REGULATION OF CUTANEOUS T CELL APOPTOSIS AND ITS RELEVANCE TO CHRONIC INFLAMMATORY SKIN DISEASE

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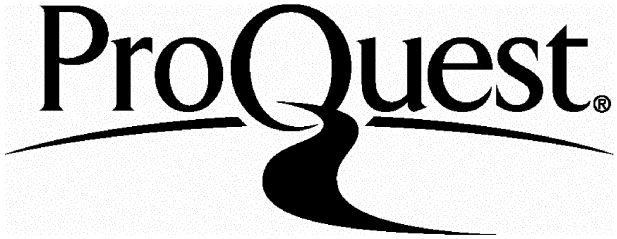

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ABSTRACT

Animal and *in vitro* studies suggest that resolution of inflammation is largely dependant on the clearance of activated T cells by apoptosis (programmed cell death). This study has sought to confirm this hypothesis *in vivo* in humans. Cutaneous PPD-induced delayed type hypersensitivity (DTH) responses were investigated in healthy volunteers, to determine features associated with both the generation and resolution of the reaction. This resolving immunological reaction was then compared to untreated chronic lesional atopic eczema (AE), to investigate whether a reduction in T cell apoptosis is involved in the generation of chronic cutaneous inflammation.

We have shown that in the DTH response, the induction phase involves not only recruitment, but also proliferation of CD4+ CD45RO+ T cells, while resolution occurs in part by induction of T cell apoptosis. Different waves of expression occurred in the two recognised groups of anti-apoptotic cytokines, at the height (IL-2R γ chain family) and during the resolution phase (IFN-β) respectively. We hypothesise that the proliferative phase and the resolution of the response are controlled by different levels of IL-2R γ chain cytokines, the presence of which promotes proliferation, while the absence of these mediators leads to apoptosis. Increased IFN-β production during the resolution phase may allow the survival of a proportion of primed T cells destined to maintain specific memory.

In chronic AE the distribution and phenotypic characteristics of infiltrating T cells were similar to those in resolving DTH responses, but low levels of T cell apoptosis were observed. IL-15 and IFN-β, members of both groups of anti-apoptotic cytokines, were expressed concomitantly. This may not only promote T cell recruitment and proliferation, but also cause excess numbers of T cells to survive in a state in which they can subsequently be reactivated without dying, and therefore contribute further to the cutaneous inflammatory response.

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ABBREVIATIONS

$\Delta\Psi m$ mitochondrial transmembrane potential
AE atopic eczema
AICD activation induced cell death
AIF apoptosis inducing factor
ANOVA Analysis of Variance
APC antigen presenting cell
β1 integrin CD29, VLA-4
β2 integrins CD11/CD18, LFA-1
Bax Bcl-associated X protein
BCG Bacille Calmette-Guerin
Bcl-2 B cell lymphoma-2 protein
Bcl-x B cell lymphoma-X protein
BSA bovine serum albumin
Ca2+ calcium
CD4 Class II MHC restricted T cells
CD8 Class I MHC restricted T cells
CD45RA+ naïve cell
CD45RO+ memory/effector cell
CD95/Fas/APO-1 pro-apoptotic cell surface receptor
CD95-L CD95 ligand
CLA cutaneous lymphocyte-associated antigen
CMV Cytomegalovirus
CTCL cutaneous T cell lymphoma
DAB diaminobenzidine
DC dendritic cell
DDC dermal dendritic cell
der.p1 *Dermatophagoides pteronyssimus* antigen 1
DISC death-inducing signalling complex
DNA deoxyribonucleic acid
DR4 Death Receptor 4
DTH delayed-type hypersensitivity
EBV Epstein-Barr virus
EC endothelial cell
EI erythema index
ELISA enzyme linked immunosorbant assay
FADD Fas-associated death domain protein
Fas-L Fas ligand
FcεRI high affinity IgE receptor
FcεRII CD23, low affinity IgE receptor
FITC fluorescein isothiocyanate
FLICE (FADD)-like interleukin-1b-converting enzyme-like protease, also known as caspase-8
FLIP FLICE inhibitory protein
GVHD graft versus host disease
HDM house dust mite
HUVEC human umbilical vein endothelial cell

