producing cells in the lesions, with relatively lower levels of Interferon-gamma (IFN-gamma) production [Van der Heijden *et al.* 1991]. Similarly, T cells in the peripheral blood of patients with AE have been shown to secrete higher amounts of IL-4 than IFN-gamma [Wierenga *et al.* 1990]. One of the important effects of IL-4 is the induction of expression of the low affinity IgE receptor (CD23) on Langerhans cells in the skin [Bieber *et al.* 1989b] and induction of similar expression on T cells isolated from peripheral blood [Sakamoto *et al.* 1992].

It is these cytokines and other soluble mediators that regulate the recruitment of the lymphocytes and monocytes from the peripheral blood thus orchestrating the inflammatory response. As well as these interactions by cell surface molecules and cytokines, adhesion molecules on endothelial cells are intimately involved in lymphocyte trafficking.

1.4.5 Adhesion Molecules

The vascular endothelial cells and keratinocytes in AE express adhesion molecules on the cell surface which are ligands for activated T lymphocytes [Cooper 1994]. This ligand to ligand interaction enables the T lymphocytes to migrate to the epidermis from the blood. Intercellular adhesion molecule-1 (ICAM-1) is expressed on keratinocytes and endothelial cells in the dermis of inflammatory conditions such as psoriasis, AE, lichen planus and mycosis fungoides and may regulate lymphocyte trafficking in the skin [Griffiths *et al.* 1995]. T lymphocytes also express the ligand lymphocyte function associated antigen-1 (LFA-1) [Griffiths *et al.* 1989]. These ligands can attach to adhesion molecules on endothelial cells lining blood vessels thus enabling T lymphocytes to leave the vessels and infiltrate the skin [Lub *et al.* 1995].

Endothelial cells express endothelial leukocyte adhesion molecule-1 (ELAM-1 or E selectin) which is a ligand for the cutaneous lymphocyte-associated antigen (CLA) expressed by the effector T lymphocytes that infiltrate inflamed skin [Santamaria *et al.* 1995]. The T lymphocytes that express these ligands are those that have recently undergone activation, perhaps in the lymph nodes, before becoming localised in the skin [Santamaria *et al.* 1995].

There are several cytokines that may induce the expression of ICAM-1 and ELAM-1 on the endothelial cells [Thornhill and Haskard 1990] leading to enhanced recruitment of leukocytes from the vasculature and extravasation and infiltration into the inflammatory milieu in lesional skin. Tumour necrosis factor

(TNF), released by mast cells and macrophages can induce ELAM [Lidington *et al.* 1998]. Interleukin-1 (IL-1), released by monocytes and keratinocytes can induce ELAM-1 and ICAM-1 [Lidington *et al.* 1998]; and the T lymphocyte derived cytokine, IL-4 potentiates ELAM-1 expression [Yao *et al.* 1996]. As described above, the cellular infiltrate in lesional skin in AE consists predominantly of T lymphocytes and also macrophages. The interaction between these cells and the release of cytokines by lymphocytes are all important in the regulation of the immunopathogenesis of AE.

1.4.6 Th1 And Th2 Cells

There are increased numbers of allergen-specific IL-4 producing CD4+ T lymphocytes in lesional skin of AE [Van der Heijden *et al.* 1991]. Furthermore increased IL-4 production by T cells is associated with reduced IFN-gamma production [Reinhold *et al.* 1991, Nakazawa *et al.* 1997]. Studies of the cytokine profile of murine T cells have revealed that T helper lymphocytes can be subdivided into type I (Th1) and type II (Th2), based on the repertoire of cytokines they produce [Romagnani 1992a]. Th2 predominantly produce cytokines IL-4, IL-5, IL-6 and IL-10 while Th1 predominantly produce IL-2 and IFN-gamma [Romagnani 1991]. Th1 cells are recognised as the mediators of delayed type hypersensitivity responses due to their ability to recruit monocytes and macrophages at the site of intracellular infections [Romagnani 1992a]. They produce IFN-gamma which can modify macrophage function by inducing the expression of Class II major histocompatibility complex cell surface antigen and stimulate the synthesis of tumour necrosis factor (TNF-alpha) [Virelizier

and Arenzana-Seisdedos 1985]. Th2 cells on the other hand are the synthesizers of IL-4 which has multiple effects on mononuclear cells, including induction of B cell IgE production [Parronchi *et al.* 1990], induction of CD23 expression on monocytes, Langerhans cells and B lymphocytes [Bieber *et al.* 1989b]. It also inhibits T lymphocyte IFN-gamma production [Pene 1989]. Thus the overall functional capacity of the T cell pool, particularly in inflammatory infiltrates will reflect the relative balance of these two subsets.

In AE, it has been suggested that there is an imbalance of this Th1/Th2 ratio in both the peripheral blood and in the lymphocytes infiltrating the skin [Bos *et al.* 1992], with a bias towards Th2 type IL-4 secreting T lymphocytes. This observation provided the rationale for the clinical trials of recombinant IFN-gamma treatment of AE [Renz *et al.* 1992] (see below).

The underlying cause of this imbalance remains unclear. However, it may be that defective monocyte control contributes to this imbalance in the cytokines secretion by T lymphocytes as there is abnormally increased cyclic adenosine monophosphate(cAMP)-phosphodiesterase activity in monocytes in AE [Holden 1990]. This defect is associated with an increased monocyte prostaglandin E_2 secretion which is inhibitory for T cell derived IFN-gamma. As aberrations in LC and macrophage function have also been reported (see above), this dysfunction of monocytes implicates this family of non lymphoid cells in the overall pathogenesis of AE.

1.4.7 Macrophages

The monocyte/macrophage series is part of the bone marrow derived mononuclear phagocyte system [Johnston 1988]. The functions of these cells include: antigen processing and presentation [Unanue and Allen 1987]; secretion of bioactive products including IL-1 [Dinarello1985], tumour necrosis factor [Beutler and Cerami 1987] complement components and bioactive lipids such as leukotrienes [Nathan 1987]; and of course phagocytosis [Johnston 1988]. They also have pro-inflammatory activity in that macrophages are recruited to sites of inflammation and release further chemotactic factors to attract further cells.

Monocytes differentiate into macrophages as they enter the tissues. Macrophages are a heterogeneous group of cells whose subsets can be defined both functionally and phenotypically [Spiteri and Poulter 1991]. Previous extensive studies have developed the use of two antibodies to subdivide macrophages further according to their phenotype. These are RFD1 and RFD7 monoclonal antibodies.

Originally described in 1986 [Poulter *et al.* 1986, Janossy *et al.* 1986], these mouse anti human monoclonals have been used by many laboratories to dissect the heterogeneity of macrophage function [Lenz *et al.* 1993, Seldenrijk *et al.* 1989, Teunissen *et al.* 1990, Zheng *et al.* 1995]. RFD1 is a mouse IgM MoAb that recognises a membrane epitope associated with antigen presentation. It is constitutively expressed on dendritic cells and on some B

lymphocytes [Poulter *et al.* 1986]. Macrophages and Langerhans cells (in the epidermis) are RFD1-ve but can be induced to express this molecule on activation [Teunissen *et al.* 1990]. RFD7 is a cytoplasmic 77kd glycoprotein associated with mature phagocytes [Poulter *et al.* 1986]. It is not expressed by dendritic cells or monocytes. Originally it was thought that the expression of these molecules was mutually exclusive, however, first in the lung, and then in other tissues a third subset of macrophages that expressed both RFD1 and RFD7 positivity was identified [Spiteri *et al.* 1992b].

Of some significance, is the fact that these phenotypically distinct subsets have been shown to exhibit functional heterogeneity. Cells with the phenotype RFD1+RFD7- act predominantly as inducers of T cell activation, while those with the phenotype RFD1-RFD7+ act as phagocytes [Poulter *et al.* 1996]. Cells expressing positivity with both MoAbs (RFD1+RFD7+) exhibit T cell suppressive activity [Spiteri *et al.* 1992b, Spiteri and Poulter 1991]. This is a dynamic group of cells where one phenotype can change into another. Such plasticity can be promoted by cytokines in the surrounding environment [Tormey *et al.* 1997]. Thus as with Th1 and Th2 T cell subsets, the overall functional capacity of the macrophage pool is dependent on the relative balance, in this case, of at least three subsets [Poulter *et al.* 1996 and Poulter *et al.* 1994].

Interestingly, LC within the dermis of AE lesions bear not only CD1 but also express RFD-1 which is expressed only by antigen presenting cells [Zachary *et al.* 1985b, Alegre *et al.* 1986]. Furthermore, RFD1+ antigen presenting cells not expressing the CD1 LC marker are also present in relatively large numbers in

AE lesions [Bos *et al.* 1986]. All of these cells strongly express HLA DR. The evidence thus clearly shows that within the cellular infiltrate of lesions of AE, not only are there are activated T lymphocytes, but also activated antigen presenting cells. Furthermore in AE, these cells may carry bound IgE [Bruynzeel-Koomen *et al.* 1986, Leung *et al.* 1987], a situation not seen in other dermatoses [Barker *et al.* 1988]. The reports of IgE present on the surface of the antigen presenting cells implicate IgE in the cell-mediated response exhibited within the skin in AE, and possibly suggest a link between the T cell mediated reactions of the lesions and the manifestations of atopy in these patients (see below).

Cells of the mononuclear phagocyte system exhibit a wide range of receptors including receptors whose exact function is unclear. These include receptors for insulin [Bar *et al.* 1977], transferrin [Spik and Montreuil 1983], thrombin [Silverstein and Nachman 1987], and fibrinogen [Mosesson 1984]. Other receptors which may be expressed on the surface include those for complement components such as C3b [Law 1988], receptors for lipoproteins [Fogelman *et al.* 1988], receptors for IgG and IgE [Ezekowitz and Gordon 1986, Gordon *et al.* 1989].

Receptors for IgE can be divided into two types, the high affinity (FcεRI) and low affinity receptor (FcεRII or CD23) [Bieber 1992, Wang *et al.* 1992, Sakamoto *et al.* 1990, Takigawa *et al.* 1991]. CD23 is expressed on peripheral blood monocytes, eosinophils, platelets and lymphocytes (predominantly B cells) [Kehry and Hudak 1989, Delespesse *et al.* 1992]. It has been shown that

CD23 in normal skin is expressed by RFD7+ macrophages and LC, but in AE there is a switch to expression by the RFD1+ inducer cells [Buckley *et al.* 1992]. In both aeroallergen patch tests and lesional skin in AE, the proportion of LC and other RFD1+ cells expressing CD23 is increased while the proportion of RFD7+ macrophages expressing CD23 is reduced [Buckley *et al.* 1992]. The emergence of CD23 on antigen presenting cells suggests that the redistribution of CD23 within macrophage subsets may be important in the pathogenesis of AE and that CD23 may be involved in antigen presentation.

1.5 LINK BETWEEN ALLERGY AND TYPE IV HYPERSENSITIVITY

The firm conclusion to be drawn from studies of the immunopathology of AE to date, is that the skin inflammation associated with this condition bears all the hallmarks of a T cell driven Type IV hypersensitivity reaction. What then are the relationships between this and the atopic status carried by patients suffering from this disease? There is no doubt that AE patients are atopic as virtually all exhibit prick test positivity to aeroallergens and have raised titres of IgE in the circulation [Stone *et al.* 1973]. The reports of IgE bound to macrophages and dendritic cells in the lesional infiltrates offer a possible clue to the interrelationship between atopic reactivity and T cell mediated immunity.

IgE is a cytophilic antibody readily binding to mast cells, basophils, some monocytes and macrophages [Sutton and Gould 1993]. This binding occurs via receptors for the Fc region of the immunoglobulin. It is the binding of IgE to

these receptors that creates the state of "sensitivity" to allergens as cross linking of the antigen binding sites of the IgE molecules by allergens, precipitates the degranulation of mast cells producing the immediate type hypersensitivity reactions characteristic of allergy. The low affinity receptors, recognised by MoAb CD23, are thought to be less involved in these allergic reactions [Sutton and Gould 1993]. However, initial studies of the distribution of IgE receptors in AE lesional skin demonstrate that it is expression of the low affinity CD23 molecules that is downregulated by treatment and not FcεRI [Xu *et al.* 1997].

Much work has been done investigating the possible role of IgE receptors in presenting antigen to T cells. It is recognised that if the IgE bound to these receptors on appropriate cells is complexed to antigen, this may promote T cell stimulation [Van Der Heijden *et al.* 1995, Mudde *et al.* 1990b] (facilitated antigen presentation). Although most studies of this mechanism have concentrated on the role of FcεRI [Bieber 1997, Maurer and Stingl 1995], this form of IgE complexed antigen presentation may occur if the IgE is bound to CD23. Indeed in AE, CD23 has been shown to be expressed by antigen presenting cells rather than macrophages [Buckley *et al.* 1992].

Evidence linking a delayed type hypersensitivity response to atopy in AE comes from the study of patch test reactions in such patients. As well as exhibiting prick test reactivity to aeroallergens, up to 40% of AE patients may exhibit a delayed type reactivity if the specific allergen is applied as a "patch" epicutaneously [Buckley *et al.* 1992, Clark and Adinoff 1989], normally

positioned on the upper back. These reactions peak at 48 hours and exhibit not only signs of erythema and induration but also mononuclear cell infiltration in the dermis [Buckley *et al.* 1992]. Immunohistological analysis of these reactions revealed them to exhibit all the features of AE lesional skin, including the aberrant expression of CD23 on antigen presenting cells [Buckley *et al.* 1993]. Importantly, when such reactions were directly compared to nickel induced contact sensitivity reactions, it was the redistribution of low affinity IgE receptors that distinguished the allergen patch test from the contact reaction [Buckley *et al.* 1993].

The possible progression from allergic reactivity to T cell mediated hypersensitivity via facilitated antigen presentation suggests therefore a link between atopy and cell mediated immunity and an underlying complexity to the pathogenesis of AE.

It goes without saying therefore that these complexities in the pathogenesis of AE, introduced above, should be taken into account when considering the treatment options. It is important in the development of effective and safe therapy to target relevant abnormalities within the immunopathogenic mechanisms observed. To decipher the parameters of immune dysregulation relevant to the clinical situation, a model can be developed whereby peripheral blood and lesional skin from patients with AE before and after effective treatment is investigated. This approach provides the opportunity, using immunocytochemical techniques, to understand the underlying pathogenic mechanisms and their relevance to severity of clinical disease.

1.6 MANAGEMENT

There are several approaches to the management of AE that vary considerably, ranging from manipulation of patient behavioural characteristics, to immunomodulatory treatment. The immunomodulatory treatment can be topical or systemic and the choice of treatment depends on severity of disease and degree of disability that the patient experiences as a result of the disease.

1.6.1 Behavioural

An important part of the management of patients with AE, as in all chronic diseases, is explanation, reassurance and discussion of the various treatment options available. Patients are advised to avoid the application of substances that dry or irritate the skin such as soaps and detergents. Advice is given to avoid other aggravating factors such as food that is known to lead to worsening of the disease in an individual. It is recommended that any attempts to follow any dietary regime should be under the supervision of a dietician [Webber *et al.* 1989].

1.6.2 Symptomatic

Frequent and liberal application of emollients to the skin is recognised as important in combatting dryness of the skin in AE. This is especially important after bathing to retard evaporative water loss from the epidermis. Antihistamines are often used as an adjunct to therapy. It is most common to

recommend those with a sedating effect as it is this central, sedative action that appears to be of benefit, rather than peripheral H_1 antagonism [Wahlgren *et al.* 1990]. Oral antibiotics are often required to treat exacerbations of eczema caused by secondary bacterial infection. For eczema herpeticum, which can be a complication of AE, either oral or intravenous acyclovir is used. Groups have tested the value of alternative approaches with varying success. For example a recent double blind placebo-controlled trial showed that the treatment of AE with essential fatty acid supplements, evening primrose oil and fish oil did not lead to clinical improvement [Berth-Jones and Graham-Brown 1993], although some studies had previously reported some benefit from such treatment [Wright and Burton 1982, Schalin-Karrila *et al.* 1987].

1.6.3 Immunomodulation

1.6.3.1 Topical Therapy

Topical corticosteroids are the mainstay of treatment of AE. Corticosteroids inhibit the expression of many pro-inflammatory cytokines by binding to gene transcription factors, upregulating the expression of cytokine inhibitory proteins, and reducing the half-life and utility of cytokine mRNAs [Brattsand and Linden 1996]. The number of Langerhans cells with IgE bound to the surface is also downregulated by treatment with topical corticosteroids [Bieber *et al.* 1989a]. This observation supports the role of IgE and the expression of its receptor on antigen presenting cells as being of relevance to the immunopathogenesis . The therapeutic range of corticosteroids used varies from mild to very potent. Side effects include thinning of the skin leading to striae atrophicae and

telangiectasia. Long-term use of topical steroids on the eyelids has been associated with the occurrence of glaucoma [Cubby 1976].

1.6.3.2 Systemic Therapy

1) Oral Corticosteroids:

Because of the failure of topical corticosteroids to control the disease in some cases, short courses of oral corticosteroids may be prescribed. Long term use is not considered advisable due to the side effects which include osteoporosis, peptic ulceration, Cushing's syndrome, glaucoma, neuropsychiatric effects, weight gain, impaired healing and easy bruising of the skin [Schimmer and Parker 1996]. Corticosteroids modulate cytokine expression by a combination of genomic mechanisms as described above. Administration of corticosteroids has been shown to decrease high peripheral blood eosinophil counts and decrease the survival rate of eosinophils by increasing apoptosis in patients with AE [Matsukura *et al.* 1997]. Methylprednisolone has also been shown to modulate circulating soluble interleukin 2 receptor levels [Sauer *et al.* 1993].

2) Ultraviolet Phototherapy:

It has been shown that treatment with psoralens and ultraviolet A (PUVA) is often effective [Atherton *et al.* 1988, Larko 1996, Sheehan *et al.* 1993, Yoshiike *et al.* 1993] in the treatment of AE. Although Broad band ultraviolet B was initially thought to be less effective in the treatment of AE [Jekler and Larko 1991], a subsequent study has shown that narrow band ultraviolet B (TL-O1) therapy is as effective as PUVA in the treatment of chronic severe AE [George *et al.* 1993]. A recent open multicentre trial showed that high dose UVA1

radiation is effective for severe exacerbations of AE [Krutmann *et al.* 1998]. However there can be long term problems involved in such therapies. For example there is a dose-dependent increase in the risk of developing squamous cell carcinoma of the skin following treatment with PUVA [Lindelof *et al.* 1991, Bruynzeel *et al.* 1991]. There also appears to be an increased risk of developing basal cell carcinomas [Bruynzeel *et al.* 1991]; and one study has shown an increase in the incidence of respiratory cancer in males and females, pancreatic cancer in males, and kidney and colonic cancer in females [Lindelof *et al.* 1991].

3) Azathioprine:

Azathioprine is a purine antimetabolite. There have been no controlled studies of azathioprine in the treatment of AE although it has been used as long-term therapy for chronic AE [Younger *et al.* 1991]. This drug inhibits cell division, and bone marrow suppression is a potential serious side effect [Min and Monaco 1991]. Other side effects include nausea, vomiting and diarrhoea [Diaso and LoBuglio 1996]. Long term immunosuppression may be the reason why certain tumours develop more commonly in patients treated with azathioprine such as non-Hodgkin's lymphoma and squamous cell carcinoma [Taylor and Shuster 1992]. However, it is highly likely that this suppression of the immune system is a necessary requirement for clinical improvement in AE.

4) Cyclosporin:

Cyclosporin belongs to the family of cyclic polypeptides derived from the fungus *Tolypocladum inflatum Gavis* and is a potent immunosuppressant

[Diaso and LoBuglio 1996]. In the last few years cyclosporin has been used increasingly to treat severe refractory AE [Sowden *et al.* 1991]. The immunosuppressant effects of cyclosporin have already been established with its use in transplantation to prevent rejection. Within lesional AE skin, treatment with oral cyclosporin, leads to a decrease in the number of activated T cells expressing the IL-2 receptor [Van Joost *et al.* 1992]. A double-blind crossover study in adults showed that treatment with cyclosporin leads to an improvement in clinical disease [Sowden *et al.* 1991] and also quality of life [Salek *et al.* 1993]. However the 8 weeks of treatment in this latter study, caused a rise in the mean serum urea and creatinine, and a significant rise in bilirubin in some patients. Thus the monitoring of blood pressure and renal and liver function tests during treatment is essential. Other side-effects reported include parasthesia, gastro-intestinal upset, gingival hyperplasia and hypertrichosis [Diaso and LoBuglio 1996, Min and Monaco 1991]. There is also a reported increase in the incidence of malignancies and lymphoproliferative disorders following cyclosporin [Ryffel 1992, Gruber *et al.* 1994, Tanner and Alfieri 1996].

5) Interferon Gamma:

Subcutaneous injections of a recombinant form of this immunomodulatory drug has been shown to be effective in the treatment of AE. A double-blind, placebo-controlled trial in adults of daily subcutaneous recombinant interferon gamma for 12 weeks showed significant clinical improvements [Hanifin *et al.* 1993]. This study showed that side-effects of headaches, myalgias and chills were common

however transient granulocytopaenia occurred in some patients. Some patients treated with interferon gamma also develop mild elevation of liver enzymes.

6) Tacrolimus:

Tacrolimus or FK506 is a macrolide antibiotic and inhibits T cell activation by binding to cytosolic protein FKBP (FK506 binding protein) [Wiederrecht *et al.* 1993]. Oral tacrolimus has been used as an immunosuppressant for the prevention of rejection in organ transplant patients and has also been associated with an increased incidence of lymphoma in transplant recipients [Ciancio *et al.* 1997]. More recently it has been found to be effective when used topically for the treatment of AE [Ruzicka *et al.* 1997, Alaiti *et al.* 1998].

7) High Dose Intravenous Immune Globulin:

Administration of intravenous immune globulin on a regular basis has been shown to reduce the need for systemic steroids in both asthma and AE [Gelfand *et al.* 1996]. The mechanism of action is unclear although it has been suggested that in AE, treatment with high dose intravenous immune globulin leads to a downregulation in IL-4 release by T cells [Jolles et al 1999].

8)Thymopentin:

There have been several studies using subcutaneous injections of thymopentin for periods of six to twelve weeks in the treatment of AE [Stiller *et al.* 1994, Hsieh *et al.* 1992, Leung *et al.* 1990]. The long term benefits of this treatment have not been clearly addressed in these studies. One of the mechanisms of

action may be the modulation of cytokine release, leading to a downregulation of IL-4 release by T cells [Hsieh *et al.* 1992, Braga *et. al.* 1996].

9) Chinese Herbal Therapy:

For centuries, Chinese herbal therapy (CHT) has been used to treat eczema in China and there are increasing numbers of herbalists that administer cocktails of CHT in Britain. A proportion of patients who do not respond to conventional therapy or develop complications do seek other less conventional forms of treatment. Indeed many patients are interested in alternative therapies.

In an effort to develop a further effective yet safe form of treatment, two double-blind, placebo-controlled studies have been undertaken. These have shown that a specific combination of 10 herbs is effective in the treatment of atopic eczema, both in adults and in children [Sheehan and Atherton 1992, Sheehan *et al.* 1992]. The most common side effects experienced include nausea, diarrhoea and abdominal bloating. Most side effects of CHT appear relatively mild, however, it is recognised that hepatitis may be a complication of treatment with CHT and there have been reports of liver damage amongst patients who have received CHT from herbalists [Perharic *et al.* 1995]. However, results of patients' liver function tests monitored during the above trials did not show any persistent abnormalities. In one study there were two children reported to have asymptomatic elevation of the liver enzyme, aspartate aminotransferase which returned to normal within eight weeks of stopping the CHT [Sheehan and Atherton 1994].

As part of the model of clinical disease variation for this thesis, a group of patients with moderate to severe AE were treated with the 10 herb preparation of CHT. This provided the opportunity to analyse samples of lesional skin and peripheral blood from patients whose disease severity changed following treatment. Thus changes in immunopathology that could be related to variations in disease severity might be identified and potentially lead to a better understanding of the immune mechanisms directly relevant to the immunopathogenesis of AE.

1.7 AIMS

There is no doubt that a full understanding of the pathogenesis of AE should provide a more rational approach to treatment and It is clear that many of the treatments that are effective in AE modify the immune response in some way. However the exact mechanism of action is not established in any of the therapies. It is important to determine specific abnormalities in the chain of pathological events within the immune system as these may provide targets for a therapeutic approach. The area of immunopathogenesis requires further investigation. In this study immunohistological techniques are used with monoclonal antibodies to define cell phenotypes. mRNA probes are used to define cytokine expression in situ.

Specific aims are:

1. To compare the decoction of Chinese herbal therapy with a freeze dried granule preparation and characterise this as a model of efficacious therapy.
2. To investigate the effect of Chinese herbal therapy on monocyte differentiation.
3. To determine whether local T cell derived cytokines in the skin influence the pathogenesis.

CHAPTER 2

A MODEL OF CLINICAL DISEASE MODIFICATION IN PATIENTS WITH ATOPIC ECZEMA USING TREATMENT WITH CHINESE HERBAL THERAPY

2. A MODEL OF CLINICAL DISEASE MODIFICATION IN PATIENTS WITH ATOPIC ECZEMA USING TREATMENT WITH CHINESE HERBAL THERAPY

2.1 INTRODUCTION

An ideal way to investigate the relationship between specific immune parameters and their relationship with disease severity is to investigate changes within the skin and peripheral blood in association with changes in clinical appearance. A dynamic model can be created whereby patients with moderate to severe AE are treated with effective therapy so that their clinical disease improves and becomes mild. Thus analysis of immunological parameters in skin and peripheral blood using immunocytological analysis both before and after such treatment provides the opportunity to develop associations between changing disease status and changing immunopathology. The aim of this approach is to identify those specific immunological alterations relevant to the clinical signs and symptoms in AE.

A proportion of patients with AE do not respond to conventional therapy and recently two double-blind placebo-controlled trials have shown that Chinese herbal therapy (CHT) is an effective treatment of atopic eczema in both children and adults [Sheehan and Atherton 1992; Sheehan *et al.* 1992].