ICAM Intercellular adhesion molecule
ICE interleukin-1β-converting enzyme
IFN interferon
Ig Immunoglobulin
IL interleukin,
IL-2-R IL-2 receptor
IL-7R IL-7receptor
IP-10 interferon-γ inducible protein
KC keratinocyte
kDa kilo-dalton
LC Langerhans cell
LCMV Lymphocytic Choriomeningitis Virus
LDA limited dilution analysis
LFA lymphocyte function associated antigen
LIT lymphocyte inhibitor of TRAIL
mCD95-L membrane bound CD95-L
MHC major histocompatibility complex
MIF migration inhibitory factors
MIP-1 α and β macrophage inflammatory proteins
MoAbs monoclonal antibodies
MR Mantoux reaction
mRNA messenger ribonucleic acid
NF-κB nuclear transcription factor κB
NGF nerve growth factor
NHS normal human serum
NRS normal rabbit serum
PBS phosphate buffered saline
PCR polymerase chain reaction
PI propidium iodide
PPD purified protein derivative
RAIDD RIP-associated Ich-1/CED-3 homologous protein with a death domain
RANTES regulated on activation normal T cell expressed and secreted
RIP receptor interacting protein
sCD95-L the soluble form of CD95-L
SSc systemic sclerosis
TBS Tris buffered saline
TCR T cell receptor
TdT terminal deoxynucleotidyl transferase
Tmix pan anti-T cell IgG MoAb mix
TNF tumour necrosis factor
TNFR1 type 1 tumour necrosis factor receptor
TRAIL TNF-related apoptosis-inducing ligand
TRICK-2 TNF-related apoptosis-inducing ligand receptor inducer of cell killing-2
TRITC tetramethylrhodamine isothiocyanate
TUNEL terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

UA unit area
UTP uridine tri-phosphate
UVR ultraviolet radiation
VCAM vascular cell adhesion molecule
VLA very late antigen
VZV Varicella Zoster Virus

1. INTRODUCTION

Although a large body of research has focused on how cutaneous inflammatory reactions are initiated, little is known about how they resolve. T cell mediated reactions are generally described as having a beginning: antigenic presentation and T cell activation; a middle: clonal expansion and differentiation into effector cells; and an end: a majority of effector cells die, whilst a few are retained as memory cells (reviewed in 1,2). In order for this sequence of events to occur, T cells need first to gain access to the site of antigenic stimulation. Thereafter, growth factors and cytokines in the local microenvironment will dictate their ability to proliferate and differentiate into effector cells. Once the antigenic stimulus has been dealt with, a majority of the expanded T cell population needs to be removed, and a minority of cells retained, to maintain immune homeostasis and T cell memory respectively. The clearance of T cells during resolution may be due both to the efflux of cells or to the death of cells *in situ.*

It is now recognised that cell numbers are controlled in a variety of biological systems by the process of cell suicide or apoptosis (3-6). This process may occur via two distinct pathways. The first involves re-ligation of the T cell receptor of activated, cycling T cells, through a secondary interaction of CD95 (Fas/APO-1) with its ligand (7,8). The second occurs following withdrawal of cytokines, such as those which signal via the γ chain of the IL-2 receptor (IL-2R). This causes downregulation of anti-apoptotic Bcl-2-family proteins relative to their pro-apoptotic counterparts and results in T cell death (4,9). The contribution of these processes to the resolution of cutaneous inflammation has not been studied. The primary aim of this study was therefore to determine factors associated with the resolution of a "normal" cutaneous immunological response *in vivo.*