In these trials of CHT, the patients were given sachets of 10 dried herbs which were boiled daily for 90 minutes in 1 litre of water. This resulted in an unpleasant smelling and tasting decoction which the patients had to drink

shortly after preparation each day. To produce a less time consuming, more palatable and more conventional preparation of this effective treatment, a freeze dried formulation of these herbs has been manufactured. Early studies have shown this to be as effective as the previous formulation. At the Royal Free Hospital, an open randomised study was designed involving administering either the new freeze dried preparation or the previous herbal decoction formulation of CHT, to patients with AE. This provided the opportunity to obtain matched skin and peripheral blood samples from patients who received treatment leading to a clinical improvement in disease.

The aims of the study were to determine the relative effectiveness of these two preparations and patient compliance. This chapter describes this study and reports the effect of these treatments on severity of AE.

2.2 STUDY DESIGN

68 patients with moderate to severe refractory AE, diagnosed according to recognised criteria [Hanifin and Rajka 1980], were recruited. The study received approval from the Royal Free Hospital Ethics Committee and all patients gave written, informed consent to take part. Patients were defined as having moderate to severe disease by assessing the extent and severity of clinical disease, severity of pruritus and chronicity of the disease. The demographic details of the patients are shown in Table 2.1. Patients included in the study were aged 16 to 65 years with a mean age of 32 years, had normal baseline full blood counts, liver function and renal function tests. Women of childbearing age agreed to take appropriate contraceptive precautions during, and for 3 months

after the trial. The patients had longstanding AE with a mean age of onset of 3 to 5 years. All the patients had used topical steroids and emollients and many had been prescribed systemic treatment, with 43 receiving courses of oral steroids and 32 ultraviolet phototherapy. The numbers of patients receiving such treatments were evenly distributed between the two groups.

Patients were excluded if the eczema was exudative or impetiginised or if they had received oral corticosteroids, immunosuppressive therapy or ultraviolet phototherapy within the previous 2 months. Patients were also excluded if they had received systemic antibiotics within the previous 2 weeks, if they had any serious concomitant illness or if they had participated in previous trials of CHT. Women who were pregnant, intending to become pregnant or were breastfeeding were excluded.

All patients were asked to maintain their current diet and topical therapy. In particular, they were instructed not to alter the frequency or potency of topical corticosteroid use during the eight week treatment period. All the patients had been using topical steroids consistently for a period of at least 1 month prior to starting treatment with CHT to maintain a stable baseline clinical appearance. Patients were randomised using random number tables into two groups.

38 patients were randomised to Group I. They were placed on therapy using the traditional method of preparing the herbs which involved boiling four sachets in 1 litre of water for 90 minutes daily for 8 weeks. The herbs were: *Ledebouriella seseloides, Potentilla chinensis, Clematis armandii, Rehmannia glutinosa, Paeonia lactiflora, Lophatherum gracile, Dictamnus dasycarpus,*

Tribulus terrestris, Glycyrrhiza glabra, Schizonepeta tenuifolia. The herbs were grown in mainland China and were prepared by methods described in the Chinese Pharmacopoeia. All herbs were screened for microbial contaminants and aflatoxin and for any heavy metal impurities such as lead, selenium and chromium. Thin-layer chromatography was performed to "finger-print" the individual herbs in each batch. The herbs were finely ground and packaged in 10 gram porous sachets. The sachets were prepared by Phytopharm Plc, Godmanchester, Cambridgeshire, UK.

The second group of randomised patients, Group II, consisted of 30 patients. They were asked to swallow the contents of 4 packets of granules daily for 8 weeks. The granules were swallowed with cold water or sprinkled on cold food by patients. The 10 herbs used were identical to those used in previous trials and underwent the same screening and identification procedure [Sheehan *et al.* 1992]. The granules were also prepared by the pharmaceutical company, Phytopharm, by boiling batches of 100kg of herbs in 250litres of water to produce a liquor which was then freeze dried. The powder was then compressed into granules which were coated with a flavourless lacquer which contained no active ingredients. Care was taken to ensure that exactly the same quantity of each herb was present in both of the preparations.

Table 2.1 Demography of patients enrolled into Group I (decoction) and Group II (freeze dried granules) in the Chinese herbal therapy study

	Group I	Group II
n	38	30
Mean age (Range)	32 years (19-65)	32 years (16-45)
Sex	20 male	18 male
	18 female	12 female
Age of onset (Range)	3 years (0-28)	5 years (0-35)
Previous oral steroids	23	20
Previous UV phototherapy	19	13

2.2.1 Patient Assessment

Patients were assessed clinically prior to starting treatment and after four and eight weeks of treatment with either form of CHT. Quantitative assessments of erythema and surface damage (i.e. papulation, vesiculation, scaling, excoriation and lichenification) were made using a standardised scoring system (Heddle *et al.* 1984) and the patients were assessed by the same person at each visit to reduce variability between different assessors.

The body surface was divided into 20 roughly equal areas, 10 on the front and 10 on the back and within each area, a score of 0 to 3 was given for the severity of erythema and severity of surface damage with 3 representing the most severe. A score of 1 to 3 was then given for the percentage area involved within each zone where 1 was <33%, 2 was 34% to 66% and 3 was >67%. A score for each erythema and surface damage was calculated for each of the 20 zones. The sum of the severity score multiplied by the area score (maximum 3x3 = 9) gave a score for each of the 20 areas assessed. The total score for erythema or surface damage was the sum of the scores from the 20 zones, the maximum being 180. The patients estimated the severity of itch and severity of their eczema before starting treatment with CHT then after 8 weeks. A linear analogue scale from 0 to 10 was used where 10 was the most severe.

Each time the patients were clinically evaluated, venesection was performed for full blood count, urea and electrolytes, serum bilirubin, aspartate aminotransferase, alanine transaminase, gamma glutamyl transferase, alkaline phosphatase, albumin, calcium and phosphate. It was established at the start of

the study that patients who showed persistently abnormal full blood counts, increased serum bilirubin or liver enzymes to above 1.5 times the normal range or other complications should be withdrawn from the trial. Furthermore, patient compliance was emphasized and it was established prior to treatment, that if on questioning, failure to take the treatment on more than 5 days in a 4 week period was reported, patients would be withdrawn. Requirement for systemic corticosteroids or antibiotics during the 8 week treatment period was also identified as a reason for withdrawal from the trial.

2.2.2 Statistics

The mean scores for both erythema and surface damage were calculated. The mean scores were compared after 8 weeks of treatment with the baseline score prior to commencing CHT using a paired t test. Similarly, the means for the patient estimates of the severity of itch and severity of their disease were compared after 8 weeks of treatment with baseline using the paired t test where significance was recorded as $p \leq 0.05$. The number of patients in each group who complained about the palatability of the formulations of CHT was compared using Chi-Squared.

2.3 RESULTS

Of the 68 patients recruited to the study, 38 patients were randomised to prepare and consume the decoction (Group I) of which 32 completed the study. 30 patients received the freeze dried granules (Group II) of whom 25 actually completed the 8 week treatment period. Of the 6 patients who did not complete the study in group I; 4 were lost to follow up, 1 withdrew after 18 days because she was admitted to hospital with a supraventricular tachycardia complicating a chest infection and 1 was unable to tolerate the taste. Of those patients whose data was not analysed in Group II; 2 were lost to follow up, 1 developed worsening of the atopic eczema, 1 withdrew due to persistent nausea and 1 withdrew due to severe diarrhoea.

2.3.1 Objective Assessment Of Clinical Disease

There was a significant decrease in mean scores for erythema and surface damage in both groups (Figs. 2.1 and 2.2). At week 0 (just prior to starting treatment with CHT), Group I patients had a mean score for erythema of 61 and for surface damage 40. For Group II patients, the mean score for erythema was 57 and for surface damage 44. After 8 weeks of treatment, the mean score for erythema in both groups was 25; and for surface damage, 15 in Group I, and 16 in Group II, ($p< 0.0001$ for erythema and $p<0.0001$ for surface damage in Group I; and $p<0.0009$ for erythema and $p<0.0004$ for surface damage in Group II). This data represents a 58% reduction in erythema scores and 61%

reduction in surface damage scores from baseline in Group I (Figs. 2.1A and 2.2A) and a 55% reduction in erythema and 63% reduction in surface damage from baseline in Group II (Figs. 2.1B and 2.2B).

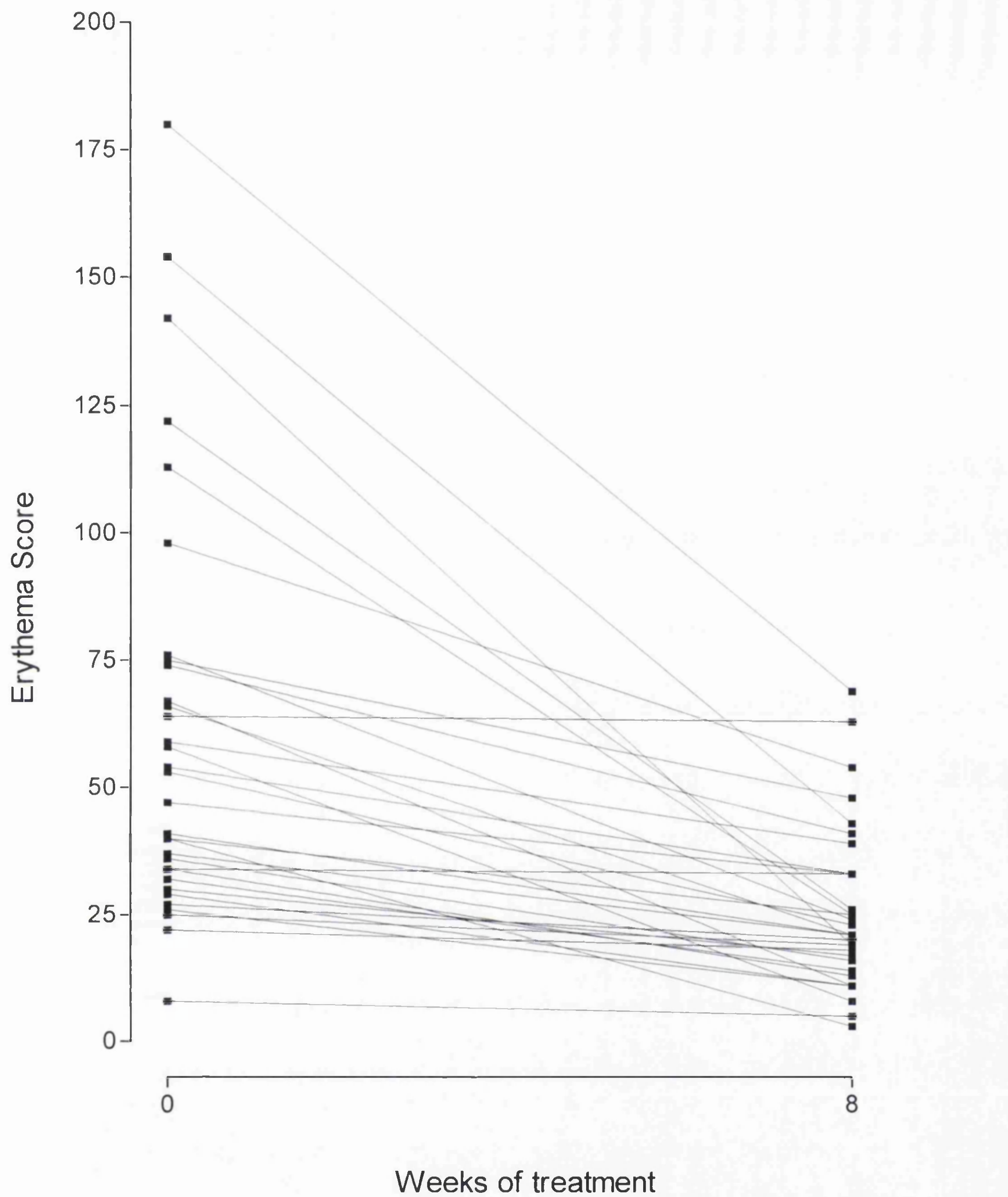

Figure 2.1(A) The erythema scores for Group I patients, who received the decoction, before and after 8 weeks of treatment with CHT. Values represent an assessment of the degree and severity of the erythema in each patient, based on an established clinical scoring system, (see methods).

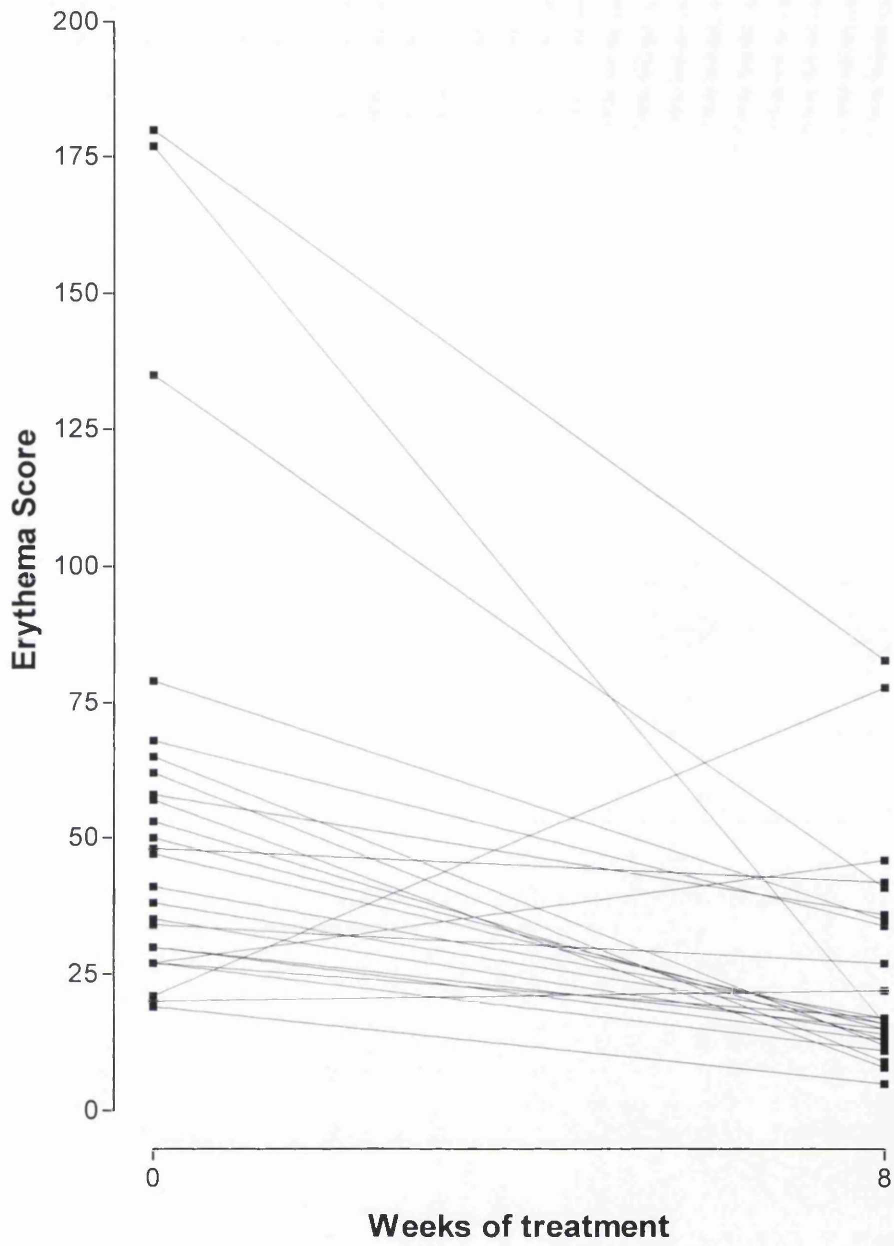

Figure 2.1(B) The erythema scores for Group II patients, who received the granules, before and after 8 weeks of treatment with CHT. Values represent an assessment of the degree and severity of the erythema in each patient, based on an established clinical scoring system, (see methods).

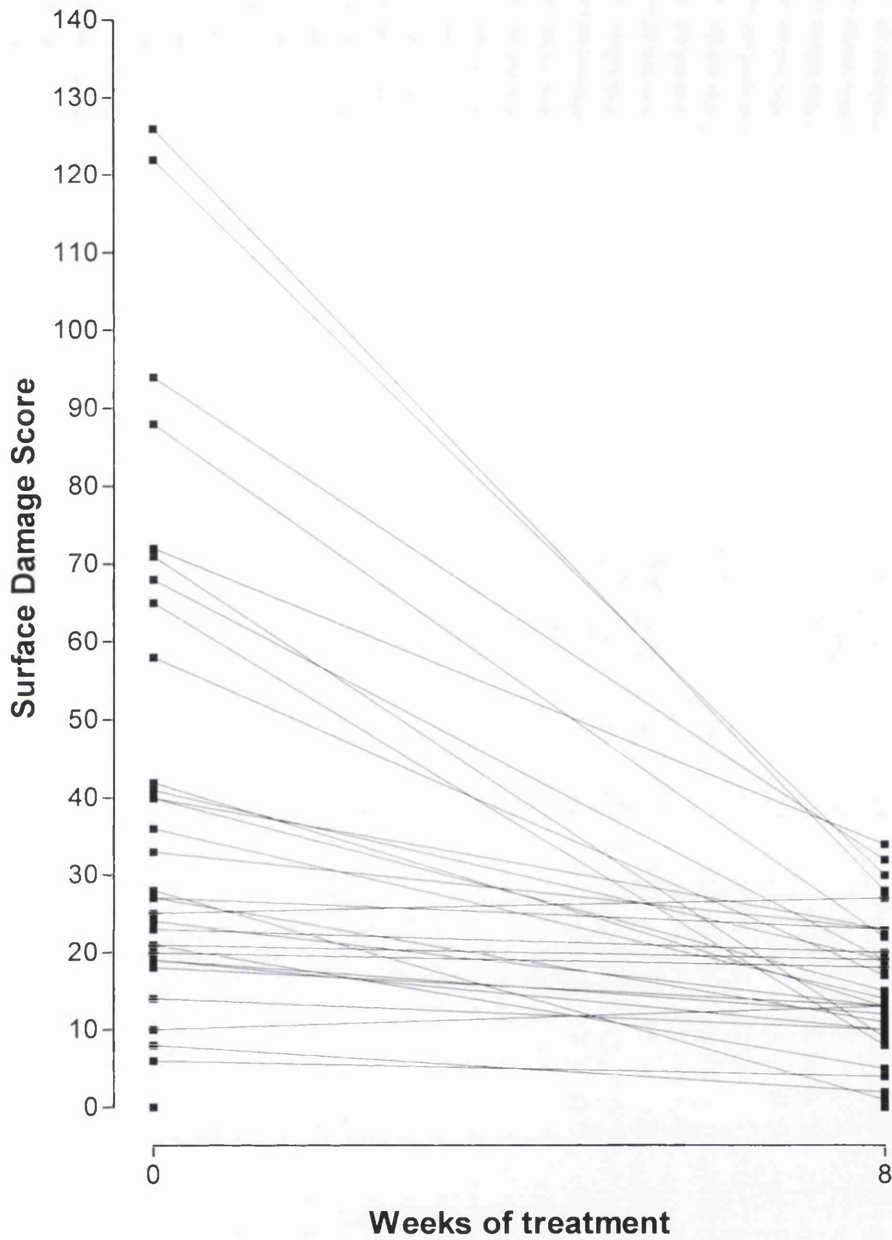

Figure 2.2(A) The surface damage scores for Group I patients, who received the decoction, before and after 8 weeks treatment with CHT. Values represent an assessment of the degree and severity of the surface damage in each patient, based on an established clinical scoring system, (see methods).

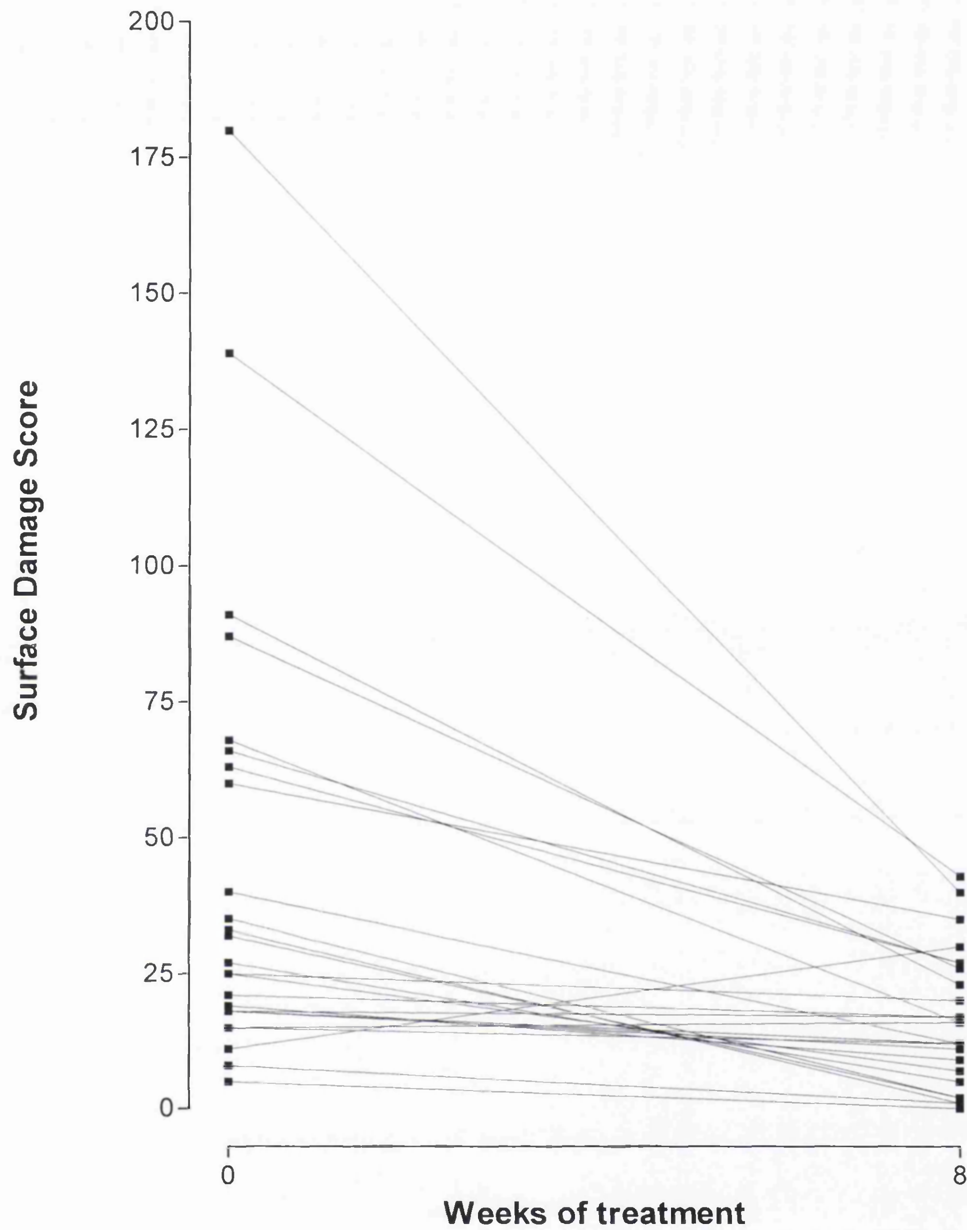

Figure 2.2(B). The surface damage scores for Group II patients, who received the granules, before and after 8 weeks treatment with CHT. Values represent an assessment of the degree and severity of the surface damage in each patient, based on an established clinical scoring system, (see methods).

2.3.2 Patient Assessment Of Clinical Disease

There were reductions in the patient estimates of the severity of their eczema and the severity of itch after 8 weeks of treatment as compared with baseline in both treatment groups. For Group I, at week 0 the mean score for itch was 6.8 and mean score for eczema 6.6. At week 8, the mean score for itch was 5.7 and for eczema 5.3. Thus there was a decrease in the patients' estimated scores for both itch and severity of eczema with treatment. However on paired analysis, this reduction was not significant for itch ($p<0.1$) but was significant for eczema ($p<0.003$). For Group II patients who received the granules, the mean score for itch was 6.3 at baseline and for eczema 6.0. After 8 weeks of treatment, the scores were 4.9 for itch and 4.4 for eczema. The decrease in scores for this group was highly significant with $p<0.0003$ for itch and $p<0.004$ for disease severity.

2.3.3 Adverse Events

Adverse events reported (Table 2.2) were gastrointestinal in nature and similar in both groups. In Group I, 4 patients complained of diarrhoea, 5 of nausea, 5 of flatulence and 1 of a bloated abdomen. In Group II, 4 complained of diarrhoea, 3 of nausea, 3 of flatulence and 2 of a bloated abdomen. Two of those patients complaining of a bloated abdomen discontinued treatment. 25 patients from Group I complained about the taste of the decoction and 7 about the lengthy preparation time. 9 patients from Group II complained about the

taste of the granules. When comparing the palatability of the two treatments , there was a significant preference for the granules with $p<0.003$.

The haematological and biochemical profiles remained normal in all but 1 patient who had received the freeze dried granules. This patient had normal liver enzymes prior to starting treatment but on biochemical analysis after 8 weeks of treatment with CHT, the aspartate aminotransferase (AST) became 78 (normal range;5-40), alanine transaminase (ALT) 116 (normal range; 5-40) and gamma glutamyl transaminase (gamma GT) 64 (normal range;10-48) with a normal bilirubin and alkaline phosphatase. Within three months of stopping the CHT, the liver function tests had all returned to normal with the exception of the gamma GT which was 56. The patient was taking carbamazepine for longstanding petit mal epilepsy. She also admitted to taking a herbal preparation called 'Kalms' sold in health shops for relieving stress a few days prior to her appointment at week 8. She was extensively investigated and found to have negative hepatitis A,B and C screen as well as a negative autoantibody screen (including Anti-nuclear factor, Anti-mitochondrial antibody, Anti-smooth muscle antibody), normal complement levels, normal immunoglobulin levels, normal clotting screen and a carbamazepine level within the therapeutic range. Interestingly, a repeated challenge with Kalms following withdrawal from the trial lead to a further rise in liver enzymes.

Table 2.2. Adverse Events Reported by Patients

Adverse Events	Number of Patients in Group I	Number of Patients in Group II
Diarrhoea	4	4
Nausea	5	3
Flatulence	5	3
Bloated abdomen	1	2

2.4 DISCUSSION

The results clearly show that the two formulations of CHT caused a marked improvement in the severity of the atopic eczema as assessed on the basis of objective erythema and surface damage scores. Importantly, the patients themselves recorded an improvement in disease severity and itch after treatment with CHT. This was true for both the decoction and granules. The results also showed no significant difference in efficacy between the two groups of patients suggesting that the freeze dried preparation is as effective as the original decoction. It was obvious however that the granules were more palatable.

There has been some concern in the literature about the safety of CHT. The Medical Toxicology Unit at Guys Hospital has reports of 11 patients developing significantly raised liver function tests whilst on treatment with CHT from 1991 to 1993 [Perharic *et al.* 1995]. Often there have been no pre-treatment measurements of liver function tests. In these reports, all the liver function tests returned to normal within 6 weeks of stopping CHT except in one patient who died, but there is some doubt as to the exact formulation of herbs that this patient received [Perharic-Walton and Murray 1992; But 1993]. During this present study one patient developed abnormal liver biochemistry results. She had received the freeze dried granules. There are three possible explanations. These changes could have resulted from a direct toxic effect of the herbs on the liver or they may have arisen due to an interaction between the herbs and carbamazepine. The third and most probable cause was the concomitant

administration of Kalms. Raised liver enzymes have been described in patients who have taken herbal preparations for relieving stress [MacGregor *et al.* 1989]. Of these three cases, two patients developed liver failure following ingestion of Kalms. Kalms contains valerian, hops, gentian, titanium dioxide and sucrose. When our patient took a further three tablets of Kalms after the liver function tests had returned to normal, (with the exception of a slightly elevated gamma GT), she developed raised liver enzymes again with AST of 102, ALT of 173 and gamma GT of 113. This strongly supports the suggestion that the Kalms caused the abnormal liver function tests in this patient.

In this study, a specific formulation of Chinese herbal therapy was given to all patients. Traditional Chinese herbalists often vary the combination of herbs that they prescribe for individual patients which they claim is tailored to different patient needs. The long term management of atopic eczema with CHT has been investigated with studies in both adults and children where patients were followed for 1 year [Sheehan and Atherton 1994; Sheehan *et al.* 1995]. Continued treatment with CHT has maintained an improvement in disease activity and in many patients, the dose can be gradually reduced. However it is important to monitor treatment closely and give rigorous attention to concomitant medication.

CHAPTER THREE

CHANGES IN CELL SURFACE CD23 EXPRESSION IN THE BLOOD AND SKIN OF PATIENTS WITH ATOPIC ECZEMA FOLLOWING TREATMENT WITH CHINESE HERBAL THERAPY

3. CHANGES IN CELL SURFACE CD23 EXPRESSION IN THE BLOOD AND SKIN OF PATIENTS WITH ATOPIC ECZEMA FOLLOWING TREATMENT WITH CHINESE HERBAL THERAPY

3.1 INTRODUCTION

The observed clinical improvement in atopic eczema (AE) following treatment with Chinese Herbal Therapy (CHT) represents a model whereby the relationship between immunopathology and clinical severity can be compared in matched samples. As the immunopathology of AE has been shown to be a complex mixture of a Type I Hypersensitivity response associated with raised serum IgE levels and a Type IV response with a mononuclear cell infiltrate in the skin, attention has focused on the IgE receptors as providing a possible link between these two mechanisms that make up this apparent paradox [Bieber 1994, Van der Heijden *et al.* 1995].

The distribution and level of expression of both the high affinity (FcεRI) and low affinity (FcεRII) receptors for IgE on cells capable of antigen presentation may contribute to the stimulation of T cell mediated mechanisms by binding of IgE/allergen complexes [Bieber 1994; Van der Heijden *et al.* 1995]. This has been shown for FcεRI by studies demonstrating facilitated antigen presentation [Maurer and Stingl 1995]. With notable exceptions [Mudde *et al.* 1995a], less evidence is available suggesting a role for the low affinity receptor. However it is dramatic changes to the expression of this moiety that are seen to occur in the lesions of atopic eczema [Buckley *et al.* 1992; Xu *et al.* 1997]. It has been shown that FcεRII in normal skin is expressed by both macrophages and

Langerhans cells [Gordon *et al.* 1989]. In atopic eczema, there is increased expression of FcεRII on dermal dendritic cells [Leung *et al.* 1987]. In both aeroallergen patch tests and lesional skin of eczema patients, the proportion of Langerhans cells and dendritic cells expressing CD23 is increased while the proportion of macrophages expressing CD23 is reduced [Buckley *et al.* 1992]. This work demonstrating the emergence of FcεRII on antigen presenting cells suggests that the redistribution of CD23 within "macrophage subsets" may be of importance in the pathogenesis of atopic eczema and that CD23 may have a role in antigen presentation to T cells.

Tissue macrophages/dendritic cells are a heterogeneous group of cells which are thought to develop from the blood monocyte as it enters the tissues. These subsets have different functional characteristics [Spiteri and Poulter 1991]. They can be subdivided by phenotype using RFD1 and RFD7 monoclonal antibodies [Spiteri *et al.* 1992b]. It has been shown that cells with the phenotype RFD1+RFD7- act predominantly as antigen presenting cells, those with the phenotype RFD1-RFD7+ act as phagocytes and those cells which are RFD1+RFD7+ act as suppressive macrophages [Spiteri and Poulter 1991; Spiteri *et al.* 1992a; Hutter and Poulter 1992]. The low affinity IgE receptors appear to be expressed predominantly by RFD1+ macrophages and Langerhans cells within lesional skin of AE [Buckley *et al.* 1992]. However, it is unclear as to whether this aberrant expression of CD23 on antigen presenting cells is confined to the skin or occurs as a result of recruitment of monocytes with increased expression of CD23 from the circulation. Recent work has shown that in atopic eczema, a higher proportion of peripheral blood monocytes

(PBM) express the CD23 antigen as compared to normal controls [Nakamura *et al.* 1991; Buckley *et al.* 1995], yet the relevance of this to the cellular interactions in eczematous lesions remains unknown. This study therefore investigated whether increased CD23 expression was a local phenomenon in lesional skin or was a result of systemic aberrations. The relevance of aberrant CD23 expression on tissue macrophages and PBM was thus investigated.

Questions addressed by this study include: Are the changes within lesional skin occurring after the monocytes have been recruited into the skin or do they reflect recruitment of abnormal monocytes from the circulation? Further, using CHT as a model of disease modification, can we link changes in CD23 expression to clinical improvement?

Thus the aim of this study was to determine whether immunological aberrations in blood and skin are altered by effective treatment with CHT since previous studies have demonstrated the clinical benefit of this treatment (see Chapter 2 and Sheehan and Atherton 1992; Sheehan *et al.* 1992).

The work investigated CD23 expression on antigen presenting cells locally in lesional skin, systemically in terms of CD23 expression on peripheral blood monocytes, and also during the differentiation of these cells into macrophages *in vitro*. Furthermore, these parameters were studied in patients with moderate eczema and subsequently (after treatment) with mild eczema.

3.2 METHODS

3.2.1 Patients

Eight patients with moderate to severe eczema and generalised skin involvement were randomly selected from the group of patients who received the granule preparation of CHT as described in Chapter 2. Demographic details are presented in Table 3.I. The median age of the patients was 30 and the patients all had longstanding eczema with an age of onset of 0 to 9 years. The patients' eczema had not been adequately controlled with topical steroids and 5 of the patients had been treated with oral prednisolone previously and 2 with ultraviolet phototherapy.

Table 3.1 Demographic details of patients who were treated with CHT in this study.

Number of patients	8
Median age (range)	30 years (18-56)
Previous treatment All systemic treatments were discontinued at least 2 months prior to this study so differences between patients were not reflected in changes in the parameters in this study .	All 8 had applied topical corticosteroids 5 had received oral corticosteroids 2 had undergone ultraviolet therapy
Median Age of onset of AE (range)	2 years (0-9)

3.2.1.1 Baseline

The clinical severity of the AE patients was assessed using a standard scoring system [Sheehan and Atherton 1992, Sheehan *et al.* 1992] at baseline and after the treatment period, (see Chapter 2 for details).

Peripheral blood (PB) was collected by venepuncture from the eight patients with AE and from eight age-and sex-matched normal non-atopic volunteers, as control samples. Those individuals in the control group had no history of atopic diseases and were non reactive to prick testing with a battery of eight common aeroallergens (house dust mite, grass, weed and tree pollen, cat, dog and horse epithelium, mould spores). Skin biopsies were taken from lesional skin from the eight AE patients.

3.2.1.2 Treatment

All eight AE patients then received treatment with Chinese herbal therapy for 8 weeks. These patients were selected from the group who had been randomised to receive the freeze dried granule preparation and not the decoction in the study described in Chapter two. It was thought at the start of the study that compliance would be better amongst this group and it was important to have consistency in the preparation of CHT used. During the 8 weeks, patients continued to apply the topical steroids that they had been using for at least one month prior to the study but were instructed not to vary the quantities to

maintain a stable state. However, any systemic therapy was discontinued at least 8 weeks prior to the study.

3.2.1.3 Post Treatment Specimens

After 8 weeks of treatment with CHT, the clinical scores of the patients were reassessed using the same scoring system as baseline. Peripheral blood was taken and skin biopsies obtained from the eight AE patients. The 4 mm punch biopsies were taken from an area of lesional skin that was adjacent to the original area biopsied on the forearm.

3.2.2 Monocyte Culture From Peripheral Blood

15 ml Peripheral blood was collected in heparinised tubes, diluted 1:1 with sterile phosphate buffered saline (PBS) and overlaid on to a density gradient; 5ml of 'Lymphoprep' (Nycomed, Oslo, Norway). This was centrifuged at 650g for 15 minutes at room temperature. The mononuclear cells layered above the Lymphoprep were removed using a pipette and washed twice with PBS. The harvested cells were centrifuged between washes for 10 minutes at 650g. After washing, the cells were suspended in 1 ml of PBS and a cell count and viability test performed. Viability was determined using trypan blue exclusion in which aliquots of cultured cells were introduced into a solution of 0.1% trypan blue in PBS and proportions of dead cells (those that take up the blue stain) were recorded after 30 seconds. All specimens showed a viability of >90%.

Culture medium was prepared by adding 1 ml of 200mM L-glutamine (GIBCO Ltd.) and 1 ml of Streptomycin (100 micrograms/ml)/ penicillin (100 micrograms/ml) (GIBCO Ltd.) and 10ml of inactivated foetal calf serum to 88ml

RPMI 1640 medium (GIBCO Ltd.). The cells were suspended in the culture medium at a concentration of 3-5x 10^6 cells per ml. 1ml aliquots of this cell suspension were placed in each well of a 24 well culture plate (Costar, Cambridge, UK) and incubated at 37°C in an atmosphere of 5% carbon dioxide in air for 2 hours. After this 2 hour period, all wells were aspirated vigorously with a pastette to remove non adherent cells. Then 2ml of fresh supplemented RPMI medium was added to each well and the plates were incubated for up to 7 days at 37°C in 5% carbon dioxide in air.

Immediately after adherence (Time 0) and after 2,5 and 7 days of culture, cells were gently scraped from triplicate wells using a pastette. After scraping, the cells were harvested by gentle aspiration using PBS at 4°C. Following centrifugation at 260g, the resulting cell pellet was resuspended in PBS. The viability was checked using trypan blue exclusion, and total cell counts performed using a haemocytometer chamber. The concentration of the monocytes was adjusted to 3-5x 10^5 cells per ml and cytospins prepared using 50 microlitre aliquots in a Shandon cytofuge II and centrifuged at 80g. Cytospins were air dried for 1 hour, fixed for 10 minutes in chloroform:acetone (1:1), wrapped in cling film and stored at -20°C until immunocytochemical staining was performed.

3.2.3 Skin Biopsies

All patients gave written consent for biopsy. This had received approval from the Royal Free Ethics committee. At week 0 and after 8 weeks of treatment,

punch biopsies of lesional skin were taken under sterile conditions from the volar aspect of the forearm using a 4mm punch biopsy (Stiefel Laboratory, UK) and 1% xylocaine (Astra Pharmaceuticals Ltd., UK) as local anaesthetic. Skin biopsy specimens were placed on "Cryoembed" medium (Bright Instrument Company Ltd., UK) on a cork disc and rapidly frozen in isopentane cooled in a bath of liquid nitrogen. Frozen specimens were stored for less than one month in liquid nitrogen at -185^{0}C until sectioning. 6 micron sections were cut in a cryostat (Bright Instrument Company Ltd., UK) and mounted on poly-l-lysine (Sigma Ltd., UK) coated slides. The microscope slides had been prepared in advance by immersing them in poly-l-lysine for 1 minute then drying for 24 hours. Once collected on to the slides, the sections were air dried for 1 hour then fixed in chloroform:acetone 1:1 for 10 minutes, wrapped with cling film and stored at -20°C and used for immunocytochemical staining within two months.

The tissue sections were removed from storage and allowed to equilibrate to room temperature before removing the cling film prior to immunohistochemical staining. The experiments were carried out in a moist chamber to prevent the sections from drying. Because of the duration of treatment, it was not always possible to investigate matched samples from the same patients simultaneously. However control sections of tonsil tissue were always used (see below) and skin samples were investigated in batches at least of four to maintain consistency of staining. Sections of human palatine tonsil were prepared as above to use as positive tissue controls during

immunocytochemical staining (see below) using the same antibodies as applied to the test sections.

3.2.4 Immunocytochemical Staining

The monoclonal antibodies (MoAb) used for determining cell phenotype are presented in Table 3.2. The appropriate dilutions and incubation periods were determined using serial tonsil sections incubated with varying concentrations of antibodies for different incubation times. Isotype controls for all MoAbs were performed on tonsil sections using irrelevant antibodies of the same class to exclude non specific staining.

Table 3.2

Monoclonal antibodies used in this study

NAME (Source)	REACTIVITY	MOLECULAR WEIGHT OF ANTIGEN	SUBCLASS OF ANTIGEN
CD25 (Dako Ltd)	Interleukin 2 receptor (alpha chain) expressed on activated T lymphocytes	55 kd	IgG2
CD45RO (Dako Ltd)	Expressed on most thymocytes, primed or memory T cells, monocytes, NK cells, some granulocytes, weakly on activated B cells	180 kd	IgG2
CD1 (Dako Ltd)	Expressed on thymocytes and Langerhans cells	43-49 kd	IgG2
CD14 (Dako Ltd)	Expressed on monocytes	55 kd	IgG1
CD23 (Dako Ltd)	FcεRII/low affinity IgE receptor	45 kd	IgG1
RFD1 (RFHSM)	Antigen expressed on antigen presenting cells		IgM
RFD7 (RFHSM)	Antigen expressed by mature tissue macrophages	77 kd	IgG1
RFDR1 (RFHSM)	Framework epitope on HLA DR molecule (MHCII antigen)	28-33 kd	IgM

RFHSM: Royal Free Hospital School of Medicine

3.2.4.1 Immunoperoxidase Technique

The immunoperoxidase method was used to identify single antigens on cells within tissue sections or cytospins. The samples were ringed with polysiloxane to retain reagents and then incubated with normal rabbit serum diluted in PBS (1:5) for 10 minutes at room temperature in a moist chamber. (This ensured that non-specific binding sites were occupied before addition of the primary antibody). The primary MoAb (diluted in PBS) was added to the sample which was then incubated in a moist chamber for 45 minutes at room temperature. The slides were then rinsed in PBS. The second layer peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako Ltd., UK code no. P161) was diluted in PBS (1:100) and normal human serum added to a concentration of 4%. This was then added to the slides and incubated for 45 minutes. The developer was prepared by dissolving 10mg of 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Ltd., UK) in 16.6ml of PBS then adding 166 microlitres of 1% hydrogen peroxide just before use. This substrate solution was added to the sample for 10 minutes. The peroxidase catalyses the hydrolysis of hydrogen peroxide and forms an oxidised product of DAB which appears as a brown compound attached to the cells. After development, the slides were washed in running tap water for 5 minutes then rinsed in distilled water before counterstaining in Harris's haematoxylin (BDH Ltd., UK)) for 2 minutes. After washing again for 5 minutes in running tap water, the samples were dehydrated by immersing for 1 minute once in 70% ethanol, twice in 90% ethanol, twice in absolute ethanol and twice in 'Citroclear' (clearing agent for histology and cytology, HD supplies UK) before finally mounting in DePeX (BDH Ltd., UK).

Cryostat sections of human tonsils were always studied in parallel to act as positive controls for each of the MoAbs used. As a negative control, some skin sections were incubated with the first layer (MoAb) replaced by the buffer solution. The incubation was then continued with the usual second layer and the development procedure.

3.2.4.2 Alkaline Phosphatase-Anti-Alkaline Phosphatase Technique

To demonstrate CD23 expression, the alkaline phosphatase-anti-alkaline phosphatase method (APAAP) was used. The primary MoAb (CD23) was diluted in Tris-buffered saline (TBS at pH 7.6) and added to the samples which were incubated for 50 minutes at room temperature in a moist chamber. After rinsing in TBS, the second layer of rabbit anti-mouse immunoglobulin (Dako Ltd., UK code no.Z259) conjugated with alkaline phosphatase was added and incubated for 45 minutes at room temperature in a moist chamber. The slides were then washed in TBS before adding the third layer, an alkaline phosphatase-mouse anti-alkaline phosphatase complex (APAAP; Dako Ltd., UK, code no. D651) for 45 minutes. The developer was prepared by dissolving 7.5mg of Fast red (Sigma Ltd., UK) and 3mg of Naphthol ASMX phosphate (the substrate) (Sigma Ltd., UK) in 10ml of 0.05 Tris-HCL buffer (pH 9.0); 2.5 mg of levamisole was also added to prevent endogenous alkaline phosphatase activity. After the developer/substrate solution was added to the sample for 15 minutes, the sections/cytospins were rinsed in TBS, washed in running tap water then counter stained with haematoxylin for 2 minutes. Finally, the slides

were mounted in PBS and glycerol 1 in 9. For the controls the first layer was replaced by TBS.

Cryostat sections of human tonsils and cytospins of B cells were used as positive controls. Skin sections incubated following the above procedure, with buffer solutions replacing the MoAbs, were used as negative controls.

3.2.4.3 Immunofluorescence Technique

To identify those macrophages with the phenotype RFD1+RFD7+, the double immunofluorescence method of staining was used. The two primary MoAbs used were of different immunoglobulin class, that is RFD1 of IgM and RFD7 of IgG class. A mixture of these reagents at appropriate dilutions was added to the slides and incubated for 45 minutes at room temperature in a moist chamber. The slides were then rinsed in PBS for 10 minutes. The second layer consisted of a mixture of goat-anti mouse IgG conjugated to TRITC (tetramethylrhodamine isothiocyanate) and goat-anti mouse IgM conjugated to FITC (fluorescein isothiocyanate) (Southern Biotechnology Associates, Inc., USA) both diluted in PBS. This was applied to the slides for 45 minutes. This second layer thus contained a mixture of anti IgG and IgM immunoglobulins which would bind to RFD7 and RFD1 respectively. The samples were washed in PBS, fixed in 4% Paraformaldehyde for 5 minutes before rinsing again in PBS and finally mounting in PBS:glycerol 1:9. On subsequent analysis with a fluorescence microscope, the RFD7/TRITC complex appeared red and the RFD1/FITC complex green.

3.2.4.4 Immunoperoxidase/APAAP Double Staining

To visualise those cells expressing two antigens the immunoperoxidase method was followed to the development stage then the indirect alkaline-phosphatase anti-alkaline phosphatase method (APAAP) was used. The immunoperoxidase method was used to identify either macrophages expressing RFD1 or RFD7 and the APAAP method to identify coexpression of CD23.

3.2.5 Analysis Of Stained Samples

Morphological parameters were assessed visually. The distribution and frequency of phenotypically distinct cell types within tissue sections was determined with a quantitative method using image analysis systems (Seescan Ltd., Cambridge). Numbers of cells expressing specific markers were counted in frame defined areas of sections. The analyser recorded the area and divided the number of cells counted by the area of the section framed. All counts were expressed as the number of cells per unit area of $1 \times 10^4 \mu m^2$. Point counts were made of cells that were positively stained in at least six random areas in duplicate sections.

Immunofluorescence preparations were counted visually using a Zeiss fluorescence microscope at a magnification of X40 with epi-illumination and selective barriers filters for FITC and TRITC.

The level of expression of CD23 and HLA DR within the skin from five of the AE patients was determined in a semi quantitative manner by measuring the relative optical density of cytochemical reaction product using the image

analyser. Multiple areas within the epidermis and dermis were framed on the computer using a circular mask (diameter 50 μm) and optical density related to the area was measured for each specimen. Care was taken to ensure that all peroxidase reactions for this analysis were performed at the same time with the same reagents. No counter staining was used. At least 50 positively stained areas were analysed. Each point analysed filled the frame at any time during quantification. Initially, the optical density was recorded for the negative control and this was taken as the threshold. The threshold value was then subtracted from all subsequent test samples to take into account background staining.

3.2.6 Statistics

As no evidence of a normal distribution was established on immunohistology data, significant differences between groups of patients before treatment was determined using the non-parametric Mann Whitney test. Median numbers of cells expressing single antigens within skin sections were calculated within each group of samples. Proportions of peripheral blood monocytes expressing RFD1 or RFD7 within a group of samples were determined and median values calculated. Using double antigen determinants, the median proportion within each of the populations also expressing CD23 was also documented. Data from individual patients in groups was compared before and after treatment using the non-parametric Wilcoxon matched pairs test. Significance was determined as $p \leq 0.05$.

The mean scores for both erythema and surface damage were calculated. The mean scores were compared after 8 weeks of treatment with the baseline score

prior to commencing CHT using a paired t test. Significance was determined as $p \leq 0.05$.

3.3 RESULTS

3.3.1 Effect Of Treatment On Clinical Disease

There were significant decreases in the score for erythema (using clinical score evaluation methods described in Chapter 2) in 6 out of 8 patients after treatment with CHT with only a moderate increase in the erythema score in 2 patients. [Fig 3.1A] Similarly there was a decrease in the surface damage scores in almost all patients [Fig. 3.1B]. Using the paired t test a significant decrease in the scores of the group was recorded for both surface damage and erythema ($p<0.05$).

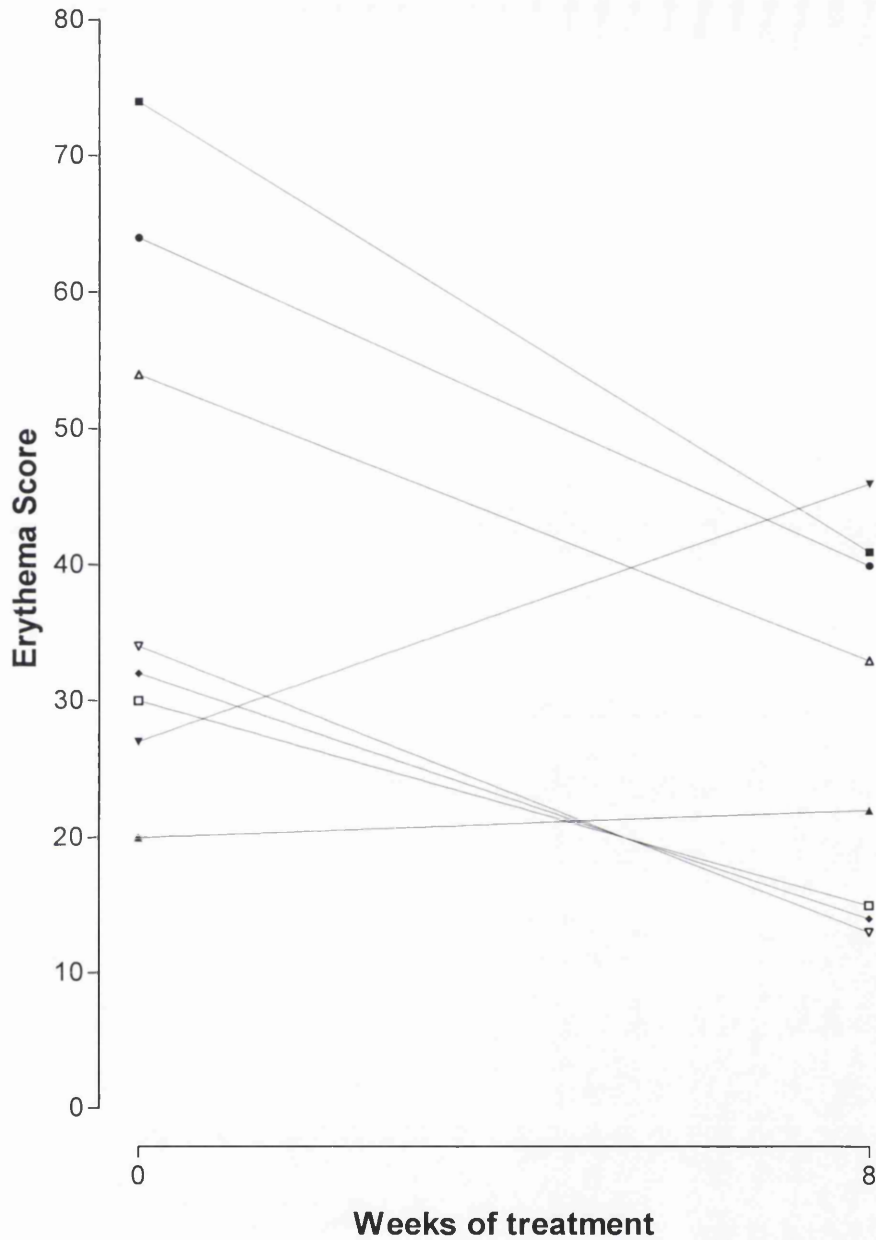

Figure 3.1(A) The erythema scores for individual patients before and after 8 weeks of treatment with CHT. Values represent an assessment of the severity of the erythema based on an established clinical scoring system, (see methods)

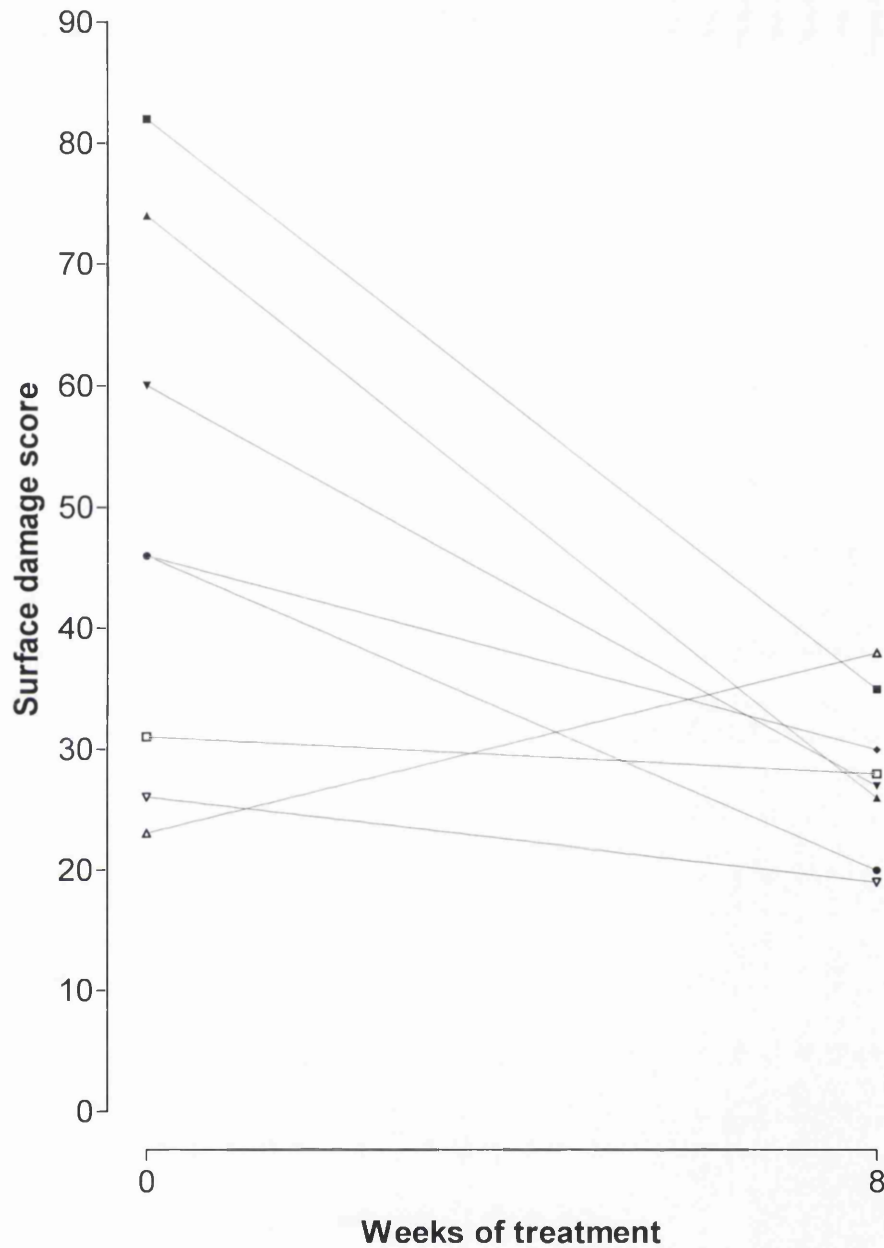

Figure 3.1(B) The surface damage scores for individual patients before and after 8 weeks of treatment with CHT. Values represent an assessment of the severity of surface damage based on an established clinical scoring system, (see methods)

3.3.2 Effect Of Treatment On Monocyte Differentiation And Comparison With Monocytes From Normal Controls

The proportions of monocytes that expressed RFD1+ and RFD7+ were quantified using immunoperoxidase over a 7 day culture period before and after treatment and compared with normal controls.