Although clearance of the expanded T cell populations is essential, certain mechanisms exist which prevent apoptosis in a proportion of T cells, allowing them to be retained and form part of the memory pool. Recent studies have

suggested that a fibroblast-derived survival factor, now thought to be IFN-β (Pilling, D., Akbar, AN., Girdlestone, J., Orteu, CH., Borthwick, JN., Scheel-Toellner, D., Buckley, CD., & Salmon, M. manuscript submitted for publication, see ref 10), can maintain previously activated, apoptosis-prone T cells in a primed but resting state (11,12). In the presence of appropriate co-stimulatory signals these cells can be re-activated without dying and may therefore represent the population destined to become memory cells at the end of a normal immunological response (12). Thus, under normal conditions, fibroblast-mediated T cell survival leads to the generation of memory cells. In contrast, in abnormal conditions, overexpression of the fibroblast factor may contribute to the persistence of a T cell infiltrate by preventing cell death (13). Both T cell memory and chronic T cell mediated inflammation may therefore be generated as a consequence of different degrees of IFN-β-mediated T cell survival. Since a number of chronic cutaneous diseases, in particular atopic eczema, are characterised by the persistence of a T cell infiltrate, the second aim of this study, was to compare the features of a resolving response with those of chronic atopic eczema which, by definition, "fails to resolve".

1.1 T CELL MEDIATED CUTANEOUS INFLAMMATION

1.1.1 DELAYED-TYPE HYPERSENSITIVITY

A number of studies of cutaneous T cell mediated inflammation have focused on the mechanisms underlying delayed type hypersensitivity (DTH) responses induced by the intradermal injection of antigens, such as inactivated pathogens or their purified protein derivatives (e.g. tuberculin) (14,15). The first descriptions of DTH responses produced under controlled conditions were those of Jenner in 1798, Koch in 1890, and Mantoux in 1910 (for historical monograph see references 15,16). The addition of adjuvants and development of the purified protein derivative (PPD) of tuberculin allowed the Mantoux test, as it became known, to be used not only diagnostically, in tuberculosis and as a test of cellular immune function, but also in research, since it is a reproducible response with well defined clinical characteristics.

1.1.1.1 Clinical features

In previously sensitised humans, the reaction may start within a few hours of injection as a white or rose coloured infiltration. Its intensity increases over the first 24 hours to reach a peak at 48-72 hours (sometimes as late as 96 hours) and usually subsides by days 7-10. At the height of the reaction, an area of erythema, induration, sometimes with associated petechial haemorrhage, and more rarely with vesiculation or central necrosis, is produced. The response is generally considered as positive if 4-9mm, and strongly positive if 10mm or more in diameter at its peak (15,17). In animals, the Mantoux reaction (MR) reaches its height earlier, at 24 hours, remains fairly marked up to 48-72 hours and persists up to at least 96 hours (15).

1.1.1.2 Immunohistology

Both *in vitro* and *in vivo* studies have investigated the kinetics and

immunohistologic features of infiltrating cells in PPD induced DTH responses. They suggest that cellular migration is biphasic, comprising an initial non-specific infiltration, which also occurs in non-sensitised subjects, and a second specific peak (15,18,19). At very early time points (6-12 hours) the majority of infiltrating cells are neutrophils (19). 12 hours after challenge, T cells and dendritic cells (DC) begin to appear around dermal blood vessels (20). In some studies, the number of epidermal Langerhans cells (LC) is also increased at this time point (20,21). Maximal numbers of infiltrating activated macrophages are present at 24 hours (20), but by 48 hours, a majority of the infiltrating cells are T cells (19,20,22). CD4+ cells exceed CD8+ cells at all time points (20,23). In contrast, few B cells have been documented in the lesions (19,20,23). Although a majority of cells are concentrated perivascularly, there is also a more diffuse infiltrate of T cells and macrophages, interspersed between collagen bundles in the upper dermis, and at later time points periadnexal cuffing (around hair follicles, sweat and sebaceous glands) is also visible (19,20,23–25). The intensity of the interstitial infiltration increases with time (up to 96 hours), but appears to show great inter-subject variability, irrespective of the clinical response (23). Epidermal changes also occur, with epidermal thickening (acanthosis) developing over the course of the MR and damaged basal KC appearing as early as 7 hours (24). By 24 and up to 96 hours, both CD4 (20) and CD8 (19,23) cells are seen infiltrating the epidermis in the vicinity of these degenerating KC.

In summary, an early infiltration of neutrophils, is followed by macrophages, dendritic cells and predominantly CD4+ T cells in MR. At later time points (studied up to 96 hours), the majority of infiltrating cells are T cells which accumulate perivascularly, and more variably in the epidermis and the interstitium. The histologic features of the reaction are thus fairly well established for the first 96 hours, and its generation is likely to involve co-operation between the different types of infiltrating cells.

1.1.2 PRIMARY AND SECONDARY CUTANEOUS IMMUNE RESPONSES

Although LC are widely regarded as the antigen presenting cells (APC) of the skin, it is not known whether they are able to take up and process antigen which has been injected intradermally. Their contribution to the induction of a primary DTH response therefore remains unclear. Another population of cutaneous DC has been described in the dermis, in both normal and diseased skin (20,26). It is not known whether these cells represent a population en-route to or from the epidermis, or a different population altogether. However, they have a distinctive phenotype (26,27) and are extremely efficient antigen presenting cells (27). Thus in primary (and secondary) responses induced by direct intradermal injection of antigen, it is likely that dermal DC (DDC) play a major role in processing and transporting antigen to lymph nodes. Thereafter, primary antigen presentation by DC in loco-regional nodes results in the activation of antigen responsive naïve T cells, which are thereby induced to proliferate and undergo transition from the naïve (CD45RA+) to memory/effector (CD45RO+) phenotype (28,29).

The response to recall antigen is thought to be mediated by CD45RO+ T cells which show a much greater proliferation in this context, than the CD45RA+ subset (2,30,31). In the very early phase of the response, however, neutrophils and then monocytes/macrophages are the predominant infiltrating cells (19,20,32). This early infiltration is likely to be directed by the production of both non-specific and specific chemotactic and pro-inflammatory factors, such as histamine, prostaglandins and chemokines. Both RANTES (regulated on activation normal T cell expressed and secreted) and MIF (migration inhibitory factors) are present in DTH responses (33,34), and a number of studies have suggested that the early production of KC derived cytokines such as IL-1 and IL-8 which are chemotactic for neutrophils, lymphocytes and, to a lesser degree, monocytes, is important in generating the response (21,35-38).

Once in the skin, activated macrophages may themselves release cytokines which amplify the response, not only by recruiting T cells but also by up- or down-regulating the production of cytokines by T cells (such as IFN-γ, IL-2 and IL-12), endothelial cells (EC) and keratinocytes (KC) (39-42). Indeed, the crucial role of macrophages in determining the cytokine microenvironment in which cells are activated is supported by the inability to mount DTH responses if macrophage function is defective (43).

Another major determinant of whether CD45RO+ cells re-encounter cutaneous antigen, and initiate a secondary response, is clearly their ability to recirculate via the skin.

1.1.3 INDUCTION

The induction phase of a DTH response to recall antigen requires that T cells are recruited to the site of antigenic stimulation and then proliferate and differentiate into a population of effector cells within the specialised microenvironment of the skin. Targeted recruitment of T cells is mediated by a homing process which involves active lymphocyte-endothelial cell (EC) recognition mechanisms (44).

1.1.3.1 Migratory advantage of CD45RO+ cells

The transition from naïve (CD45RA+) to memory/effector (CD45RO+) cell appears to be crucial in determining a T cells homing potential. CD45RO+ T cells predominate in a variety of chronic inflammatory conditions and may, per se, have an enhanced ability to bind to and migrate across vascular endothelium at sites of inflammation (45-48). In Mantoux reactions (MR), *in vivo* studies have shown that CD4+CD45RO+ CD29+ (β1 integrin) T cells migrate preferentially into the lesions (49,50). The degree of differentiation of CD45RO+ cells is also important, since highly differentiated CD45RO+

CD45RB ^{lo} cells (51), which express high levels of β1 (CD29, VLA-4) and β2 (CD11/CD18, LFA-1) integrins (30,50), migrate better across cultured EC than less differentiated CD45RA+ or CD45RO+RB^{hi} cells (52,53).