3.3.2.1 Proportions Of RFD1+ Peripheral Blood Monocytes

At Time 0, peripheral blood monocytes (PBM) from patients with atopic eczema contained a significantly higher proportion of RFD1+ cells (median 14%), compared to normal controls (median 5% $p<0.05$) [Fig 3.2A]. The proportion of PBM expressing RFD1 in AE patients (median 14%) did not significantly change after treatment with CHT (median 10%), although the range of data in samples taken after treatment was very large [Fig. 3.2A]. After 5 days of culture, increased proportions of RFD1+ cells were seen in all samples [Fig. 3.2A].

The difference between the proportions of RFD1+ PBM in AE patients and normal controls was maintained after 5 days of culture ($p<0.01$), but this difference was lost after 7 days ($p>0.05$) [Fig. 3.2A]. In the group treated with CHT, there was no marked difference in the proportion of RFD1+ PBM compared with the untreated group at either 5 or 7 days of culture, however there was a dramatic increase in variability of results, [Fig. 3.2A].

At Day 7, a median of 43% of monocytes from patients with AE before treatment and 28% of monocytes from normal controls expressed RFD1

($p>0.05$). There was no significant difference in the proportions of monocytes expressing RFD1 before treatment (median 43%) and after treatment with CHT (median 44%) [Fig. 3.2A].

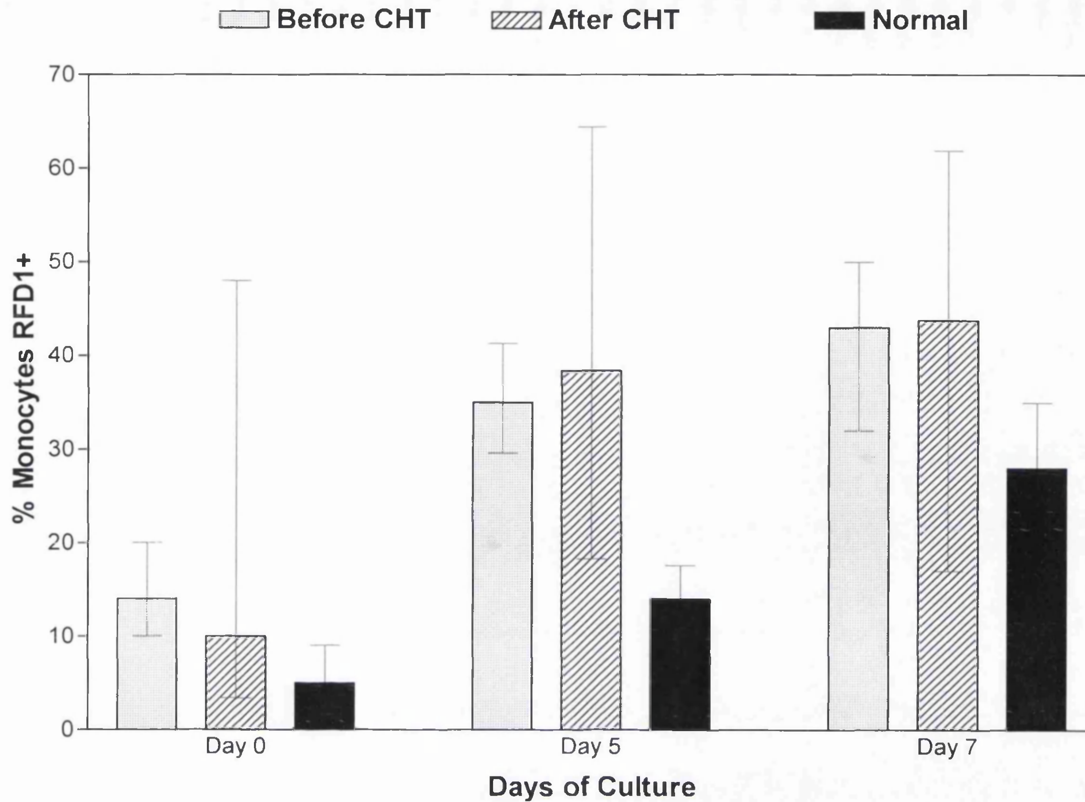

Figure 3.2(A) The proportions of RFD1+ monocytes from patients with AE, before and after 8 weeks treatment with CHT, and from normal controls. Data was obtained at harvest (Day 0) and after 5 and 7 days of culture. All columns represent median proportions of cells staining positively using the indirect immunoperoxidase method (see Text). Ranges are represented by the vertical lines.

3.3.2.2 Proportions Of RFD7+ Peripheral Blood Monocytes

Similar analysis was performed on cells expressing the RFD7 antigen. At Time 0, there was no significant difference in the proportion of monocytes from AE patients expressing the RFD7 antigen compared with normal controls: median 13% in AE and 10% in normals [Fig. 3.2B]. However, after treatment with CHT, a significant increase in the proportion of monocytes expressing the RFD7 antigen was observed (median 27%).

Following culture for 5 and 7 days, the monocytes obtained from AE patients both before and after treatment showed a more rapid increase in the expression of RFD7 antigen compared to monocytes from normal controls [Fig. 3.2B]. At day 5, a median of 26% of monocytes harvested from AE patients before treatment expressed RFD7, while a median of 39% of monocytes harvested after treatment were RFD7+. In contrast only 11% of monocytes from normal controls showed RFD1 positivity at day 5.

However, by day 7, no significant difference in the percentage of monocytes expressing RFD7 was seen in either of the samples taken from patients with AE, with a median of 35% expressing RFD7 before and 49% after treatment ($p>0.05$). In the control cultures 28% of monocytes expressed RFD7 after 7 days of culture [Fig. 3.2B]. This was also not significantly different to the AE groups.

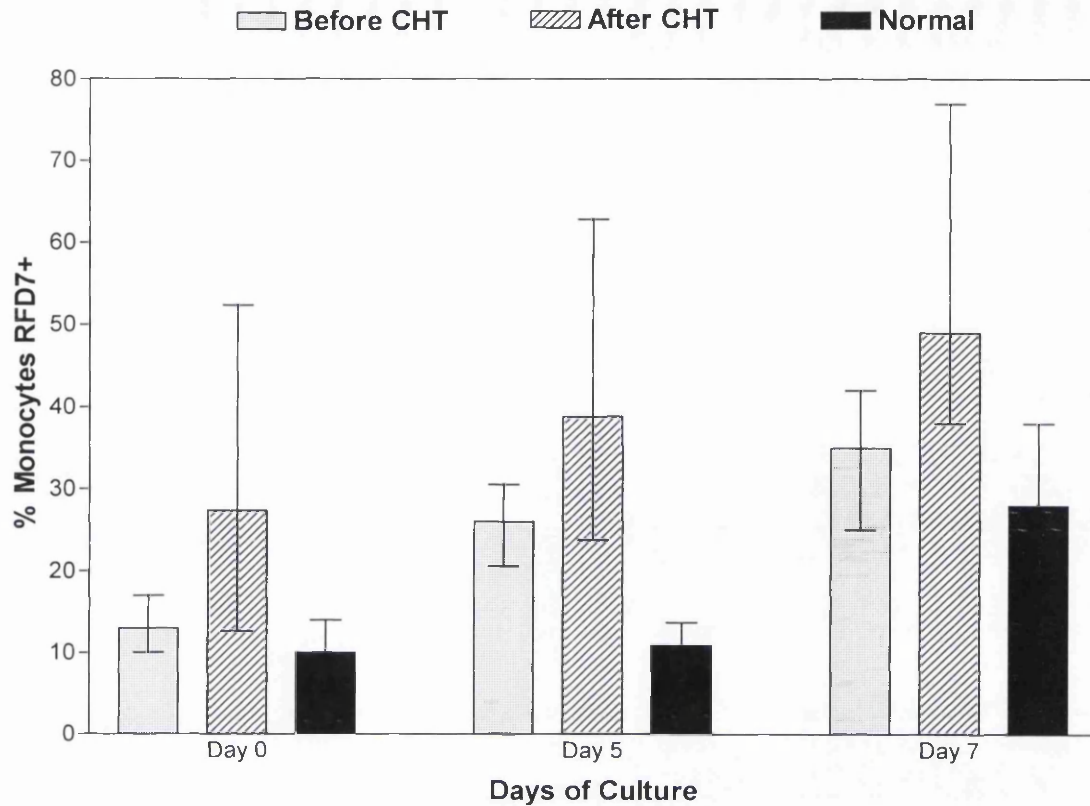

Figure 3.2 (B) The proportions of RFD7+ monocytes from patients with AE, before and after 8 weeks treatment with CHT, and from normal controls. Data was obtained at harvest (Day 0) and after 5 and 7 days of culture. All columns represent median proportions of cells staining positively using the indirect immunoperoxidase method (see Text). Ranges are represented by the vertical lines.

3.3.2.3 Macrophage Subset Phenotype

Double immunofluorescence analysis revealed that 7 days of culture produced a RFD1+/RFD7+ dominance in the control population (median 46%) compared to <30% of cells expressing either RFD1 or RFD7 positivity [Fig. 3.3]. No such dominance was seen in the differentiating subsets of monocytes obtained from AE patients prior to treatment (median values: RFD1+=34%, RFD7+=33%, RFD1+/RFD7+=32.5%). Monocytes obtained from AE patients post treatment showed a dominance of RFD1+ cells (median 47%) compared to a median of 26% RFD7+ cells and 27% RFD1+/RFD7+. On analysis however none of these differences reached statistical significance.

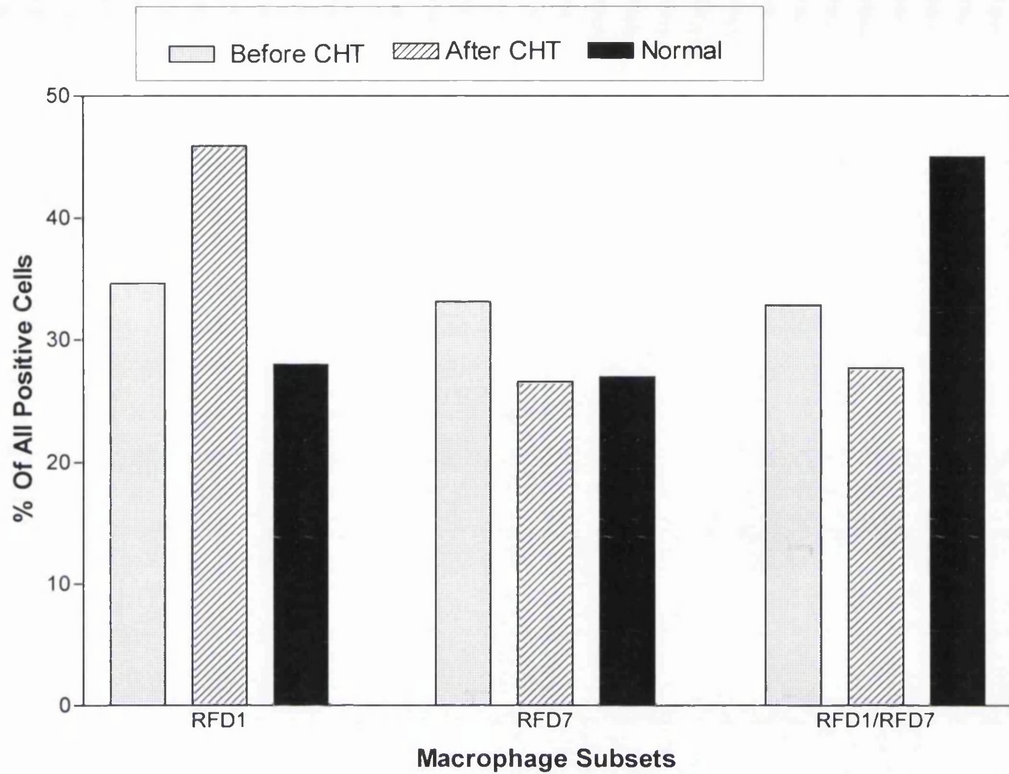

Figure 3.3 The proportions of monocytes expressing RFD1 and RFD7 from patients with AE, before and after 8 weeks treatment with CHT and from normal controls. Data was obtained at Day 7 of culture. The results presented are median proportions of all positively staining cells using immunofluorescence techniques for RFD1, RFD7 or cells that were positively stained for both RFD1 and RFD7. No significant differences were found between groups.

3.3.3 Effect Of Therapy On CD23 Expression By Differentiating Monocytes

Similar cultures were performed to investigate the effect of treatment on the expression of CD23 on both RFD1+ and RFD7+ peripheral blood mononuclear cells.

Following initial harvest (after 2 hours adherence) a median of 70% of RFD1+ cells from samples obtained from patients with atopic eczema were seen to express CD23 [Fig. 3.4A]. This percentage was significantly higher than that seen in samples from normal controls (median 10%) ($p<0.0001$). The proportion of RFD1+ cells expressing CD23 was significantly reduced by therapy to a median of 42% ($p<0.05$).

As these cells were allowed to differentiate in culture, there continued to be a marked difference between the proportions of RFD1+ cells expressing CD23 in AE, both pre-treatment and post-treatment, compared with control samples that were much lower. However the difference in the proportions of RFD1+ PBM expressing CD23 between the pre-treatment and post-treatment samples was gradually lost during the 7 day culture period [Fig. 3.4A]. At Day 7, the median pre-treatment value was 80% and post-treatment 76%. Both however were significantly raised compared to the normal controls (median 40%) ($p<0.01$ in both cases) [Fig. 3.4A].

When the analysis above was repeated on the RFD7+ population within the monocyte pool, the percentage of RFD7+ cells co-expressing CD23 in samples

from untreated patients was significantly higher than that in monocytes derived from normal controls. However, there was no significant difference observed between the treated and untreated AE groups at any time of culture.(Fig. 3.4B)

At day 0, after the initial harvest, a significantly higher proportion of RFD7+ cells expressed CD23 with a median of 82.5% from patients with AE compared with only 15% in normal controls ($p<0.001$). However there was no significant difference observed when samples taken following treatment were analysed (median 62.5%; $p>0.05$).

At Day 7 of culture, the untreated group had higher proportions of RFD7+ cells expressing CD23 (median 84%) compared with normal samples (median 38%) ($p<0.05$). By day 7 of culture, there remained no significant difference in the proportions of RFD7+ cells coexpressing CD23 in the patients with AE before treatment (median 84%) and after treatment with CHT (median 58%). Also, the post treatment values were not significantly different to results from normal controls (median 38%) ($p>0.05$).

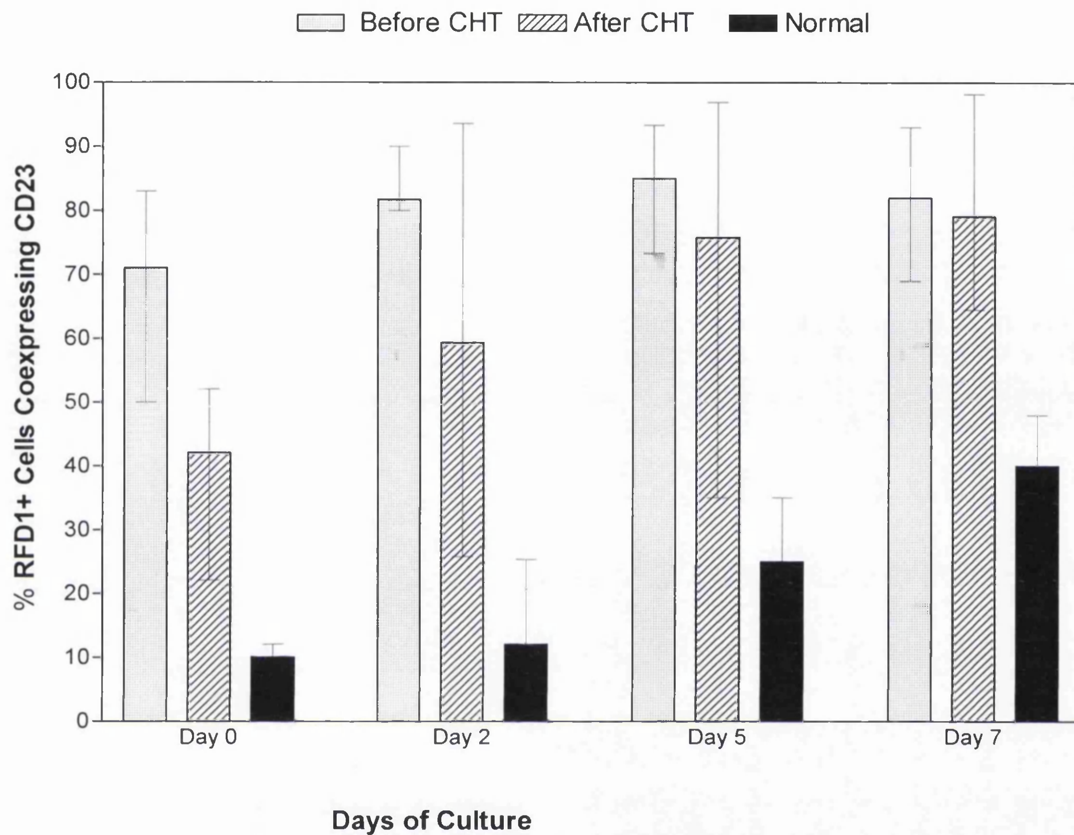

Figure 3.4 (A) The proportions of RFD1+ monocytes coexpressing CD23 from patients with AE, before and after 8 weeks treatment with CHT and from normal controls. Data was obtained at harvest (Day 0) and after 2,5 and 7 days of culture. The results presented for (A) and (B) are proportions of monocytes that stained both for RFD1 with the immunoperoxidase method and CD23 with alkaline phosphatase anti-alkaline phosphatase. The columns represent median values and ranges are expressed as vertical lines for all data.

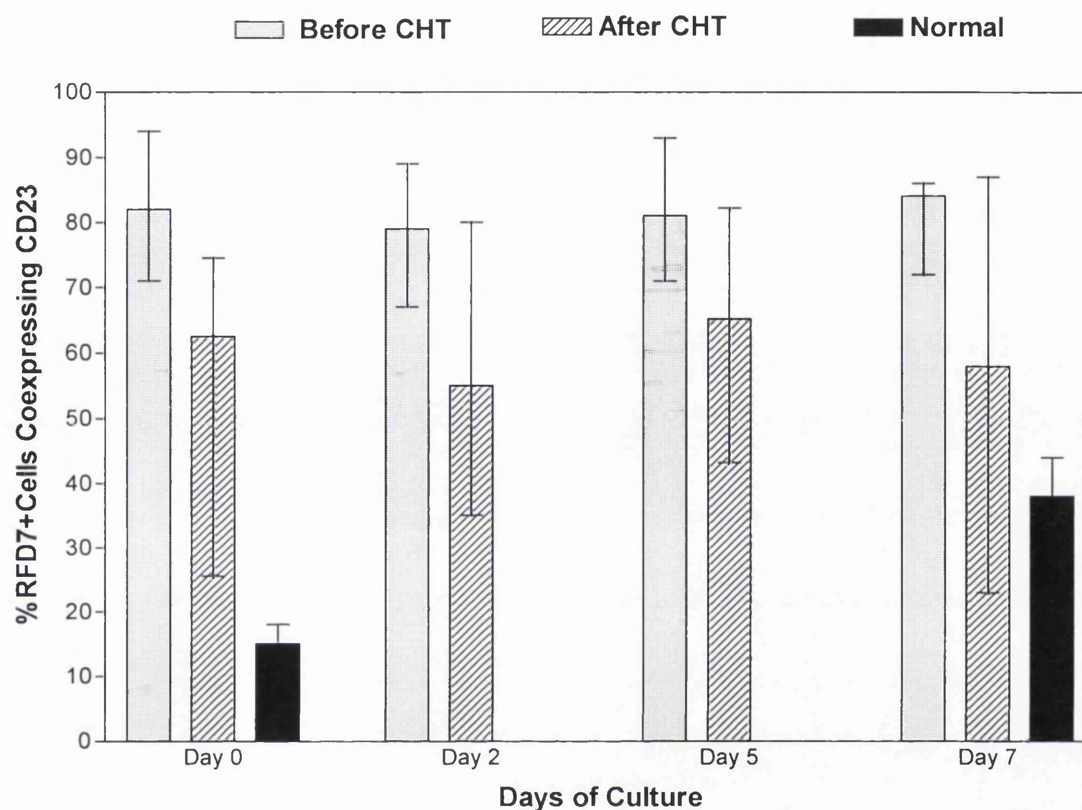

Figure 3.4 (B) The proportions of RFD7+ monocytes coexpressing CD23 from patients with AE, before and after 8 weeks treatment with CHT and from normal controls. Data was obtained at harvest (Day 0) and after 2,5 and 7 days of culture. The results presented for (A) and (B) are proportions of monocytes that stained both for RFD1 with the immunoperoxidase method and CD23 with alkaline phosphatase anti-alkaline phosphatase. The columns represent median values and ranges are expressed as vertical lines for all data.

3.3.4 Effect Of Therapy On T Cells And Antigen Presenting Cells Within Lesional Skin

Once PBM differentiation and CD23 expression had been investigated, the cells within sections of lesional skin were analysed. This analysis included an investigation of numbers of T lymphocytes, antigen presenting cells and CD23 expression in lesional skin before and after treatment with CHT.

The median number of T lymphocytes within lesional skin that expressed activation marker CD25 (the IL-2 receptor) at Time 0 was 1.7 cells per 10^4 μm^2 and after treatment this was reduced to 0.86 cells per 10^4 μm^2 ($p<0.05$). At baseline there was a median of 2.97 CD45R0+ cells (Memory T cells) per 10^4 μm^2 and after treatment these levels were reduced to 2.06 cells per 10^4 μm^2 [Fig 3.5].

Similarly, the number of antigen presenting cells within lesional skin was downregulated by treatment. At baseline, the median number of RFD1+ cells was 2.16 cells per 10^4 μm^2 and after treatment this was reduced to 1.17. The median number of CD1+ cells (Langerhans cells) at baseline was 3.87 cells per 10^4 μm^2 and after treatment this was downregulated to 2.17 . The number of cells expressing RFDR1 (an epitope on HLA DR) was also reduced by treatment with a median of 3.44 cells per 10^4 μm^2 at baseline and 2.13 cells per 10^4 μm^2 after CHT. [Fig. 3.5]

Thus, these results show that within lesional skin, treatment leads to significant reductions in the number of T lymphocytes expressing the IL-2 receptor. In

addition the number of Langerhans cells and macrophages as well as HLA DR+ cells all decreased ($p<0.05$) clearly showing that the number of activated T cells and antigen presenting cells within the inflammatory infiltrate in the skin had been downregulated by treatment.

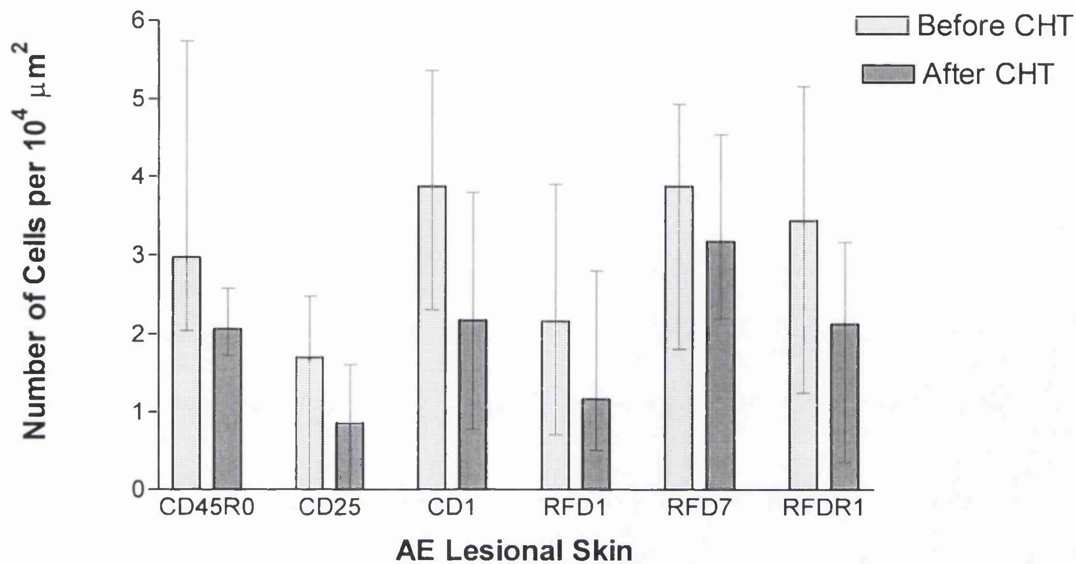

Figure 3.5 The numbers of cells in lesional skin from patients with AE before and after 8 weeks treatment with CHT. Results are for numbers of cells using immunoperoxidase methods expressing CD45RO, CD25, CD1, RFD1, RFDR1, RFD7 and cells expressing CD23 using alkaline phosphatase anti-alkaline phosphatase techniques. The values presented are median numbers of cells per 10^4 μm^2 with ranges as vertical lines.

3.3.5 Effect Of Therapy On CD23 Expression By Antigen Presenting Cells In Lesional Skin

A significant reduction in the numbers of RFD1+ cells within lesional skin expressing CD23 was observed after efficacious therapy with CHT (median 1.2 cells per 10^4 μm^2 before treatment and 0.67 cells per 10^4 μm^2 after treatment) [Fig. 3.6], ($p<0.01$ paired analysis). Similar reductions were also seen in the numbers of RFD7+ macrophages coexpressing CD23 (median 0.7 cells per 10^4 μm^2 before and 0.5 cells per 10^4 μm^2 after treatment) ($p>0.05$ paired analysis) and CD1+ Langerhans cells expressing the low affinity IgE receptor (median 0.5 cells per 10^4 μm^2 before and 0.2 cells per 10^4 μm2 after treatment $p<0.05$ paired analysis) [Fig 3.6].

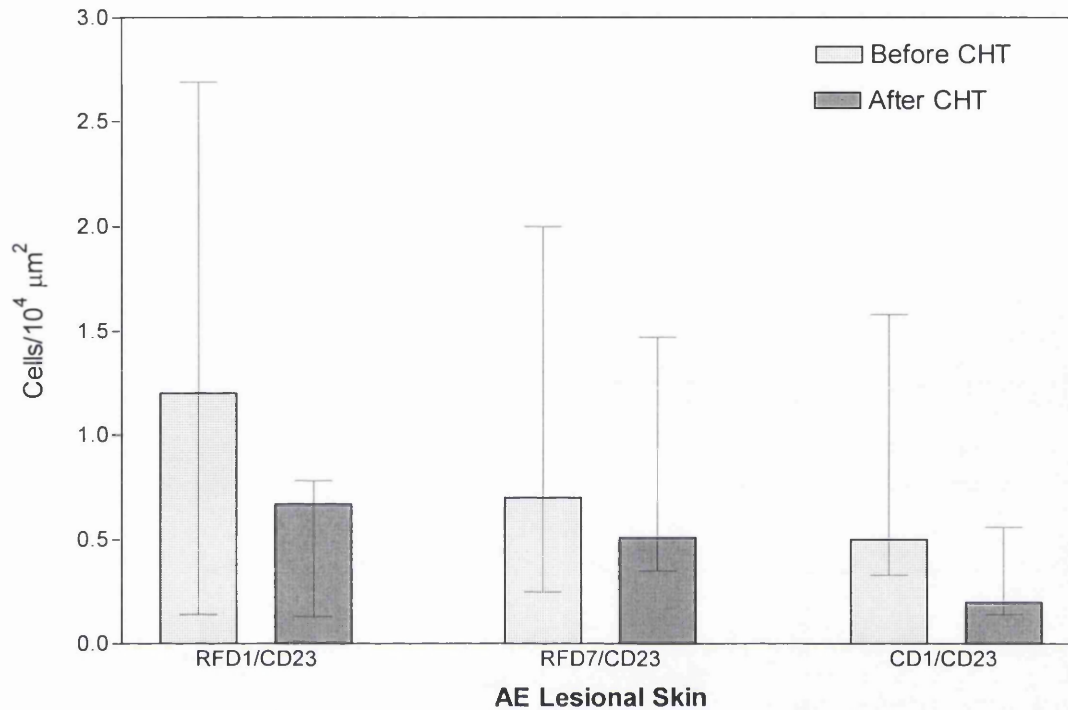

Figure 3.6 The number of antigen presenting cells expressing CD23 in lesional skin from patients with AE before and after 8 weeks treatment with CHT. Results are for positively stained cells using immunoperoxidase methods for RFD1, RFD7 and CD1 followed by alkaline phosphatase anti-alkaline phosphatase stain to identify cells coexpressing CD23. The values presented are median numbers of cells per 10^4 μm^2 with ranges shown as vertical lines.

3.3.6 Effect of Therapy on Level of Expression of CD23 and HLA DR in Lesional Skin

Measurement of the relative optical density (ROD) of the reaction product on the inflammatory cells of lesional skin from five of the AE patients before and after treatment revealed a significant reduction in the relative level of expression of CD23 on these cells in lesional skin (ROD 43 before treatment and ROD 30 after treatment, $p<0.05$) (Wilcoxon matched pairs) [Fig. 3.7A]. Furthermore, treatment lead to a reduction in the level of expression of MHC Class II antigens [HLA DR] on the inflammatory cells in the lesional skin (ROD 21.5 before and ROD 16.5 after CHT with $p<.01$) [Fig.3.7B].

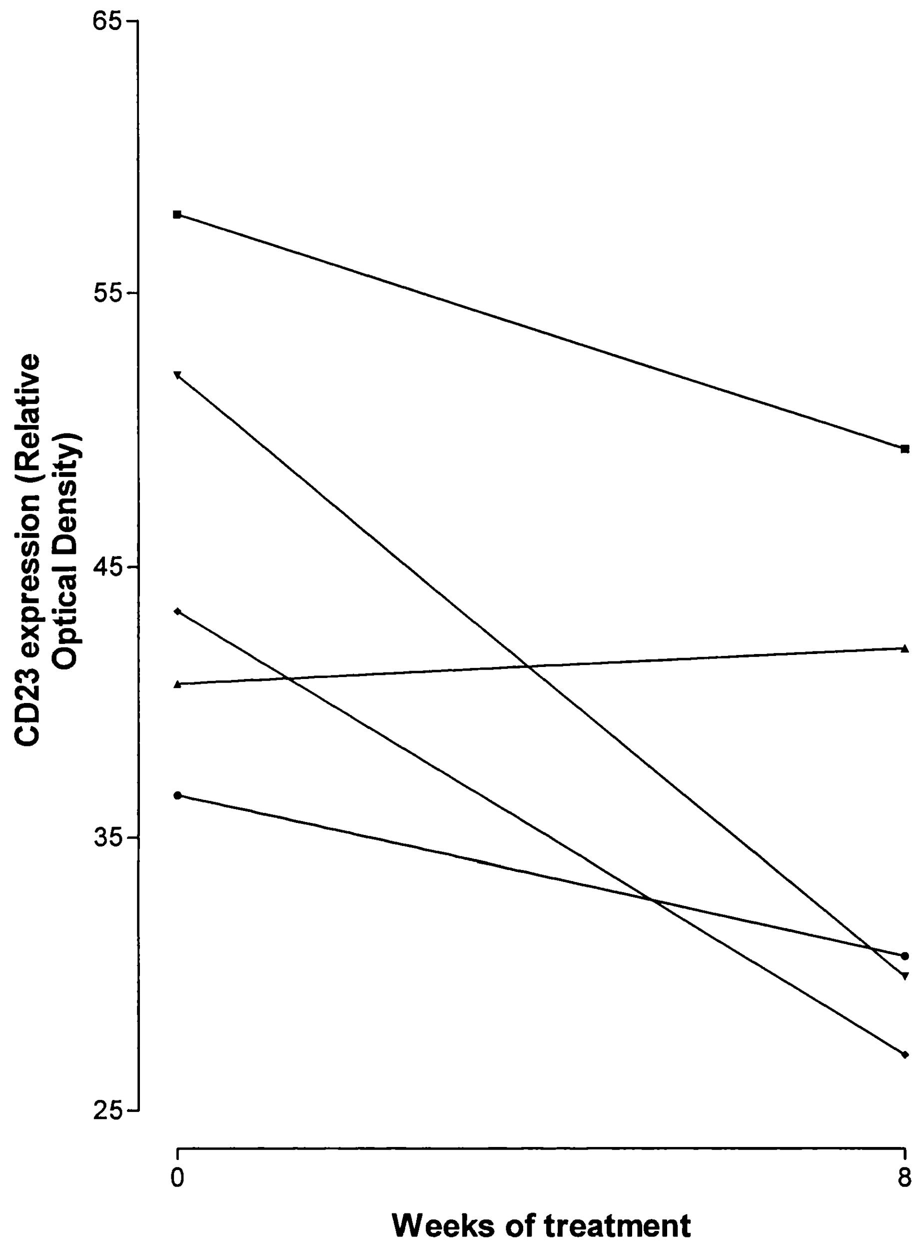

Figure 3.7(A) The effect of efficacious treatment on the expression of CD23 in lesional skin from patients with AE. Median relative optical density values given for each patient before and after 8 weeks treatment with CHT.

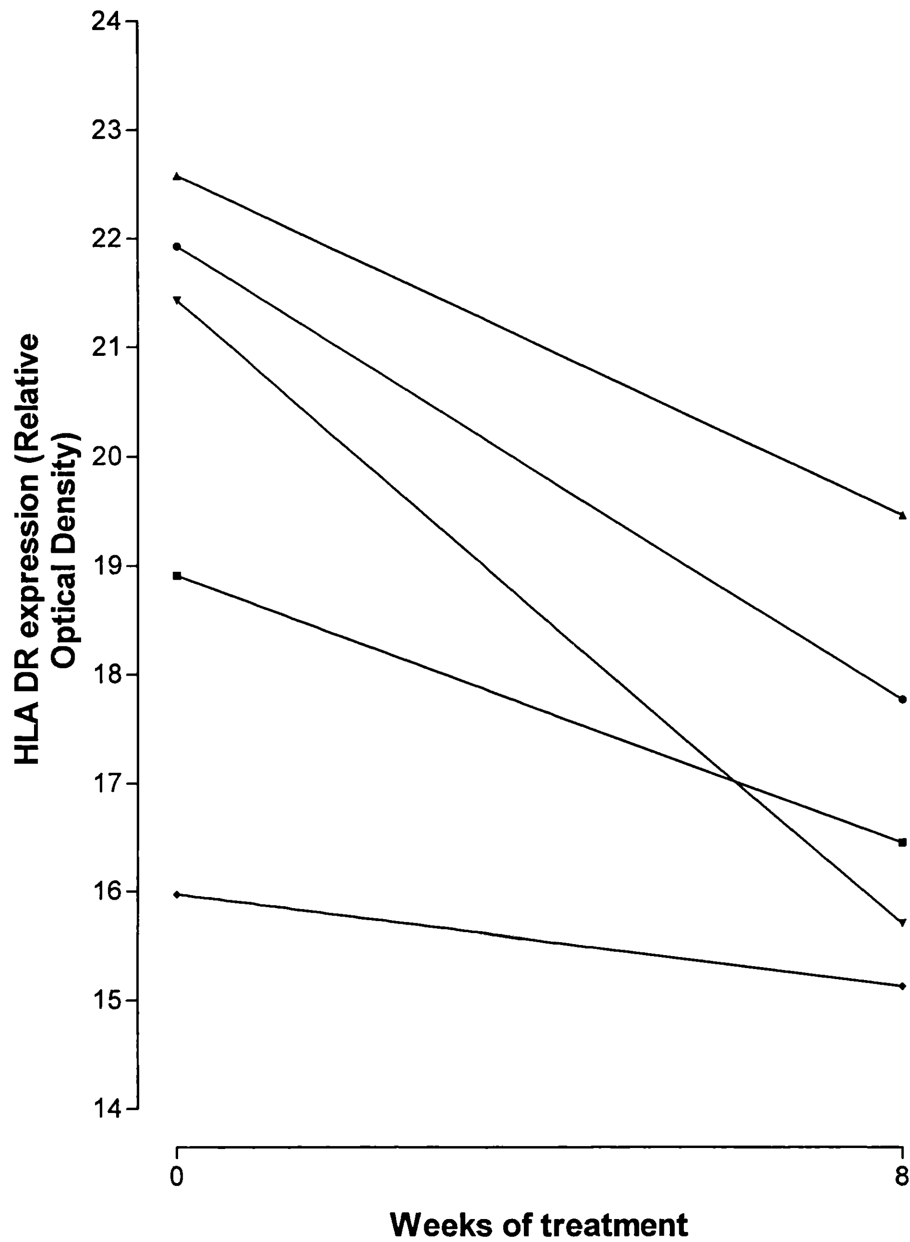

Figure 3.7 (B) The effect of efficacious treatment on the expression of HLA DR in lesional skin from patients with AE. Median relative optical density values given for each patient before and after 8 weeks treatment with CHT.

3.4 DISCUSSION

This study shows that efficacious treatment of atopic eczema may be associated with changes in the differentiation and function of cells of the macrophage-monocyte lineage. Clearly monocytes from patients with AE undergo aberrant monocyte differentiation *in vitro* compared with similar cells obtained from normal non-atopic controls.

There is a more rapid expression of differentiation markers RFD1 and RFD7, and an increased proportion of monocytes expressing CD23 in AE patients compared with normals throughout the 7 day culture period. Treatment of AE patients leads to a downregulation of CD23 expression by those monocytes expressing RFD1 at day 0 of culture although this difference was not maintained through the 7 day culture period. Although this suggests there may be abnormal monocyte phenotype in AE compared with normals, this is only significantly altered by treatment at day 0 of culture.

Notably, these changes are more evident in lesional skin where treatment leads to marked decreases in CD23 expression by RFD1+ cells, RFD7+ cells and Langerhans cells as well as causing decreased levels of expression of CD23. Thus CHT appears to affect tissue bound tissue bound CD23 more than monocytes. This suggests that downregulation of expression of CD23 by mature antigen presenting cells in the skin is associated with clinical improvement.

As might be expected, clinical improvement is associated with a decrease in the inflammatory infiltrate with decreased activated T lymphocytes and antigen presenting cells. Specifically, the number of CD23+ cells and the level of expression of this receptor in the lesions is down regulated by therapy. The aberrant CD23 expression and abnormal differentiation of circulating monocytes obtained from AE patients although altered, remains abnormal even when studied in samples taken after successful therapy. These data therefore support the suggestion that clinical improvement is more associated with local changes to the immune mechanisms in the skin rather than with systemic effects on the circulating monocyte pool. Importantly, these results further support the suggestion that CD23 expression may be associated with the clinical severity of atopic eczema.

In atopy, it has been documented that there are higher numbers of monocytes, macrophages, eosinophils, platelets and B lymphocytes expressing the low affinity IgE receptor [Nakamura *et al.* 1991]. The role of this receptor and its contribution to the immunopathogenesis of eczema is unclear. However, raised levels of IgE in the peripheral blood of AE patients are considered related to type I hypersensitivity and specific IgE can bind to antigen presenting cells via these IgE receptors. This could result in antigen bound to this immunoglobulin promoting T cell proliferation. Thus raised IgE and aberrant IgE receptor expression in AE may link type I hypersensitivity with the changes in the skin associated with type IV hypersensitivity. Increases in both high affinity (FcεRI) and low affinity (FcεRII) IgE receptors have been demonstrated on Langerhans cells in AE [Grabbe *et al.* 1993, Schmitt *et al.* 1990]. Thus both could contribute

to T cell stimulation in the skin via IgE bound antigen. However it is CD23 expression that is shown to be reduced by efficacious therapy (this study) whereas the number of cells expressing FcεRI have been shown not to change, [Xu *et al.* 1997]. This dichotomy could be taken to indicate a more significant role for CD23 in the pathogenesis of AE. But see also Maurer and Stingl 1995, Klubal *et al.* 1997.

This study shows that a larger proportion of monocytes from patients with atopic eczema express CD23 compared with normals. One possible explanation for this is the action of the cytokine Interleukin 4. Interleukin 4 (IL-4) upregulates the expression of CD23 on PBM and macrophages [Bieber *et al.* 1989b] and IL-4 also induces the synthesis of IgE by B cells [Mudde *et al.* 1995a]. Interleukin 4 is a cytokine released by Th2 type cells which are found to be present in increased numbers in AE [Van der Heijden *et al.* 1991] The local presence of this cytokine could stimulate the expression of CD23 by macrophages and is investigated in Chapter 4. CD23 is involved in B cell growth and differentiation, and stimulates further IgE synthesis by B cells that have been stimulated by Il-4. Thus upregulation of CD23 may be considered pro-inflammatory in patients with AE. The presence of CD23 on antigen presenting cells in lesional skin from patients with atopic eczema is of importance. Langerhans cells and dendritic cells within AE lesional skin are known to express IgE molecules on their surface [Barker *et al.* 1988]. CD23 could thus have a role in antigen presentation in the following ways: Complexes of allergen attached to IgE can bind to CD23 on antigen presenting cells and lead to antigen specific T lymphocyte proliferation. This is known as facilitated antigen presentation [Van Der Heijden *et al.* 1995]. Another role of CD23 may

be as the IgE receptor in IgE mediated allergen presentation by B cells [Mudde *et al.* 1995a].

This study shows that not only is there a larger proportion of antigen presenting cells expressing CD23 in lesional skin as well as increased expression of this molecule, but also clinical improvement leads to a down regulation of expression of this receptor. These results thus imply that CD23 expression is of relevance to the immunopathogenesis of AE. As it is not possible to investigate CD23 expression on RFD1+RFD7+ cells simultaneously (this triple phenotype could not be achieved in immunohistology), the relevance of expression of CD23 on "suppressive macrophages" is unknown. It is the raised expression of CD23 on RFD1+ inductive cells that is however consistently associated with increased inflammation.

It is well documented that the dermal mononuclear cell infiltrate in atopic eczema shows a dominance of activated T lymphocytes [Zachary *et al.* 1985a]. Such activation may be promoted by antigen presenting cells as part of a chronic type IV hypersensitivity reaction . Interestingly, comparison of patch test reactions in AE patients and those with contact sensitivity (a recognised T cell mediated reaction) showed similar immunopathology [Buckley *et al.* 1992]. The only difference between these two reactions was a significant increase in CD23 expression on Langerhans cells and dermal dendritic cells in the atopic patch test reactions, thus suggesting that this phenomenon may be allergy associated. This present study shows no significant overall decrease in the proportion of PBM expressing CD23 in matched samples from subjects showing clinical improvement . This suggests that the increases of CD23 seen

in the samples of lesional skin represent a local response rather than a systemic aberration leading to change in CD23 expression. Such results promote the possibility that it is the local environment rather than systemic factors that cause imbalance within mature macrophage populations and CD23 expression. As marked changes to monocyte differentiation have been shown to be promoted by T cell derived cytokines [Tormey *et al.* 1997], further experiments investigated these factors in lesional skin before and after effective treatment.

CHAPTER 4

IL-2 AND IL-4 EXPRESSION IN LESIONAL SKIN OF PATIENTS WITH ATOPIC ECZEMA AND MODULATION BY TREATMENT

4. IL-2 AND IL-4 EXPRESSION IN LESIONAL SKIN OF PATIENTS WITH ATOPIC ECZEMA AND MODULATION BY TREATMENT

4.1 INTRODUCTION

The previous chapter has shown that in the present studies conducted in patients with AE, immunologic changes identified within the skin rather than changes in peripheral blood are related to clinical severity. To investigate the cellular microenvironment within the skin and intercellular signalling between lymphocytes and macrophages, cytokine expression was investigated using in situ hybridisation and immunocytochemical techniques.

There are significant differences in the cytokine profiles released by different populations of T lymphocytes. The T cell-mediated inflammatory infiltrate in the lesions of AE has been found to contain CD4+ aeroallergen specific T cell clones that *in vitro* produce IL-4 [van der Heijden *et al.* 1991]. Mouse T helper or CD4+ lymphocytes have been subdivided into T helper type 1 and T helper type 2 on the basis of the cytokines they secrete [Romagnani 1992a]. Th1 cells produce predominantly IL-2, IL-12 and IFN-gamma whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10, [Romagnani1991, Romagnani 1992a].

One of the relevant features of IL-4 is its ability to stimulate the cell surface expression of the CD23 antigen as well as increased release of soluble CD23 [Bieber and Delespesse 1991, Delespesse *et al.* 1991]. The last chapter revealed that there is aberrant expression of CD23 in AE both on circulating monocytes and in lesional skin. An important finding has been that treatment leading to clinical improvement resulted in a decrease in the numbers of cells expressing CD23. This current chapter describes studies designed to investigate whether the downregulation of CD23+ cells or CD23 expression is associated with changes in IL-4 production locally within the skin.

This study uses in situ hybridisation to determine the level of expression of IL-2 mRNA and IL-4 mRNA in lesional skin from AE patients before and after treatment with CHT. IL-4 levels are related to CD23 expression and represent a marker cytokine for Th2 type cells. IL-2 was measured for comparison and as a marker of Th1 type cells. The detection of levels of both cytokines was used to determine whether any change was associated with 'real' change in expression or rather a possible switch in the cytokine profile of local T cell populations. This approach also provided the opportunity to investigate whether there is a relationship between the local production of these cytokines by T cells and the clinical severity of AE.

4.2 METHODS

4.2.1 Patients

Lesional skin biopsies were taken from 5 patients with moderate to severe eczema with generalised skin involvement and from three normal volunteers. The patients with AE were randomly recruited from the 'granules' group of patients described in Chapter 2. Demographic details are presented in Table 4.I. The median age of the patients was 25 and the patients all had longstanding eczema with an age of onset ranging from infancy to 9 years. All the patients had eczema which had not been adequately controlled with topical corticosteroids and 2 of the patients had been treated with oral prednisolone previously and 2 with ultraviolet phototherapy. Skin biopsies were taken at baseline and after 8 weeks of treatment with CHT. During the 8 weeks, the patients continued to apply the topical steroids that they were using prior to the study however any systemic therapy was discontinued at least 8 weeks prior to the study. The clinical severity of the patients was assessed, at baseline and after 8 weeks, using a standard scoring system (Heddle *et al.* 1984), as described in Chapter 2.

Table 4.1 Demographic details of patients who were treated with CHT in this study.

Number of patients	5
Median age (range)	25 years (18-56)
Previous treatment All systemic treatments were discontinued at least 2 months prior to this study so differences between patients were not reflected in changes in the parameters in this study .	All 5 had applied topical corticosteroids 2 had received oral corticosteroids 2 had undergone ultraviolet therapy
Median Age of onset of onset (range)	2 years (0-9)

4.2.2 Biopsies

All subjects gave written consent for biopsy. At week 0 and after 8 weeks of treatment, punch biopsies of skin were taken under sterile conditions from eczematous lesions on the volar aspect of the forearm using a 4mm punch biopsy (Stiefel Laboratory,UK) using 1% xylocaine (Astra Pharmaceuticals Ltd.,UK) as the local anaesthetic. The same technique was used for the site controlled biopsies from the non-atopic volunteers . The skin was stored and sectioned as described in Chapter 3.

4.2.3 Immunoperoxidase Technique

The immunoperoxidase method was used for identifying the total number of T lymphocytes using single antibody staining as described previously in Chapter 3.

4.2.4 Cytokine Staining Using A Modified Alkaline Phosphatase Technique

The technique for staining cells that are secreting IL-4 used a mouse monoclonal antibody requiring freshly cut tissue and special fixation techniques before following a modified biotin streptavidin alkaline phosphatase technique. Fresh 6 micron sections were cut using a cryostat then air dried for 1 hour

before ringing with polysiloxane. The sections were then fixed in a pre cooled mixture of methanol:acetone 1:1 at -20^{0}C for 10 minutes and then rinsed twice for 30 seconds in PBS. The first layer was prepared by diluting the mouse anti-human antibody for IL-4 (Genzyme, UK) in 0.05% bovine serum albumin (BSA) (Sigma Ltd, UK) in PBS at 4^{0}C then 100μl of this solution was added to each section and incubated overnight in a moist, covered chamber for 16 hours at 4^{0}C. The following day, the slides were washed in TBS (pH 7.6) for 2 minutes. The biotinylated second layer was prepared as anti-mouse IgG (Vector Labs, UK) in PBS-BSA, with 1:100 dilution. This was added to the sections which were then incubated for 1 hour at room temperature in a moist chamber. The sections were then rinsed in TBS for 2 minutes. Next, a solution of 1% streptavidin-alkaline phosphatase (Vector Labs., UK) dissolved in PBS-BSA was added to the samples and incubated at room temperature for 1 hour. The substrate was prepared by dissolving 0.02g fast red (Sigma Ltd., UK) in a solution of 0.01g Naphthol ASBI phosphate (Sigma Ltd., UK) in 20ml Tris buffered HCL (pH 8.2) and 400μl Dimethyl formamide (Sigma Ltd., UK); 500μl of levamisole (Sigma Ltd., UK) was added to prevent endogenous alkaline phosphatase activity. The substrate was added to the samples for 20-30 minutes. The slides were then washed in tap water for 2 minutes. Following this, the samples were counter stained in Mayer's haematoxylin (Sigma Ltd., UK) for 3 minutes, washed in running tap water for 2 minutes then rinsed in

distilled water twice for two minutes. Finally, the slides were mounted in PBS and glycerol 1:9.