1.1.3.2 CLA / E-selectin interactions in cutaneous homing

Another receptor, the cutaneous lymphocyte-associated antigen (CLA) (54-56), is also upregulated on T cells during the CD45RA+ to CD45RO+ transition in skin-associated peripheral lymph nodes (29,50) and is present on up to 70% of the predominantly CD45RO+ T cells at sites of cutaneous inflammation (46,47,50). CLA is the major ligand for, and binds specifically to, a vascular adhesion molecule, E-selectin (55,57) which is upregulated, in part by TNF-α and IL-1β, at sites of cutaneous inflammation (58,59). Accordingly, studies employing suction blisters produced over DTH lesions have shown that CLA+ CD45RO+CD4+ cells migrate preferentially into the lesions (49,50). Furthermore, plasma levels of soluble E-selectin correlate with disease activity in atopic eczema (60), and T cells mediating cutaneous diseases express high levels of CLA. Thus, disease-related, antigen-specific T cell clones, from a patient with atopic eczema sensitive to the house dust mite antigen der.p1, express very high levels of CLA and exhibit specific E-selectin binding (61). In addition, T cell stimulation with bacterial superantigens (both staphylococcal and streptococcal exotoxins) thought to be involved in the pathogenesis of cutaneous diseases such as atopic eczema and guttate psoriasis (62) produces an IL-12 dependant significant increase in the numbers of CLA+ T cell blasts (63).

1.1.3.3 Molecular interactions in extravasation

The specificity of the homing process is determined by multistep sequential engagement of adhesion and signalling receptors on lymphocytes and vascular endothelial cells (reviewed in 44,64,65). Recruitment involves four separate steps: 1) transient and reversible primary adhesion, 2) rapid lymphocyte “activation”, 3) stable but potentially reversible “activation”

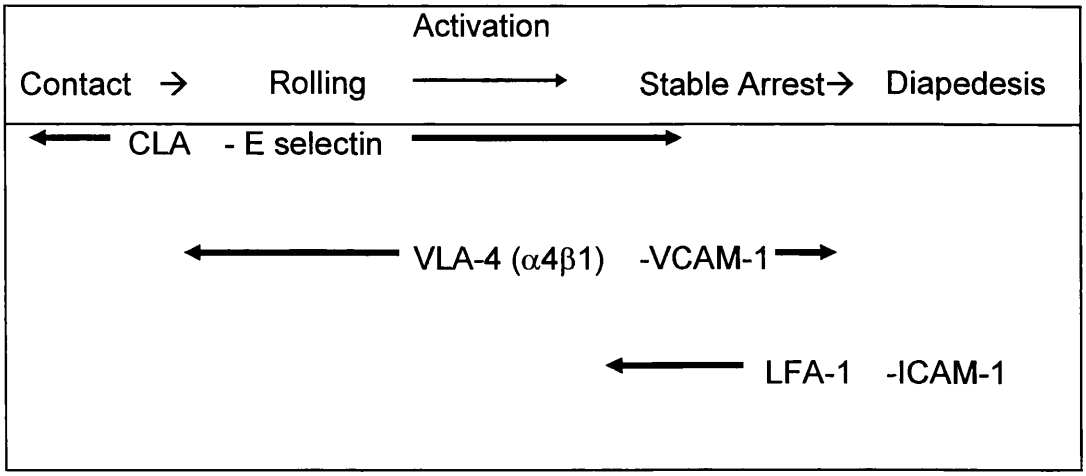
dependent "arrest" and 4) diapedesis (44,66).

The first step is mediated by selectins such as CLA and E-selectin, and appears to be the most important in determining which tissue (e.g. lung vs. skin) lymphocytes home to (67). In step 2, rolling cells are "activated" by chemokines, leading to alterations in integrin function (68). Activated integrins then bind to their EC counter-receptors and mediate step 3 (67,69). The reversibility of each of these steps is crucial, such that in the absence of appropriate signalling for the subsequent step, cells can be released. If on the other hand, appropriate signals are present, stable arrest is followed by extravasation. The lymphocyte homing receptors and EC ligands involved in T cell extravasation in the skin are summarised in Table 1.1.

1.1.3.4 Differential recirculation of naïve and memory cells

The process of extravasation is therefore, a critical regulatory point, and is responsible, at least in part, for the different recirculation pathways exhibited by naïve and memory/effector cells. Thus, under normal conditions, naïve T cells recirculate predominantly through secondary lymphoid tissues (e.g. lymph nodes, tonsil and spleen) where they are exposed to antigen collected from epithelial surfaces, somatic tissues and blood within the context of the local microenvironment (30,70). This allows antigen-induced activation and differentiation, as well as removal of auto-reactive cells to occur. In contrast, memory and effector cells, which also traffic through secondary lymphoid tissues, are in addition able to access and recirculate through extralymphoid effector sites such as inflamed skin and joints (30,70-72). This allows them to return to sites of original antigenic stimulation where they are best adapted to function.