The monoclonal antibodies (MoAb) used are presented in Table 4.2. The appropriate dilutions and incubation periods were determined using serial tonsil sections incubated with varying concentrations of antibodies for different incubation times. Isotype controls for all MoAbs were performed on tonsil sections using irrelevant antibodies to exclude non specific staining.

Cryostat sections of human tonsils were used as positive controls and for negative controls instead of using the antibody for IL-4, the first layer was replaced by the buffer solution.

Table 4.2 Monoclonal antibodies used in this study

NAME	REACTIVITY	SUBCLASS
anti-IL-4 (Genzyme, UK)	Recombinant antibody binds to IL-4 produced by thymocytes, subsets of T lymphocytes, mast cells, basophils	IgG1
CD3 (RFHSM)	Pan T lymphocyte marker	IgG1

RFHSM: Royal Free Hospital School of Medicine

4.2.5 In Situ Hybridisation

Until hybridisation with the probe was complete, autoclaved diethyl pyrocarbonate treated water was used and all glassware had been baked for 12 hours at 180°C prior to use. Sterile gloves and pipettes were also used. These precautions were all taken to reduce the risk of introducing contaminants especially RNA and thus prevent non-specific interactions. Stored sections were allowed to equilibrate to room temperature and were then fixed in 4% paraformaldehyde (Sigma Ltd., UK) for 15 minutes just prior to commencing the hybridisation procedure.

The slides were washed for 10 minutes with phosphate buffered saline (pH 7.4) containing 5mM magnesium chloride (PBSM) (BDH Ltd., UK) then immersed for 10 minutes in PBSM containing 0.25% Triton X-100 (Sigma Ltd., UK) and 0.25% Nondet P-40 (Sigma LTD.,UK) for 10 minutes, all at room temperature. Then the slides were rinsed twice for 5 minutes in PBSM before dipping in 20% acetic acid (Sigma Ltd., UK) at 4°C for 15 seconds. They were again washed in PBSM only at 4°C. Slides were then incubated in 20% glycerol (Sigma Ltd., UK) for 1 hour at room temperature. Following this, the slides were immersed twice in saline sodium citrate (SSC) (3M NaCl, 0.3M trisodium citrate) (BDH Ltd., UK) (pH 7.0) before probe hybridisation. The digoxigenin labelled oligonucleotides to identify mRNA of IL-4 and IL-2, and the poly-dT probes (R&D Ltd., UK) were

dissolved in a hybridisation solution which contained 0.6M sodium chloride (Sigma Ltd., UK), 30% formamide (Sigma-Aldrich Ltd., UK) and 150 microlitres sonicated salmon sperm DNA. This was applied to the skin sections and covered with a glass coverslip. Initial incubation was performed for 20 minutes on a preheated tray at 70°C. The sections were then transferred to an incubator for 16 hours at 37°C. The probes used are listed in Table 4.3.

The post hybridisation washes removed excess unbound probe and reduced background signal by removing any loosely bound probes. Four 15 minute washes in quadruple strength saline sodium citrate (SSC) were performed at room temperature, during which time the coverslips were gently removed. The slides were then washed in double strength SSC for 20 minutes at 60°C then one fifth strength SSC for 20 minutes at 42°C. These processes were followed by two washes at room temperature, the first in one tenth strength SSC for 5 minutes and the second in double strength SSC for 10 minutes. The slides were then rinsed at room temperature for 10 minutes in 5% Triton X-100 dissolved in a solution of trinitro monotetrazolium (TNMT) (0.1M Tris HCL pH 7.5, 0.1M NaCl, 2mM $MgCl_2$, 0.05M Triton X-100). All samples were then immersed in 3% Bovine serum albumin (BSA) (Sigma Ltd., UK) dissolved in TNMT for 1 hour at room temperature. Then again the slides were washed in TNMT twice for 10 minutes at room temperature.

The next steps involved detection of the bound probe. The sections were incubated overnight for 16 hours at room temperature with anti-digoxigenin antibody (Boehringer, UK) dissolved in 1% BSA in TNMT. The slides were then rinsed twice for 10 minutes in TNMT before rinsing twice for 5 minutes in 10ml of 1mM magnesium chloride (BDH Ltd., UK) dissolved in Tris-HCL buffered saline (NBT) (pH 9.6), all at room temperature. The developer, 5-bromo-4-chloro-3 indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) (Sigma Ltd., UK) dissolved in deionised water, was added to the sections and covered with a coverslip overnight for 16 hours at room temperature. The following day the slides were washed in deionised water and the coverslips were gently removed during the wash. The slides were finally mounted in PBS/glycerol 1:1.

Negative controls were sections incubated with hybridisation solution only. In order to demonstrate that the target nucleic acid was RNA, some tissue sections were incubated with RNAse before application of the probe. The RNAse degrades all mRNA in the sections therefore the resulting positive staining would represent non-specific binding of the probe. For the positive control, sections were incubated with digoxigenin labelled poly-dT oligonucleotide (R&D Ltd., UK) which detects all mRNAs thus resulting in positive staining. The probes used are listed in Table 4.3.

Table 4.3 DNA probes used in study

PROBE (digoxigenin labelled)	REACTIVITY
IL-4 DNA probe (R&D, UK)	Single stranded anti-sense oligonucleotide probe complementary to mRNA for the IL-4 gene
IL-2 DNA probe (R&D, UK)	Single stranded anti-sense oligonucleotide probe complementary to mRNA for the IL-2 gene
poly-(dT) probe (R&D, UK)	Single stranded oligonucleotide, identifies total cellular mRNA

4.2.6 Quantification

4.2.6.1 Analysis Of Stained Sections

The positively staining cells in the epidermis and dermis were quantified using an Olympus microscope at a magnification of X40 connected to a computerised image analyzer to define and measure framed areas of section visualised on a colour monitor. Point counts were made of cells that were positively stained in at least six areas. The results were expressed as the mean number of cells per unit area ($10^4\ \mu m^2$).

4.2.6.2 Analysis Of In Situ Hybridisation Products:

The optical density of the mRNA for IL-2 and IL-4 was measured semi-quantitatively using a computerised image analyser (Seescan Ltd., UK). Specific cells were defined and measured after first setting the threshold of relative absorption, using the control preparation as zero. At least 50 cells were counted and the median optical density calculated per section.

4.2.7 Statistics

The median values of optical densities and mean clinical scores were each compared before and after treatment using Wilcoxon matched pairs and

Students' paired t test respectively. Median optical densities before treatment were compared with normal controls using the Mann Whitney test. The numbers of T cells and numbers of cells secreting IL-4 before and after treatment were also compared using non parametric statistics. Significance was defined as $p \leq 0.05$.

The mean scores for both erythema and surface damage were calculated. The mean scores were compared after 8 weeks of treatment with the baseline score prior to commencing CHT using a paired t test. Significance was determined as $p \leq 0.05$.

4.3 RESULTS

4.3.1 Effect Of Treatment On Clinical Scores

The scores for erythema and surface damage in the five patients decreased following treatment for 8 weeks with CHT resulting in a significant decrease in clinical scores; $p<0.05$ using paired analysis. [Fig. 4.1A and Fig. 4.1B]

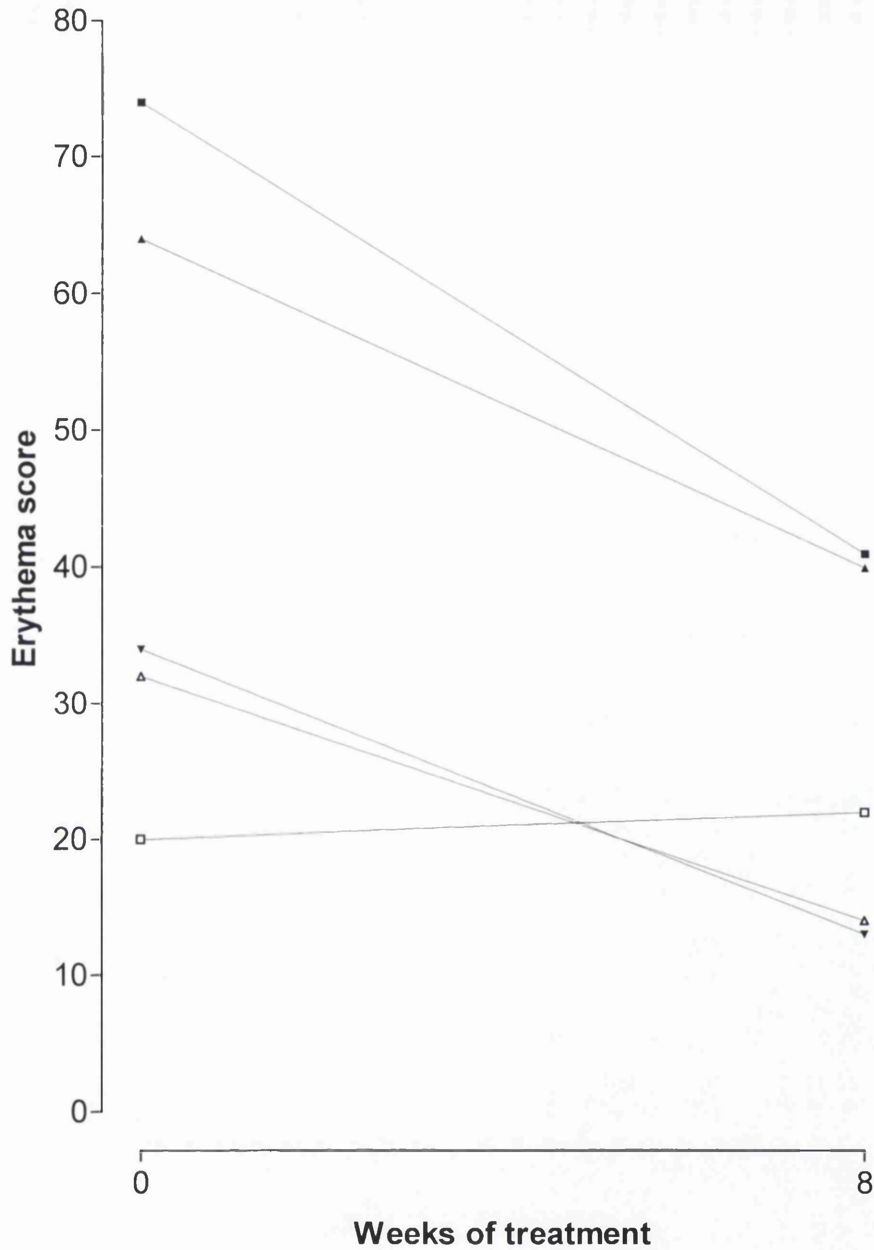

Figure 4.1(A) The erythema scores from patients with AE before and after 8 weeks of treatment with CHT. Values represent an assessment of the degree and severity of erythema based on an established clinical scoring system, (see methods).

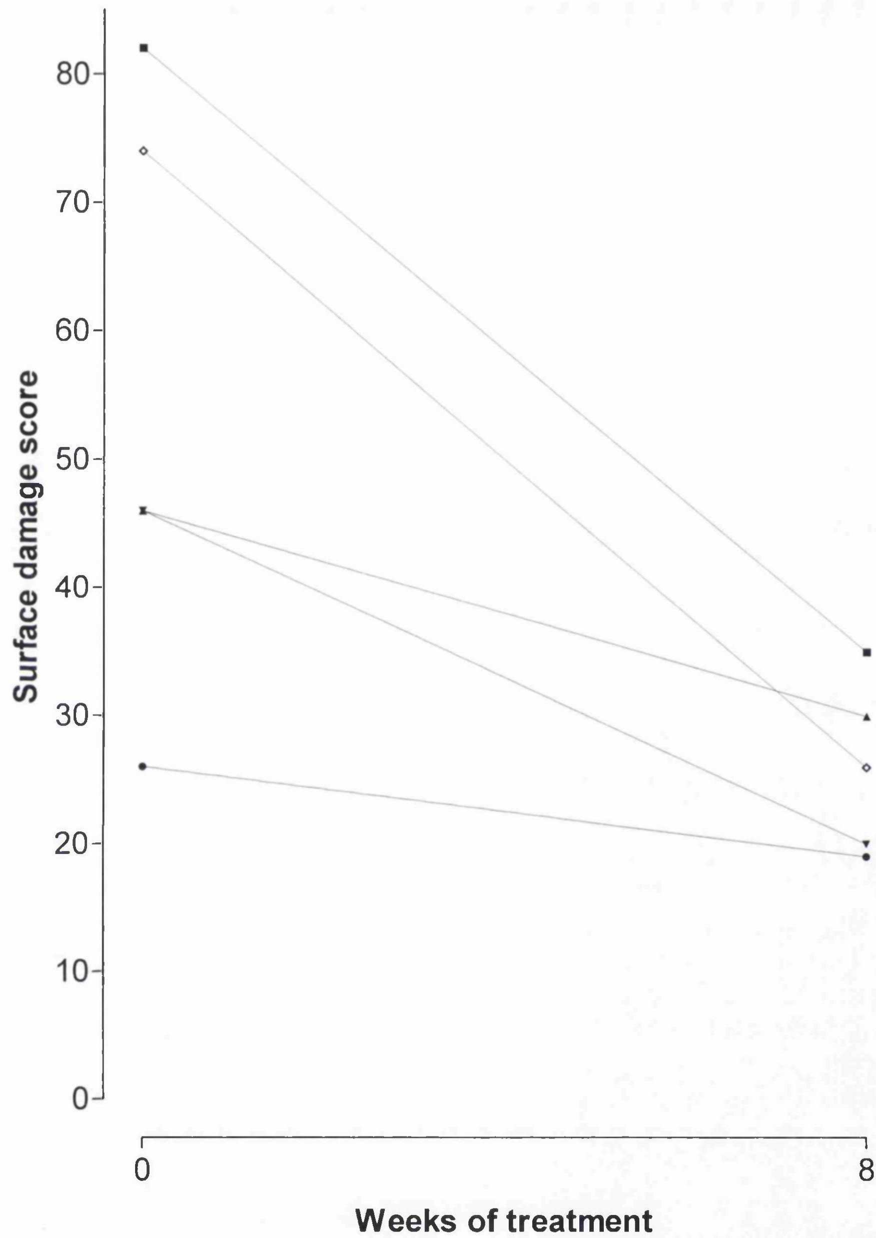

Figure 4.1(B).The surface damage scores from patients with AE before and after 8 weeks of treatment with CHT. Values represent an assessment of the degree and severity of surface damage based on an established clinical scoring system, (see methods).

4.3.2 Effect Of Treatment On IL-4 mRNA In Lesional Skin And Comparison With Normal Controls

The relative optical density (ROD) of the IL-4 mRNA in lesional skin from patients with AE (median ROD 39) was significantly greater than that in the skin of normal controls (median ROD 24.9, $p<0.03$). [Fig 4.2] After 8 weeks of treatment with CHT, there was a marked downregulation of IL-4 mRNA expression to a median relative optical density of 18.7 and this was significant with $p<0.01$. [Fig 4.3]

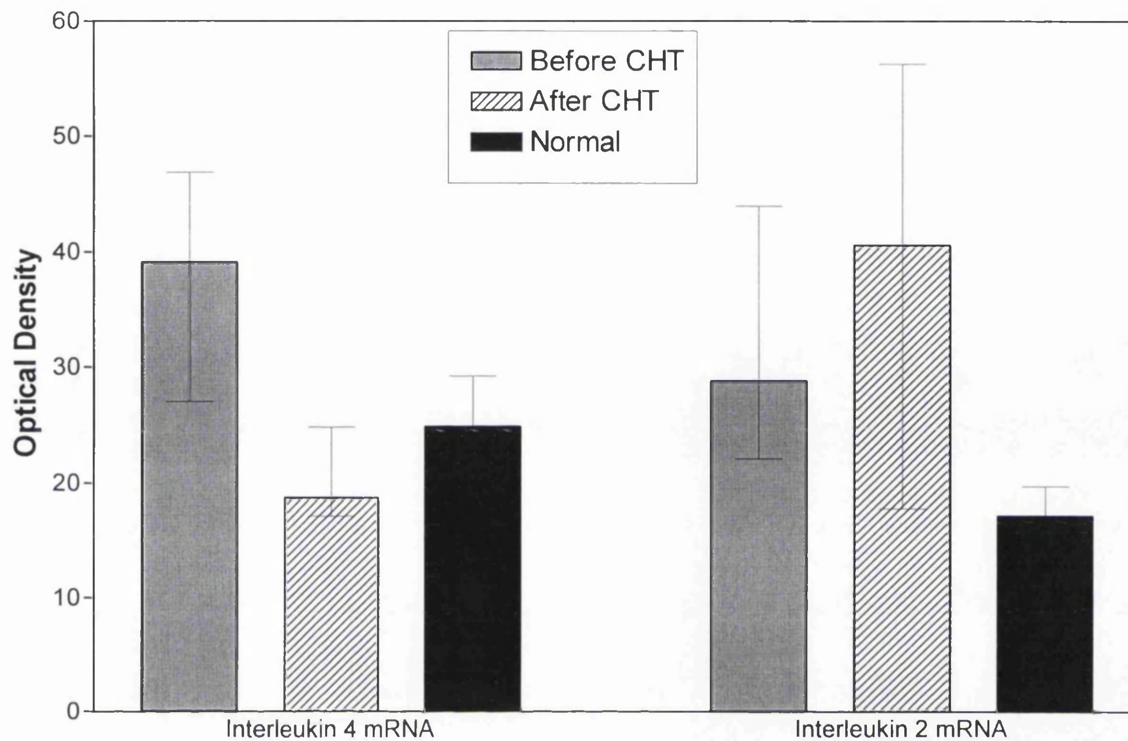

Figure 4.2 The level of expression of mRNA for IL-4 and IL-2 in lesional skin of patients with AE before and after 8 weeks of treatment with CHT and in skin from normal controls. The columns represent median relative optical densities recorded of formazan product of reaction of cells following in situ hybridisation. Ranges are represented as vertical lines.

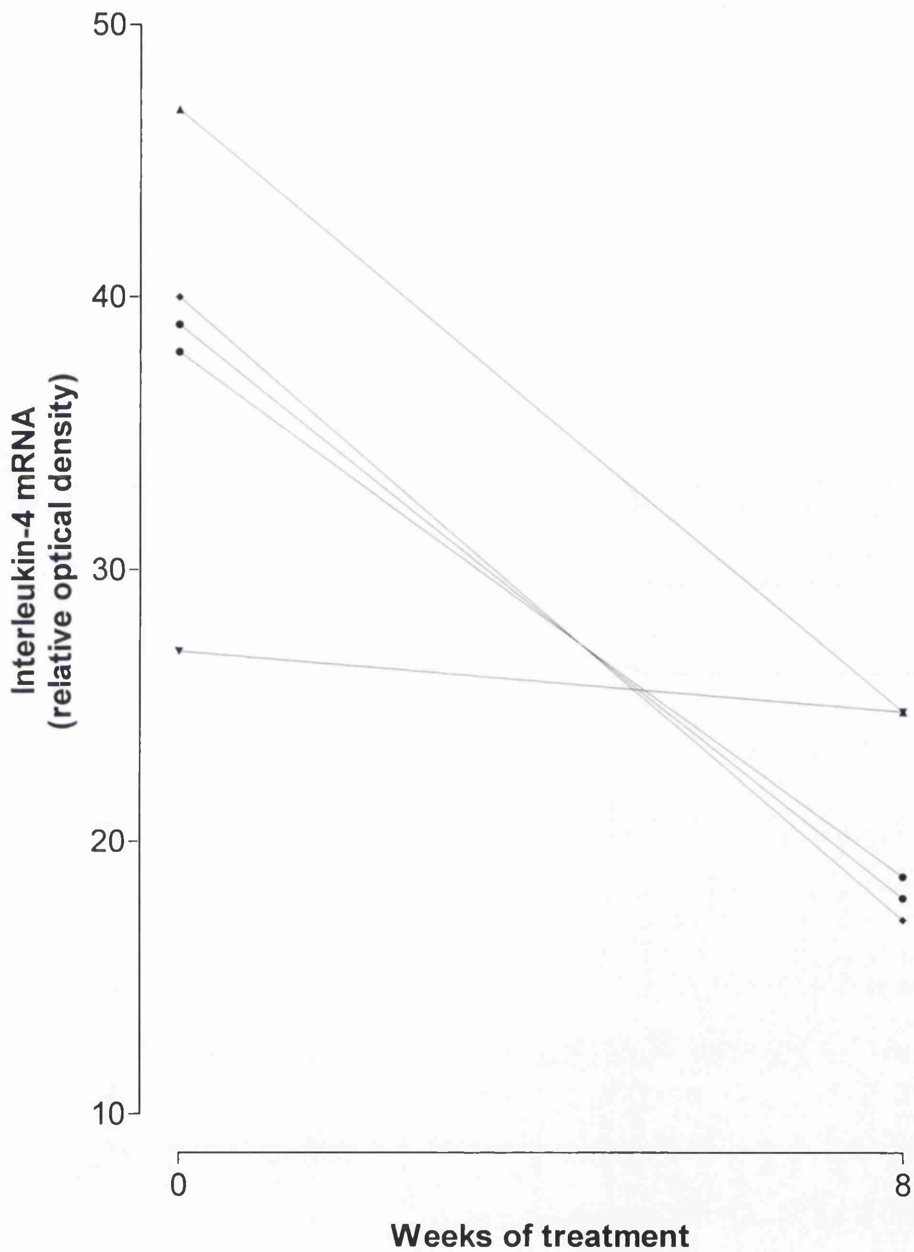

Figure 4.3 The relative optical densities recorded of reaction product identifying cell associated IL-4 mRNA following in situ hybridisation in lesional skin of 5 patients with AE before and after 8 weeks of treatment with CHT.

4.3.3 Effect Of Treatment On IL-2 mRNA In Lesional Skin And Comparison With Normal Controls

Analysis of levels of mRNA for IL-2 showed that in lesional skin from patients before treatment, there was more IL-2 mRNA (median ROD 28.8) than in normal skin (median relative optical density 17.2, $p<0.05$), although this difference was not as marked as for IL-4 mRNA. [Fig 4.2] In 4 out of 5 patients, there was a rise in levels of mRNA for IL-2 after treatment, however this did not reach statistical significance because of the 1 sample in which there was a decrease (median ROD 40.6, $p>0.3$). [Fig 4.4]

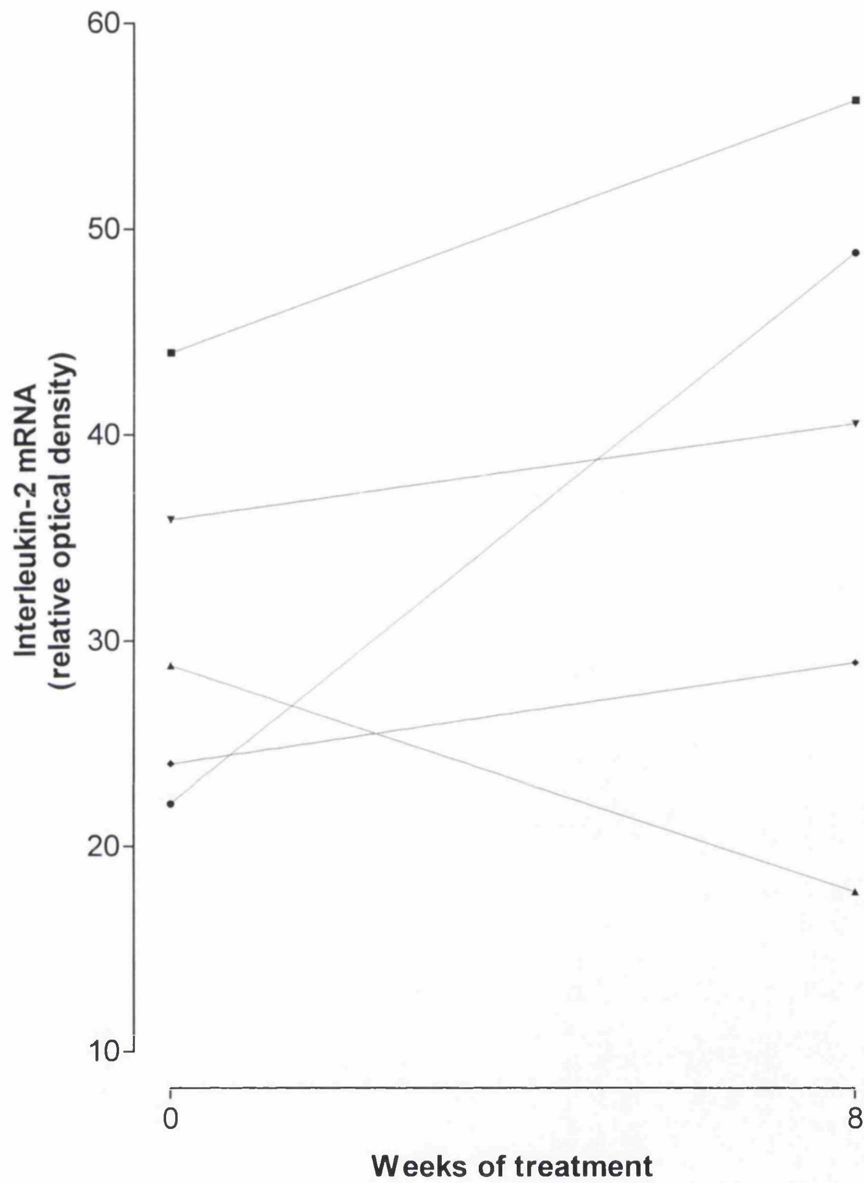

Figure 4.4 The relative optical densities recorded of reaction product of cell associated IL-2 mRNA following in situ hybridisation in lesional skin of 5 patients with AE before and after 8 weeks of treatment with CHT

4.3.4 Effect Of Treatment On Numbers Of T Lymphocytes In Lesional Skin

Numbers of lymphocytes that stained positively with CD3 (pan T cell marker) were compared in lesional skin before and after treatment. Treatment lead to a downregulation of numbers of T cells present with a median of 9.9 cells per 10^4 μm^2 before treatment and 6.2 cells per 10^4 μm^2 after treatment ($p<0.04$). [Fig 4.5]