Table 1.1 LYMPHOCYTE-ENDOTHELIAL CELL INTERACTIONS INVOLVED IN MEMORY T CELL EXTRAVASATION IN THE SKIN[1]



[1] CLA cutaneous lymphocyte-associated antigen; VCAM vascular cell adhesion molecule; VLA very late antigen; LFA lymphocyte function associated antigen

1.1.3.5 Antigen specificity

Although it is now known that skin homing CD4+ CD45RO+ CLA+ T cells accumulate in PPD induced DTH responses (46,47,50), it is not clear what proportion of antigen specific cells are recruited. Studies employing limited dilution analysis (LDA) have suggested that they account for 1% or less of infiltrating T cells in DTH responses (73,74). This would imply that most of the CD45RO+, HLA-DR+ T cells are not antigen specific, but rather reflect preferential homing of activated bystander cells to sites of inflammation. However, a recent observation in mice suggests that although antigen specificity does not influence migration into inflamed tissue, only antigen specific cells are retained (75). Thus, although non-specific activated cells may traffic through the lesions and serve to amplify the response, antigen-specific cells may be the predominant effector cells. This suggestion is further supported by a more recent study employing tetramers of MHC class I molecules containing viral peptides, in LCMV (lymphocytic choriomeningitis virus) infected mice (76). This showed that 50-70% of the expanded CD8+ T cell population detected at the peak of the responses to primary and secondary infection were LCMV-specific, and that previously employed LDA methodology underestimates the frequency of antigen specific T cells by 20-100 fold (76). The issue of antigen specificity in DTH responses has not yet been resolved, however, with the development of multivalent MHC class II peptide complexes (77,78) it may soon be possible to determine the proportions of antigen-specific T cells infiltrating the skin more accurately.

1.1.3.6 Cytokine dependent induction of proliferation

Once recruited antigen-specific T cells need to be induced to proliferate and differentiate into effector cells, in order for the efficient removal of antigen to occur. After antigenic activation, cytokines are required to drive cells into cycle. The cytokine that is best characterised for progression of activated T cells into cycle is IL-2 (79). More recently, it has been shown that IL-15 (80-83) and IL-7 (84) can also trigger proliferation of activated T cells, and that in

mice, IL-7 is required for normal clonal expansion of mature peripheral T cells both to antigen-specific and -independent stimuli (85,86). Both IL-2 and IL-15 exert their proliferative effects via the β chain of the IL-2 receptor complex, whereas IL-7 induced proliferation is mediated via the IL-7 receptor (IL-7R) α chain (83,85). These cytokines are produced by different cell types. IL-7 is stromal cell-derived and in the skin it is expressed by epidermal keratinocytes (87). IL-15 is predominantly synthesised in macrophages (81,83) but has also been detected in dendritic cells, including LC (88,89), and epidermal KC (89). In contrast, T cells are the major source of IL-2 (79). The co-ordinated expression of cytokines by these different cell types may therefore be involved in the induction of T cell proliferation in DTH responses.

In addition, cytokines such as type I interferons (90) and combinations of IL-2 +TNF-α +IL-6 (91) have been shown both *in vitro* and *in vivo* to cause bystander (non antigen-specific) T cell proliferation, activation and differentiation into effector cells. It is also possible therefore, that the DTH response is amplified as a result of cytokines produced by specific T cells and macrophages causing activation, proliferation and cytokine production within bystander resting cutaneous CD45RO+T cell populations. Although previous studies have shown that a majority of cells accumulating in DTH lesions have undergone recent proliferation (74), there is little information regarding whether cells proliferate in these lesions *in situ*.