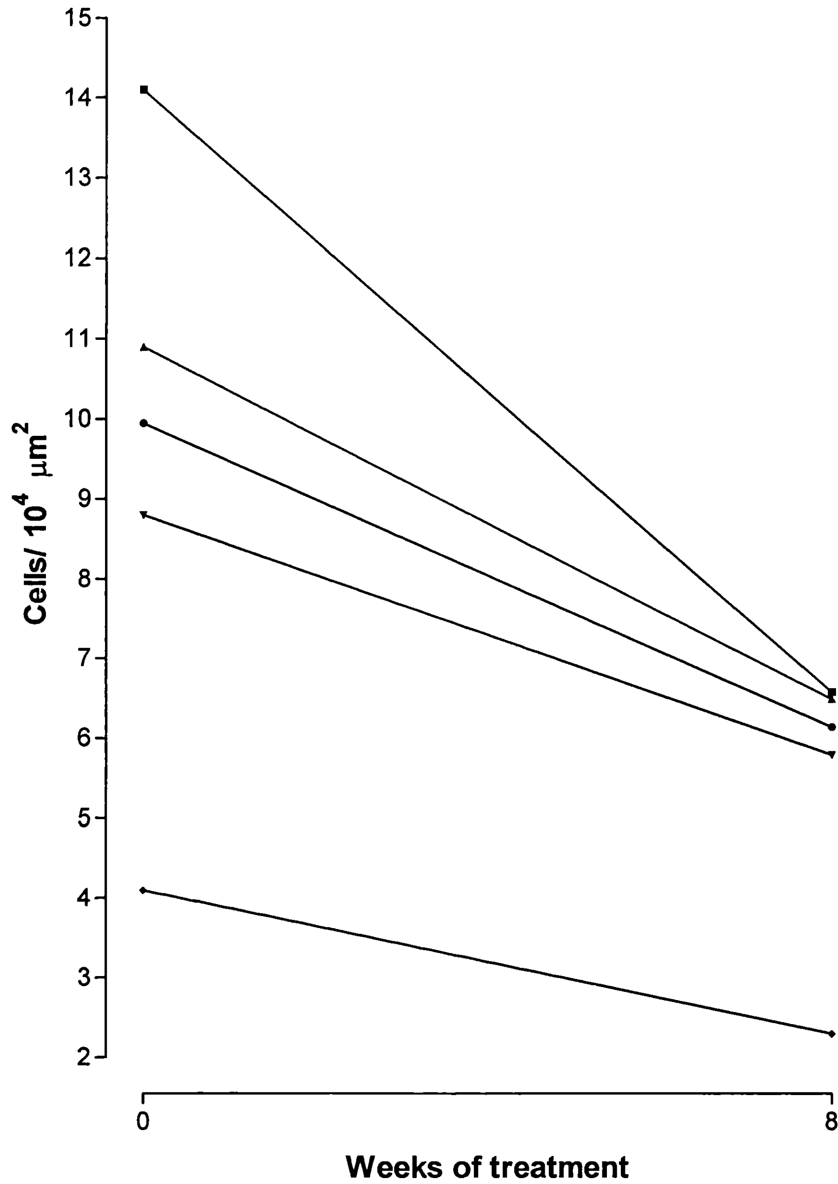

Figure 4.5 The number of T lymphocytes in lesional skin of patients with AE before and after 8 weeks of treatment with CHT. Results are for positively stained cells with immunoperoxidase method using the monoclonal antibody CD3. The values represented are numbers of cells per 10^4 μm^2

4.3.5 Effect Of Treatment On The Number Of Cells Staining Positively For IL-4

As the level of expression for the mRNA for IL-4 decreased with treatment, it was necessary to confirm that there was a downregulation in the number of cells staining positively for IL-4. The median number of positively staining cells before treatment was 4.0 cells per 10^4 μm^2 and 2.7 cells per 10^4 μm^2 after treatment ($p<0.001$) [Fig 4.6]. This may, however be explained by an absolute loss in the number of T cells. (See above)

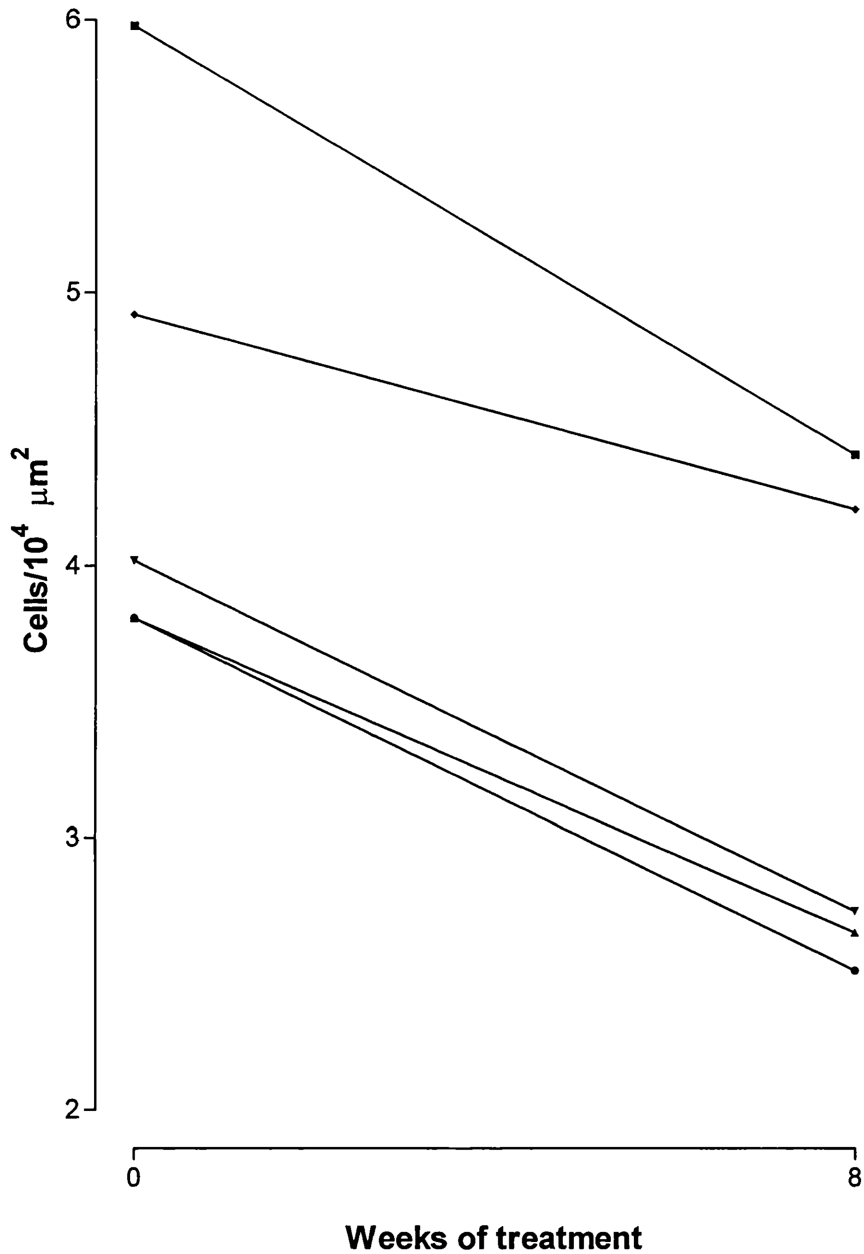

Figure 4.6 The number of cells expressing IL-4 in lesional skin of patients with AE before and after 8 weeks of treatment with CHT. The results are numbers of lymphocytes per $10^4 \mu m^2$ that stained positively using an anti-IL-4 antibody and a modified alkaline phosphatase technique.

4.4 DISCUSSION

This study has shown that in AE lesions, there is increased expression of the mRNA for the Th2 cytokine IL-4 and this is downregulated by treatment. This is associated with improvement in clinical disease. Using in situ hybridisation mRNA levels for the cytokine have been recorded which relate to the capacity of the cells to secrete IL-4. Also, by using a MoAb for IL-4, actual numbers of cells expressing IL-4 can be quantified. Treatment leading to a reduction in disease severity downregulates both mRNA for IL-4 and numbers of cells expressing IL-4. Not surprisingly, the results also show that an improvement in clinical disease is associated with a decrease in the absolute numbers of lymphocytes present in the inflammatory infiltrate within lesional skin. Thus, it is not possible from these investigations to conclude whether the marked decrease in IL-4 producing lymphocytes is due to a selective downregulation of Th2 lymphocytes, decreased secretion of IL-4 from the lymphocytes or simply a result of the decrease in total numbers of T cells. However, the downregulation in IL-4 mRNA to near normal levels clearly suggests that this cytokine is more relevant to disease severity than the Th1 cytokine IL-2 where the levels of mRNA are not significantly affected by treatment, although there is no data of numbers of cells that express IL-2. It may be relevant nevertheless that in 80% of our subjects, treatment lead to an upregulation of IL-2 mRNA implying that there may be a switch from Th2 to Th1 cells as clinical disease improves. This

also supports the hypothesis that it is predominantly Th2 cells that are selectively downregulated as clinical severity is reduced.

CHAPTER 5

GENERAL DISCUSSION

5. GENERAL DISCUSSION

This study has quantified the level of disease severity and parameters of immunopathology in samples of lesional skin from AE patients before and after efficacious therapy, and thus it has been possible to dissect out aspects of immune dysfunction that appear related to clinical severity of the disease.

Aberrations within the balance of immunocompetent cell populations in samples of lesional skin from patients with AE have been identified. Culture studies have shown that monocytes from patients with AE differentiate more rapidly compared with similar cells from normal controls and increased numbers express differentiation markers associated with the phenotype associated with antigen presentation. Furthermore the study demonstrates a dysregulation in the expression of the low affinity IgE receptor (CD23) on subsets of macrophages. Using phenotypic markers it is shown that in AE antigen presenting cells of the skin express these receptors; whereas in normal skin tissue phagocytes contribute the major population of CD23+ cells. This suggests that in AE, CD23 may be associated with antigen presentation. Evidence is presented that these abnormalities are principally promoted by the local environment rather than being the sequelae of a systemic immunologic problem. The results also suggest that in this local environment, the balance of

cytokine production by T cells may promote aberrations in monocyte differentiation and CD23 expression.

Eczema is a very common dermatological condition which is of great importance both as a health care problem and economically, in that it is associated with a considerable degree of morbidity which may affect work habits. Despite the high incidence of the disease, the pathogenetic mechanisms involved are poorly understood and the current therapeutic options are not curative. The therapeutic approach ranges from the use of topical emollients and allergen avoidance strategies, to the use of topical corticosteroids and potent immunomodulatory drugs such as cyclosporin A [Sowden *et al.* 1991] and Interferon gamma [Hanifin *et al.* 1993]. Even cytotoxic drugs such as azathioprine have been used [Younger *et al.* 1991].

Systemic treatment is limited by potentially serious side effects discussed in Chapter one. Osteoporosis, hypertension and diabetes mellitus are examples of the risks with long term steroid therapy [Schimmer and Parker 1996]. Cyclosporin has been shown to cause changes at the microscopic level in the kidneys of all patients treated over a period of two years and arteriolar hyalinosis and interstitial fibrosis are seen on renal biopsy [Zachariae *et al.* 1998]. Long term use of both cyclosporin and azathioprine have been associated with lymphoproliferative and skin malignancies [Taylor and Shuster

1992, Jones *et al.* 1996, Sieber 1977]. Ultraviolet therapy increases the risk of the subsequent development of squamous cell carcinomas of the skin [Bruynzeel *et al.* 1991] and involves the inconvenience for patients of two to three visits each week to a phototherapy unit during treatment. Gamma interferon currently needs to be administered under controlled circumstances subcutaneously within the hospital setting thus limiting its application.

Chinese herbal therapy has been used successfully by Chinese practitioners for many centuries, without rigorous clinical trials. However, more recently a 10 herb preparation has been used in two double blind placebo controlled studies in adults and children to treat moderate to severe recalcitrant AE [Sheehan *et al.* 1992, Sheehan and Atherton 1992]. During these trials, the side effects were generally mild and gastrointestinal in nature and the treatment was shown to be effective. A major limitation of the original decoction was the daily time consuming method involved in its preparation. This is markedly different from conventional treatments, and continued compliance with the ritual of preparation would be difficult for many patients. The initiation of a clinical trial of CHT within the Department of Dermatology at Royal Free Hospital offered the opportunity to biopsy AE patients before and after this potentially efficacious therapy, thus determining which aspects of immunopathology may be associated with clinical severity. This study compared the use of the original

decoction, with the associated drawback of laborious preparation time, with a freeze dried granule preparation which is easily taken on a regular basis.

The current study was able to confirm the previous reports of efficacy [Sheehan *et al.* 1992, Sheehan and Atherton 1992], and offered in addition convincing evidence that the freeze dried granule preparation of the herbs was as effective clinically as the original decoction. Perhaps more importantly it was able to show that the granule preparation was better tolerated by the patients and produced no greater side effects than the decoction. General compliance is essential in any long term therapy. In asthma for example much effort is taken up in monitoring compliance [Schmier and Leidy 1998]. With this study in progress therefore, it provided an ideal opportunity to obtain a patient group who clinically changed from a state of more to less severe eczema after receiving Chinese herbal therapy.

As well as good compliance and the availability of 'before and after' samples, attempts to relate immunopathological changes with clinical improvement required a reproducible method of quantifying the clinical condition. Although quantitative and semi-quantitative techniques are available for measuring immunopathological parameters, clinical status measurement often relies heavily on subjective judgement.

The clinical severity of the eczema in our patient group was assessed using a scoring system which measures the area of skin involved, and the severity of erythema and surface damage (papules, lichenification, vesicles); a system described previously, [Heddle *et al.* 1984]. Although subjective, good reproducibility was recorded, provided a full set of scores at all body sites was taken into account [Xu 1998]. Other scoring systems also take into account patients' symptoms such as pruritus leading to sleep disturbance [Costa *et al.* 1989, Sowden *et al.* 1991, Rajka and Langeland 1989]. These scoring systems are also subjective in nature yet are well accepted by the medical and scientific community [Finlay 1996]. Results reported here using this body scoring system confirm previous reports of the clinical efficacy of CHT [Sheehan *et al.* 1992, Sheehan and Atherton 1992, Sheehan and Atherton 1994, Sheehan *et al.* 1995].

Previous studies in this [Buckley *et al.* 1992, Buckley *et al.* 1993, Xu *et al.* 1997, Zachary *et al.* 1985b] and other laboratories [Maurer and Stingl 1995, Mudde *et al.* 1995a, Bruynzeel-Koomen *et al.* 1986, Bieber 1997] have revealed specific dysregulation in immunological parameters in AE. In particular aberrant expression of CD23 [Buckley *et al.* 1992, Mudde *et al.* 1995a] and production of IL-4 [Van der Heijden *et al.* 1991, Mudde *et al.* 1992] have been implicated in the pathogenesis. In order to identify whether the CD23 expression in the

lesions and the control of its expression by the Th2 cytokine, IL-4, are components of the pathogenetic mechanisms leading to clinical disease, this thesis used a dynamic clinical model. This study investigated changes in vivo wherein patients with moderate to severe clinical disease were treated with effective treatment resulting in clinical improvement. This provided the opportunity to investigate specific parameters and their relationship to disease severity. Ultimately, the future targets of immunotherapy may be identified by revealing those immune parameters that are directly related to clinical disease severity.

Chronicity of disease was also a consideration and in this study all patients had disease which had not remitted for at least 1 year prior to starting treatment with CHT. Clinical scores were assessed before and after 8 weeks treatment with CHT. At these times, peripheral blood was collected and lesional skin biopsies taken. There is a relationship between clinical severity and immunopathology as demonstrated by the observed decrease in the numbers of T lymphocytes and antigen presenting cells in the lesions when there is clinical improvement after therapy. Work in this laboratory [Buckley *et al.* 1992] has also shown that in both non lesional skin and lesional skin, histologically there is a mononuclear cell infiltrate. There are however decreased numbers of T cells and antigen presenting cells in non lesional compared with lesional skin suggesting that the

clinical involvement of the skin is reflected by an increased mononuclear infiltrate on a microscopic level.

AE is deemed an allergic condition where the body's immune system responds inappropriately to environmental allergens, for example cat or dog dander, pollen, or house dust mite excrement. By definition therefore this clinical condition is manifest only in atopic subjects. In such individuals, reactivity to these environmental allergens generates raised serum IgE levels. Such raised IgE titres [Stone *et al.* 1973] then predisposes atopics to immediate type hypersensitivity reactions involving degranulation of mast cells and eosinophils. Although AE and other clinical conditions such as hayfever and asthma are associated with a state of atopy, this immune dysregulation alone is not the cause of these clinical problems. Indeed, many subjects exhibiting atopic reactivity do not exhibit AE [Jones *et al.* 1975]. Up to 40% of the population show atopic reactivity if tested yet only 7 to 10% will exhibit clinical symptoms. Furthermore cases of AE have been reported where circulating IgE levels may be normal, while elevated levels of IgE are found in other conditions such as lymphoma [Sundstrom *et al.* 1988], Job's syndrome [Donabedian and Gallin 1983], parasitic infection [Bell 1996] and hypergammaglobulinaemia [Jako *et al.* 1997] without any concurrent evidence of AE. Thus high IgE titres are not pathognomonic of atopy and bear no relation as to whether eczema is present or not. Other immunopathogenic features must therefore be present. The

development of the lesions in the skin cannot be based upon the inconsistently raised levels of IgE in the peripheral blood or the type 1 response to environmental allergens. Thus there is a need to investigate in more detail other possible features of immune dysfunction within the skin and peripheral blood of AE patients which may represent further targets for therapeutic intervention.

This study has confirmed the work of others, that within the skin of patients with AE, a mononuclear cell infiltrate is found, dominated by T cells and antigen presenting cells. The reaction exhibits the characteristics of a type IV delayed hypersensitivity reaction [Zachary *et al.* 1985a]. Because of this, it is now generally accepted that this disease is also associated with dysfunction within the T cell mediated immune response network. However, the underlying pathogenesis remains obscure. Macrophages and T cells are recognised as crucial players in many other if not all chronic inflammatory diseases such as sarcoidosis [Poulter 1990, du Bois 1990], rheumatoid arthritis [van den Berg and van Lent 1996], ulcerative colitis [Allison *et al.* 1988] and asthma [Poulter and Burke 1996].

The skin is a common site for immune dysregulation to be manifest as a clinical problem. Other chronic skin conditions such as bullous pemphigoid, pemphigus vulgaris and dermatitis herpetiformis are characterised by antibody or immune complex deposition promoting inflammatory reactions [Jordon *et al.* 1985]. In

these cases underlying immunological defects are clear. For example in pemphigus, circulating IgG autoantibodies directed against the keratinocyte desmosomal proteins form local deposits resulting in loss of adhesion between epidermal cells and intraepidermal splitting [Korman 1990]. Yet it is thought to be both a loss in cell adhesion as result of activation of plasmin as well as activation of the complement cascade by local autoantibody deposition that results in the inflammatory process which destroys intercellular connections within the epidermis. The circulating autoantibodies are also known to correlate with the disease activity, yet we do not understand what promotes the formation of the autoantibody.

In contact dermatitis it has been recognised for many years that the erythema and induration of the skin are a result of a T cell mediated delayed type hypersensitivity reaction promoted by hapten-protein conjugates forming after contact with a variety of small molecular weight chemicals. In contrast, in AE, the antigens promoting the T cell reaction cannot be identified within the skin lesions which makes it difficult to understand what is promoting the T cell reaction in AE.

It would be reasonable to suggest that an understanding of the links between atopic reactivity and the chronic T cell reactions in the skin may unravel this complex situation. With raised circulating IgE and IgE-allergen complexes, it is

reasonable to determine whether there is any increase in the IgE receptors that can bind these complexes within the skin. To this end, this study has sought evidence linking these two phenomena by investigating the distribution and regulation of the expression of IgE receptors on antigen presenting cells in the skin.

Aberrant T cell activation in AE lesions has been demonstrated previously with an excess of activated T cells within the cellular infiltrate [Soter 1989, Bos *et al.* 1992]. There are increased numbers of dendritic cells and Langerhans cells; cell types that have primary roles in antigen presentation although there is no evidence of actual increased antigen presentation within the lesions [Zachary *et al.* 1985a]. Eosinophils also accumulate in some but not all AE lesions [Leiferman 1989, Soter 1989]. This would suggest that an increased accumulation of eosinophils is not a prerequisite for the manifestation of this disease. Interestingly the levels of eosinophilic cationic proteins in peripheral blood has been reported to correlate with disease severity [Czech *et al.* 1992, Halmerbauer *et al.* 1997]. Increased expression of IgE receptors has also been demonstrated on dendritic cells within the lesional skin [Grabbe *et al.* 1993, Schmitt *et al.* 1990].

This study has confirmed that there are increased numbers of dendritic cells, with increased numbers of cells that express the low affinity IgE receptor in

lesional skin in AE. Also there are aberrations in local cytokine production. These features describe an environment where aberrant CD23 expression on antigen presenting cells could promote T cell activation via facilitated antigen presentation [van der Heijden *et al.* 1995]. The increased expression of IL-4 within the lesions demonstrated in this study supports the suggestion that it is local IL-4 that promotes CD23 expression on the dendritic cells, as IL-4 promotes CD23 expression on circulating B cells and monocytes [Alderson *et al.* 1994, Delespesse *et al.* 1991].

Most previous work has been conducted on samples from existing lesions . Such an approach fails to establish whether observed aspects of immune dysfunction are causative of the clinical lesions or effects of the emergence of pathology promoted by other abnormalities. This study has attempted to resolve this problem by comparing the immunopathology in biopsies of lesional skin sampled when the AE is recalcitrant to treatment, to matched samples taken and investigated after clinical improvement has been achieved by efficacious therapy. The approach rests with the premise that any move towards 'normal' identified within an immunologic parameter that is associated with clinically defined improvement is likely to have contributed to the original pathogenic process promoting the disease.

Such an hypothesis is seen to hold in other allergic diseases. In eosinophilic allergic aspergillosis (EAA), circulating precipitins to allergens may be present in both symptomatic and asymptomatic patients [Johnson *et al.* 1989] which suggests that the presence of these precipitins is related to the disease but not necessarily to the disease severity. Lymphocytosis in the bronchoalveolar lavage of EAA patients is present in symptomatic patients but declines when treatment is given or patients are protected from exposure to allergens [Johnson *et al.* 1989] suggesting that the T cells are relevant to active disease. Study of the inflammatory reaction in the lung that promotes the restrictive defect in this condition, consistently shows lymphocyte infiltration and activation while no definite evidence for a pathogenic role for precipitins has emerged.

Immunohistological studies have focused attention on several aspects of immune dysregulation found in AE. These include: the distribution of IgE receptors [Maurer and Stingl 1995, Buckley *et al.* 1992, Mudde *et al.* 1995b, Bieber 1997]; changes to the macrophage and dendritic cell populations in the skin [Bos *et al.* 1986,]; and the local influence of cytokines derived from Th1 and Th2 T lymphocytes [van der Heijden *et al.* 1991, Bos *et al.* 1992, Mudde *et al.* 1992].

Earlier studies in these laboratories identified aberrant expression of CD23 on antigen presenting cells as being characteristic of AE lesions and distinguished

these from those of contact sensitivity reactions, despite the fact that both are dominated by T cells [Buckley *et al.* 1992]. This work revealed that the main difference between lesional skin in AE and a patch test with a contact allergen is the switch in the expression of CD23 from effector macrophages to inducer macrophages. This study has investigated further the relevance of this aberrant CD23 expression to clinical disease severity.

From the results presented here it can be seen that there is a significant reduction in numbers of CD23+ cells in all the different macrophage subsets with treatment. Importantly, an overall decrease in the level of expression of CD23 was also seen. It is of relevance to note there is also a decrease in both the number of HLA DR+ cells and in the level of expression of this MHC class II antigen which is expressed by antigen presenting cells. In the lesions of AE, there are clearly increased numbers of antigen presenting cells, identified by MoAb RFD1. The absolute numbers of RFD1+ cells and Langerhans cells decrease in association with clinical improvement which suggests that there is either a decrease in recruitment or an increase in removal of these cells from the skin. This study has addressed the issue of whether there is aberrant monocyte recruitment from the peripheral blood and subsequent differentiation in vitro.

With the knowledge that some monocytes express CD23, the possibility emerges that dysregulation of immune interactions within the lesions is a consequence of recruitment of circulating monocytes that already exhibit aberrant CD23 expression. Alternatively, the local environment within the skin may exert a dominant influence on monocyte differentiation and the relative expression of FcεRII on macrophage subsets as they mature. By directly testing this possibility, the current work has confirmed that there is aberrant monocyte differentiation in vitro when cells taken from patients with AE are compared with normal non atopic subjects.

Macrophages can be divided into functionally distinct subsets which mature from a common monocyte origin [Johnston 1988]. The RFD1+ macrophages are inducer cells and associated predominantly with antigen presentation [Poulter *et al.* 1986, Spiteri and Poulter 1991], the RFD7+ cells are phagocytic effector cells [Spiteri and Poulter 1991, Spiteri *et al.* 1992b], and those expressing both RFD1 and RFD7 exhibit a T cell suppressive function [Spiteri *et al.* 1992a, Poulter and Burke 1996]. Over a 7 day culture period, monocytes from atopic eczema patients exhibit a more rapid differentiation as measured by early expression of the cell surface antigens, RFD1 and RFD7. However efficacious treatment did not lead to any significant changes in this pattern of differentiation.

There was however an increased proportion of monocytes expressing CD23 in the AE patients compared with the normal controls throughout the culture period. Furthermore, treatment lead to a downregulation of the proportion of RFD1+ monocytes that expressed CD23 during the first few days of culture although this difference was lost by day 7. These results suggest that aberrant monocyte differentiation in AE is present amongst those monocytes that exhibit surface antigen RFD1 (a marker associated with antigen presentation) [Poulter *et al.* 1986, Spiteri and Poulter 1991]. Notably, in cells from AE patients, RFD1+ monocytes that express CD23 maintain higher proportions throughout the culture period than equivalent cells from normal controls. Also, the proportion of RFD1+/CD23+ cells is downregulated by efficacious treatment. These observations support the suggestion that aberrant expression of CD23 on APCs (RFD1+ cells) may contribute to pathogenic changes in AE.

There are however two types of IgE receptors: the high affinity (FcεRI) and low affinity (FcεRII or CD23) [Bieber 1992, Wang *et al.* 1992, Sutton and Gould 1993, Maurer and Stingl 1995]. Of these, more is known of the function of the high affinity receptor. FcεRI is expressed by Langerhans cells, mast cells and other basophils in normal individuals [Ravetch and Kinet 1991, Sutton and Gould 1993]. In atopic subjects, peripheral blood monocytes also express this

receptor [Maurer *et al.* 1994]. It is accepted that cross linking by allergen of IgE molecules bound to mast cells and basophils via FceRI triggers cellular degranulation releasing pro-inflammatory mediators; this being the basis of immediate type hypersensitivity reactions [Ishizaka 1989]. It has been suggested that FcεRI on antigen presenting cells has a role as an allergen-focusing receptor in facilitated antigen presentation [Bieber 1997, Maurer and Stingl 1995].

Circulating IgE-allergen complexes are detected in AE. The possibility exists therefore that these IgE complexes if entering the skin can bind via Fc receptors to APC within the skin, such as Langerhans cells and macrophages. This may result in the subsequent presentation of antigen to T cells. It is known that binding of IgE-allergen complexes enables T cell activation at antigen concentrations that are 100-1000 fold lower than with non complexed antigens [van der Heijden *et al.* 1995]. This process may thus form the link in pathogenesis between raised IgE levels seen in most AE patients [Stone *et al.* 1973], and the T cell stimulation seen in the skin lesions. With notable exception [Mudde *et al.* 1995a, van der Heijden *et al.* 1995], there has been less systematic investigation of the role of CD23 in antigen presenting mechanisms.

CD23 is an integral membrane protein and is a member of the calcium-dependent lectin family of proteins [Delespesse *et al.* 1991]. Membrane bound CD23 (mCD23) can be cleaved at the cell surface to form a soluble form of the receptor (sCD23) which can also bind IgE [Delespesse *et al.* 1989]. Ligands, other than IgE, have been reported for CD23 [Sutton and Gould 1993]. One example is the CD21 molecule which is the receptor for the complement component C3b [Fearon 1993]. CD23 has also been implicated as having a role in cell adhesion as its structure is similar to the selectin family of adhesion molecules [Sutton and Gould 1993]. Another function of both forms of CD23 is to promote B cell growth and differentiation [Delespesse *et al.* 1992]. In this regard, sCD23 has been found to enhance IgE production by B cells thus sCD23 may be contributing to the increased IgE seen in peripheral blood by enhancing production of this immunoglobulin by B cells. Both forms of CD23 are thought to have a role in antigen presentation, as antigens can be internalised by phagocytosis or endocytosis by these molecules [Sutton and Gould 1993]. Interestingly there have been reports of increased levels of sCD23 in AE patients [Takigawa *et al.* 1991, Muller *et al.* 1991, Reddy *et al.* 1992, Bujanowski-Weber *et al.* 1992]. CD23 is known to be expressed on a wide variety of cells including lymphocytes, monocytes, eosinophils, platelets, natural killer cells and Langerhans cells [Delespesse *et al.* 1991, Kehry and Hudak 1989]. In atopics, there is a well documented increase in the number of

peripheral blood mononuclear cells (PBM) that express CD23 [Nakamura *et al.* 1991].

Although most studies investigating facilitated antigen presentation have concentrated on the binding of IgE-allergen complexes to FcεRI [Maurer and Stingl 1995], there are reports of low affinity receptors being involved in similar mechanisms of T cell stimulation [van der Heijden *et al.* 1995, Mudde *et al.* 1990a]. Although FcεRII may normally only play a minor role in facilitated antigen presentation, this could be as a consequence of their normal distribution within the macrophage populations being restricted to effector phagocytes [Buckley *et al.* 1992], where they may act to promote opsonisation. In AE, expression of FcεRII has been shown to switch to dendritic cells expressing the phenotypic characteristics of APCs [Buckley *et al.* 1992]. Under these circumstances, the capacity of FcεRII to bind IgE-allergen complexes and promote facilitated antigen presentation may be significantly enhanced. Facilitated antigen presentation has been demonstrated in vitro to involve CD23 [van der Heijden *et al.* 1995]. In these studies, IgE-allergen complexes were formed with Der p 2 (housedust mite antigen) and IgE by pre-incubation of Der p 2 with atopic sera containing allergen-specific IgE. Epstein-Barr virus transformed B cells, of which 60-80% expressed CD23, were used to bind

these complexes. When the IgE-allergen complexes were incubated with these CD23 expressing B cells as well as T cells, this resulted in T cell proliferation. Interestingly, when sera was used with radioallergosorbent tests (RAST) showing lower Der-p concentrations, this resulted in reduced T cell proliferation as compared with the use of sera giving higher IgE titres on RAST testing. There is therefore some rationale for the view that T cell activation via FcεRII expressing dendritic cells binding IgE-allergen complexes, may form the basis for the chronic cutaneous lesions of AE.

To support this hypothesis, evidence is needed that the local milieu within the skin is such that it will promote and stimulate CD23 expression on dendritic cells. Further, there needs to be evidence that IgE is bound to these potential APCs. As far as this latter point is concerned there are several studies showing the presence of IgE in lesions of AE [Bruynzeel-Koomen *et al.* 1986, Leung *et al.* 1987], including reports that this immunoglobulin is present on the surface of dendritic cells [Barker *et al.* 1988, Leung *et al.* 1987]. Regarding the local milieu and CD23 expression; it is important to look to the work that has focused on the dominance of the Th2-type T cells in these lesions [van der Heijden *et al.* 1991, Mudde *et al.* 1992]. For T cells to interact with macrophages there must be communication via soluble mediators released by these cells.

Th2-type T cells exhibit a cytokine repertoire that includes IL-4 [Del Prete *et al.* 1994]. This cytokine has been shown to function as a chemotactic factor for basophils [Schleimer *et al.* 1992], and as a promoter of CD23 expression, [Bieber *et al.* 1989b, Alderson *et al.* 1994, Delespesse *et al.* 1991]. Indeed, IL-4 has shown to induce CD23 on PBM [Dierks *et al.* 1994, Krauss *et al.* 1993, Alderson *et al.* 1994] in a dose dependent manner. There is also evidence that these latter functions may be linked.

Another role for CD23 in antigen presentation is in IgE mediated allergen presentation by B cells [Kehry and Yamashita 1989, Mudde *et al.* 1995a]. IgE bound via the IgE receptor on antigen specific B cells encounters antigen/allergen thus resulting in activation of the B cell. The antigens are then processed and presented to specific T cells in association with MHC II and if the responding T cell is of the Th2 type, the B cell is induced to switch to produce antigen specific IgE. Such IgE mediated antigen presentation could thus contribute to the overproduction of IgE and the presence of increased numbers of Th2 type cells, which in turn would lead to raised IL-4 production [Romagnani 1991]. Thus a vicious circle may be initiated that drives the Th2 dominated T cell activation responsible for skin inflammation in AE.

The use of paired biopsies taken pre and post treatment where clinical improvement occurred, makes it possible in the present study to link severity of

disease with raised expression of CD23. If local IL-4 production is responsible for regulating CD23 expression, a fall in IL-4 post treatment would be predicted. Using methods of in situ hybridisation, it has indeed been possible to demonstrate an association between local IL-4 production, (as recorded by levels of mRNA) and clinical severity. Of some interest was the further observation that tissue levels of IL-2 mRNA were increased following therapy. The cytokine IL-2 is produced by Th1-type rather than Th2-type T cells [Del Prete *et al.* 1994]. This observation opens up two possibilities: either there is positive stimulation of the Th1 subset with downregulation of Th2 cells; or that CHT, (effective therapy), is associated with a shift of Th2 to Th1 cells i.e. with a repertoire from IL-4 production to IL-2 production.

The results presented thus further our understanding on three points. Firstly, it is confirmed that CHT is an effective therapeutic option in cases of atopic eczema. This may be of particular importance in those cases where the patients appear resistant to conventional therapy. Secondly evidence is presented to support the hypothesis that CHT therapy promotes a modulation of immunological mechanisms both directly and indirectly. Thirdly by identifying concurrent changes in clinical status and immunopathology, evidence towards the immune basis of AE emerges.

The cause and effect relationships between these phenomena remain obscure. Together with existing knowledge however the results enable a working hypothesis to be proposed. The lesions of AE are characterised by macrophage subset imbalance, aberrant expression of IgE receptors and over stimulation of Th2 type cells in the skin.

A hypothesis is proposed whereby:

1/ Aberrant monocyte differentiation leads to the expression of CD23 on inductive cells.

2/ IgE-allergen complexes bind to CD23 on these inductive cells

3/ Inappropriate antigen presentation causes local stimulation of Th2 type cells

4/ Local production of IL-4 is induced

5/ This further promotes CD23 expression

Thus while exposure to allergen occurs, abnormal IgE titres exist and this process will be self perpetuating.

It is important to emphasize that this hypothesis is speculative. However such a pathogenic process fits the facts as currently understood. Further, this study has revealed for the first time that a therapeutic agent which modulates monocyte differentiation also causes reduced Th2 type activity and reduced CD23 expression in vivo. There is of course no direct evidence that these effects are related. Caution should also be exercised in interpreting the *in vivo*

data as changes to immunopathology are inevitably a dynamic process and this interpretation is based on two time points where biopsies were taken. Nevertheless there is a logic to the hypothesis proposed above as it satisfies recent observations of dysregulation in IL-4 production [van der Heijden *et al.* 1991], T cell subsets [Romagnani 1992a] and IgE receptor expression [Grabbe *et al.* 1993, Schmitt *et al.* 1990] in AE.

Recent observations of the role of apoptosis in regulating chronic inflammatory reactions [Orteu *et al.* 1998] need to be considered. There would appear to be a very real possibility that persistent stimulation of T lymphocytes within chronic inflammatory conditions may not be due to continued stimulation but due to a failure of reactive cells to be driven into programmed cell death. This possibility requires further investigation in AE.

This thesis has confirmed that this ten herb extract of CHT is an effective treatment for AE however it would be interesting to investigate whether these herbs separately could be an effective treatment alone. Although in China, polypharmacy has been in practice for centuries so it would be unlikely that the herbs individually would be as effective clinically. Long term studies of both clinical efficacy of CHT and importantly long term safety of treatment would be essential.

Further study of immunopathology of sequential investigations of skin immunopathology at several time points over a treatment period could corroborate this work especially if deterioration in clinical disease was found to show a change in immune parameters returning to that associated with more severe disease. It would be interesting to repeat the studies with several treatment modalities such as cyclosporin and azathioprine in addition to CHT.

There is no doubt that a full understanding of the pathogenesis of AE should be the basis of the approach to treatment. It is clear that many of the treatments that are effective in AE modify the immune response in some way. However the exact mechanism of action is not established in any of the therapies. It is important to determine specific abnormalities in the chain of pathological events within the immune system as these may provide targets for a therapeutic approach. The area of immunopathogenesis requires further investigation. In this study immunohistological techniques were used with monoclonal antibodies to define cell phenotypes and mRNA probes used to define cytokine expression in situ.

This study investigates which of the abnormalities noted above are modulated by treatment with CHT in a cohort of patients. This aims to help provide a link between Type I Hypersensitivity (apparent in peripheral blood) and Type IV

hypersensitivity (mediated in the skin). The study investigates more closely the increased presence of CD23+ antigen presenting cells in lesional skin in AE and investigates further the increased Th2 lymphocyte activity in AE.

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7. PUBLICATIONS/PRESENTATIONS ARISING FROM THIS THESIS

7.1 PUBLICATIONS

1. Association of immunological changes with clinical efficacy in atopic eczema patients treated with Traditional Chinese Herbal Therapy (Zemaphyte).
J.Int Archives of Allergy and Immunology 1996;109:243-249.
Latchman Y, *Banerjee P*, Poulter LW, Rustin M, Brostoff

2. Changes in CD23 expression of blood and skin in atopic eczema after Chinese herbal therapy
Clinical and Experimental Allergy 1998;28:306-14.
Banerjee P, Xu X-J, Rustin MHA, Poulter LW.

3. Modulation of immune mechanisms in the skin of atopic eczema patients by Chinese herbal therapy.
Br J Dermatol 1997;136:54-59.
Xu X-J, *Banerjee P*, Rustin MHA, Poulter LW.

4. A new more palatable formulation of Chinese herbal therapy in the treatment of atopic eczema in adults. (In press)
Clinical and Experimental Dermatology
Banerjee P, Rustin MHA.

5. Traditional Chinese herbal therapy (Zemaphyte) for atopic eczema induces IL-10 and TNF-alpha production in vitro.
(submitted to J of Allergy and Clinical Immunology)
Latchman Y, Chow J, *Banerjee P*, Rustin M, Brostoff J.

7. Analysis of cytokines IL-2 and IL-4 expression in situ in lesional skin from patients with atopic eczema and their relationship with disease severity. (in preparation)
Banerjee P, Xu X-J, Condez A, Rustin MHA, Poulter LW.

8. Effects of Chinese herbal Therapy on cytokine mRNA Expression in the skin of atopic dermatitis patients. (in press)
Clinical and Experimental Immunolo gy
Xu X-J, *Banerjee P*, Poulter LW, Rustin MHA.

9. Serum soluble CD30 reflects symptomatic disease activity in both atopic eczema and atopic asthma
(submitted to European J of Resp Medicine)
Leonard C, *Banerjee P*, Tormey VJ, Burke CM, Poulter LW.

7.2 ABSTRACTS

1. Efficacy of a new palatable formulation of Chinese herbal therapy as a treatment of atopic eczema.
Br J Dermatol 1994: 131 (suppl 44): 26.
Banerjee P, Rustin MHA.

2. Macrophage differentiation and cytokine production in atopic dermatitis.
J Invest Dermatol 1995; 104(4): 683.
Banerjee P, Xu X-J, Poulter LW, Rustin M.

3. Relationships between CD23 expression on non-lymphoid cells and disease activity in atopic eczema.
J Invest Dermatol 1995; 105(3):516.
Banerjee P, Xu X-J, Poulter LW, Rustin MHA.

4. Clinical and immunological changes following successful treatment of atopic eczema with a new formula of Chinese herbal therapy.
American Academy of Dermatology, New Orleans, February 1995.
Banerjee P, Xu X-J, Poulter LW, Latchman Y, Brostoff J, Rustin MHA.

5. T cell subset activity and circulating levels in patients with atopic eczema.
Br J Dermatol 1996; 134(3): 570.
Banerjee P, Xu X-J, Poulter LW, Rustin MHA.

6. Relationships between Interleukin 4 production and CD23 expression in the lesional skin of patients with atopic eczema.
J Invest Dermatol 1996; 107(3): 467.
Banerjee P, Xu X-J, Poulter LW, Rustin MHA.

7. Relationships between disease severity with Th2 activity and CD23 epression in lesional skin of patients with atopic eczema.
Br J Dermatol 1997; 136:54-59.
Banerjee P, Xu X-J, Rustin MHA, Poulter LW.

8. Immunohistochemical changes in the skin of patients with atopic eczema treated with Chinese herbal therapy.
Br J Dermatol 1994; 131(2): 442.
Xu X-J, *Banerjee P*, Poulter LW, Rustin MHA.

9. Effects of Chinese herbal therapy on cytokine mRNA expression in the skin of atopic dermatitis patients.
The Second Asia Pacific Congress of Allergology and Clinical Immunology.Taiwan, 18-22 November, 1995.
Xu X-J, *Banerjee P*, Rustin MHA, Poulter LW.

10. CD30 as a marker of Th2 activity in atopic individuals.
Irish Thoracic Society Annual Scientific Meeting, Belfast, November 1995.
Leonard C, *Banerjee P*, Tormey V, Faul J, Poulter LW, Burke CM.

11. Changes in cell surface markers with atopic eczema patients treated with Zemaphyte - Traditional Chinese Herbs. *International Meeting on Allergy and Clinical Immunology, Sweden, June 1994.*
Brostoff J, Latchman Y, *Banerjee P*, Rustin M.

7.3 ORAL PRESENTATIONS

1. ***The British Association of Dermatology; July 1994, London***
Efficacy of a New Palatable Formulation of Chinese Herbal Therapy as a Treatment of Atopic Eczema.

2. ***The American Academy of Dermatology; February 1995, New Orleans***
Clinical and Immunological Changes Following Successful Treatment of Atopic Eczema with a New Palatable Formula of Chinese Herbal Therapy.

3. ***Eczema and Contact Dermatitis Conference for the Royal College of Nurses; October 1995, London***
Chinese Herbal Therapy in the Treatment of Atopic Eczema.

4. ***First European Atopic Dermatitis Workshop; Feb 1996, Utrecht***
Dendritic cells in Atopic Dermatitis and their Expression of Low Affinity IgE Receptors.

5. ***The British Society of Investigative Dermatology; March 1996, Glasgow***
T Cell Subset Activity and Circulating CD30 Levels in Patients with Atopic Eczema.

6. ***The British Society of Investigative Dermatology; April 1997, Bristol***
Relationships Between Disease Severity with Th2 Activity and CD23 Expression in Lesional Skin of Patients with Atopic Eczema.

7.4 POSTER PRESENTATIONS

1. ***The British Society of Investigative Dermatology; September 1994, Bath***
Immunohistochemical Changes in the Skin of Patients with Atopic Eczema Treated with Chinese Herbal Therapy.

2. ***The Society of Investigative Dermatology; May 1995, Chicago***
Macrophage Differentiation and Cytokine Production in Atopic Dermatitis.

3. ***The European Society of Dermatological Research; September 1995, Vienna***
Relationships Between CD23 Expression on Non-Lymphoid cells and Disease Activity in Atopic Eczema.

4. ***The European Society of Dermatological Research; September 1996, Amsterdam***
Relationships Between Interleukin 4 Production and CD23 expression in the Lesional Skin of Patients with Atopic Eczema.

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APPENDIX A

The following paragraphs should be read in the context of the Main Introduction within section 1.4 Immunopathology (page 31).

CUTANEOUS BASOPHIL HYPERSENSITIVITY

It has been documented that aqueous aeroallergen extract applied as a patch test to the mildly abraded skin on the back of patients with AE can induce an eczematous reaction (Mitchell *et al.* 1982) . Histologically these lesions consist of an inflammatory infiltrate with basophils, eosinophils , mononuclear cells and neutrophils and this is a form of cutaneous basophil hypersensitivity (CBH) (Dvorak 1976, Askenase 1977). Basophils have also been less frequently noted in contact hypersensitivity and in late phase reactions in human skin (Dvorak & Mihm 1972, Solley *et al.* 1976, deShazo *et al.* 1982). In guinea pigs CBH is considered to be lymphocyte dependent (Bast *et al.* 1971). In humans it has been shown that systemic plasma infusion or local intradermal serum injection from an atopic to a non atopic patient can transfer the ability to elicit the immediate and delayed type reactions in the skin to aeroallergens (Mitchell *et al.* 1984). In humans the presence of basophils in the patch test response can only be induced by local transfer of AE serum to non atopic recipients and not by systemic infusion of plasma prior to patch testing with the house dust mite antigen (Mitchell *et al.* 1984). However purified antibody to house dust mite (HDM) antigen when transferred lead to eosinophil and not basophil recruitment at patch test sites. This suggests that basophil recruitment is not mediated by sensitisation of mast cells with IgE alone and perhaps this involves a combination of T cell cytokines as well as locally released mediators by basophils.

In chronic AE mast cells have been noted in the mononuclear infiltrate within the dermis (Mihm *et al.* 1976). Basophils and eosinophils are seen less frequently unlike in CBH. It has been suggested that basophil recruitment occurs early on in the formation of the AE lesions and that this is followed by an increase in the numbers of mast cells (Mihm *et al.* 1976). Aeroallergens applied to the skin can induce a delayed type response with the features of CBH. Mitchell *et al.* have also demonstrated that repeated application of high concentrations of allergen to the same site results in the basophils seen in the initial infiltrate being replaced by mast cells (Mitchell *et al.* 1986). In the latter study the five patients were known to have high serum IgE levels and IgE-allergen complexes as well as showing a positive patch test response with the HDM antigen. At day 2 of patch testing with the HDM antigen erythema, papules, induration and exudation was seen macroscopically and histologically the cellular infiltrate included basophils, eosinophils, mononuclear cells and neutrophils that are the features of CBH. The HDM antigen was reapplied every 2 days for 10 days in 4 patients. By day 10 the centre of the lesion had become paler and there was surrounding erythema and scaling. In 3 of the 4 patients the numbers of mast cells within the lesions expanded by 50% and there was a fall in the number of basophils. It was suggested that the re-application of the HDM antigen to the same site mimicked the constant presence of HDM in the patients' environment so was more representative of

APPENDIX B

The following paragraphs should be read in the context of the study design and patient assessment in Chapter 2 (page 56).

The null hypothesis of the study (detailed in chapter 2) was that there was no difference between the two preparations of CHT used in the study; that is, both the granules and the decoction were equally effective. The primary measure of efficacy in this study was the percentage change in the clinical scores for erythema and surface damage. The power of the study was calculated to determine the numbers of patients that needed to be recruited to test the null hypothesis. Assuming a standard deviation of 50% and a two sided significance level of 5%, a sample size of 30 patients in each group is sufficient to detect a relevant difference of 30% with a power of 90%. The numbers of patients used in the clinical trial were thus adequate. For the current study a random selection of 5 to 8 patients were recruited to serve as a model of changing clinical status. The significance of the changes in each parameter demonstrates sufficient power.

Patients' compliance was assessed by asking the patients at each visit whether they had missed any treatments and asking them to return all unused CHT treatment at the end of the trial. It was established at the start of the study that failure to take treatment on more than 5 days in a 4 week treatment period would lead to withdrawal from the study. Poor compliance did not lead to withdrawal of any patients from the study. Throughout the study all patients were asked to continue with the same topical corticosteroids that they had been using during the 1 month prior to the study. As no new topical corticosteroids were introduced during the study or in the month leading up to the study, any differences in clinical score during the study could not be attributed to the topical corticosteroids. This could have been supported further by giving patients diary cards to record details and quantities of topical treatments used during the study.

This was an open randomised parallel group study. An alternative design would have been to have a either a double or single blinded study and a crossover study. The difficulties involved in blinding both the patient and the observer is that both the appearance and packaging of the CHT preparations have obvious differences in that one is a large quantity of CHT sachets and the other is a smaller volume of CHT granules. One way to overcome this would have been to introduce placebo preparations for both the granules and decoction. However this would require larger patient numbers and recruitment is more difficult when patients are aware that they may receive a placebo. Another alternative is to blind the observer so only the patient is aware which of the two CHT preparations he has taken. Particularly the person who took the skin biopsy could have been blinded if patients from both groups had been used. If the study had been a crossover study, the duration would have been much longer thus there is an increased risk of patients dropping out of the trial. Another disadvantage of the crossover design is that the washout period between the treatments is of a variable length of time and it is difficult to accurately plan the length of time required for the clinical scores to reach

APPENDIX C

The significance levels represented in the monocyte culture studies seem fair in Chapter 3 (page 91-101). No longitudinal analysis using ANOVA were required as multiple comparisons of the same data were not made simultaneously.

The advice of Dr Caroline Sabin in the Department of Epidemiology at the Royal Free Hospital NHS Trust is gratefully acknowledged.

APPENDIX D

The following paragraphs should be read in the context of the Final Discussion and expand the discussion of the high (FcεRI) and low affinity IgE (FcεRII) receptors (page 158).

The high affinity IgE receptor is a member of the immunoglobulin super family and on mast cells is present as a tetramer consisting of one α-chain, one β-chain and a gamma-chain homodimer (Ravetch & Kinet 1991). Normally only monocytes and Langerhans cells co-express the FcεRI α-chain (which binds IgE) and the gamma-chain (the signal- transducer) (Maurer & Stingl 1995). In AE it has been shown that there is increased expression of FcεRI on dendritic cells in the epidermis of both lesional and non lesional skin (Wollenberg *et al.* 1996). Using flow cytometry these cells were shown to lack CD1a and the Birbeck granule, features of classical Langerhans cells (Wollenberg *et al.* 1996). It is not apparent whether these cells have a stimulatory function contributing to an amplification of the inflammatory reactions in the skin or have a downregulating function. It has been suggested that FcεRI is the predominant receptor for IgE in lesional skin in AE (Klubal *et al.* 1997). In this latter *in vitro* study by Klubal *et al* , pre-incubation of acid treated AE skin with anti- FcεRI lead to lack of cellular uptake of IgE. The role of FcεRI on mast cells is clearly to bind IgE-allergen complexes and promote degranulation responsible for the type I hypersensitivity response. The role of FcεRI on monocytes and Langerhans cells is less clear. *In vitro* studies have shown that FcεRI on antigen presenting cells can function as an allergen focusing receptor resulting in 100-1000 fold amplification of autologous T-cell clones (Ravetch & Kinet 1991). This allergen specific T-cell clone proliferation was shown to be reduced by the addition of anti- FcεRI and not by anti- FcεRII by one group (Ravetch & Kinet 1991) which suggests that FcεRI is the relevant IgE receptor involved antigen presentation.

There is increasing evidence to support the role of the low affinity IgE receptor or CD23 in AE. FcεRII is structurally very different from other Fc receptors including FcεRI in that structurally it is a type 2 integral membrane protein (Sutton & Gould 1993). The N terminal cytoplasmic tail can exist in either of two forms FcεRIIa and FcεRIIb. FcεRIIa is expressed on antigen activated B cells and mediates endocytosis by B cells. FcεRIIb is inducible by IL-4 on macrophages, monocytes, platelets, eosinophils, platelets, Langerhans cells, B and T cells, natural killer cells and is involved in IgE mediated phagocytosis by cells such as monocytes and eosinophils. Both types of FcεRII have a large C terminal extracellular region containing a C-type lectin which is present in the selectin family of adhesion molecules (Sutton & Gould 1993).

There is evidence showing increased numbers of peripheral blood mononuclear cells expressing CD23 and this is predominantly as a result of increased CD23 expression by B cells, monocytes and macrophages (Nakamura *et al.* 1991). It has even been shown that the number of circulating B cells expressing CD23 is related to disease severity in AE with >50% of

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