1.2 THE ROLE OF APOPTOSIS IN IMMUNE RESOLUTION

Once the antigenic stimulus has been cleared, resolution may occur either as a result of T cell efflux from the lesions, or as a result of *in situ* cell death. Although the clinical response usually resolves within 7-10 days, the time-course of histologic resolution in MR is largely unknown. Whereas some studies have suggested that the overall number of infiltrating cells begins to fall by 96 hours (20,22), others have noted a progressive increase in perivascular cell numbers and density up to this time (23). Few studies have followed the response beyond 96 hours. Those that have suggest that after 14 days, collections of epithelioid cells, giant cells and lymphocytes, resembling tuberculoid granulomata, are present in the lowermost part of the dermis (14). The processes by which activated T cells are removed and inflammation is resolved have not been fully characterised. Although a proportion of T cells may emigrate from the lesions at the end of a DTH response (74), it is likely, that the control of cell numbers predominantly reflects a balance between cell proliferation and cell death.

It is now recognised that cell numbers can be controlled by the process of cell suicide or apoptosis (3-6). This type of cell death occurs following re-ligation of the T cell receptor of activated, cycling T cells through a secondary interaction of CD95 (Fas/APO-1) with its ligand (activation induced cell death) (7,8), or via TNF-α /TNFR1 (type 1 tumour necrosis factor receptor) interactions (92,93). Apoptosis may also be induced by the withdrawal of cytokines which signal via the common γ chain of the IL-2R, including IL-2, IL-15,and IL-7 (4,9). This is of particular relevance, since these cytokines are also involved in induction. Thus, both induction and resolution may be driven by different waves of production of the same group of cytokines.

1.2.1 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF APOPTOSIS

The term apoptosis is derived from classical Greek and means "falling away". Cells dying by apoptosis have characteristic morphological and biochemical features which are highly conserved throughout ontogeny. The morphological features include plasma membrane blebbing, volume contraction, nuclear and cytoplasmic condensation, and fragmentation of chromosomal DNA into 180 base-pair nucleosomal units as a result of endonuclease activation (94-97). Biochemical features may be subdivided into mitchondrial manifestations, namely dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$) and oxidation of the inner membrane; and "post-mitochondrial" changes which include exposure of phosphatidylserine residues on the plasma membrane, vacuolisation of the cytoplasm with extrusion/oxidation of reduced glutathione, increase in cytosolic Ca2+, and nuclear DNA fragmentation (reviewed in 98,99).

The process of apoptosis can be subdivided into three functionally distinct phases: initiation, effector and degradation. The first phase is dependant on the nature of the initial apoptotic stimulus (e.g. cytokine deprivation, CD95/CD95-ligand interactions, TNF/TNF-R interactions, sub-necrotic damage by toxins or irradiation) . The effector and degradation phases are common to all apoptotic processes, although only the degradation phase is beyond regulation.

The most important determinant of the effector phase is a disruption of mitochondrial membrane function which occurs as a result of the dissipation of the mitochondrial $\Delta\Psi m$, causing opening of pores in the mitochondrial membrane and the release of apoptogenic proteins such as Cytochrome c and apoptosis inducing factor (AIF). This is a common manifestation of the apoptotic process, irrespective of cell type and induction stimulus, and marks

the point of no return at which cells are committed to die. Thus, agents which stabilise the mitochondrial $\Delta\Psi m$ can prevent apoptosis, whereas factors which disrupt it, can induce apoptosis (reviewed in 98-101).

1.2.2 ANTIGEN-DRIVEN CELL DEATH

Removal of cells by apoptosis may therefore occur "passively" as a result of a lack of stimulation by antigen or cytokines, or "actively" via receptor/ligand interactions. This second mechanism operates in situations of antigen excess, and is predominantly mediated by the interactions of CD95 and type 1 tumour necrosis factor-receptor (TNF-R1) with their ligands. In this context, T cell apoptosis may serve to prevent excessive immune stimulation and damage to the host. (reviewed in 7,102,103).

1.2.2.1 CD95

CD95 was initially described by two independent groups in 1989, as a cell surface molecule that could transduce an apoptotic signal in various human cell lines (104,105). It is a 45kDa transmembrane protein belonging to the TNF and nerve growth factor (NGF) receptor superfamily, which are characterised by cysteine-rich extracellular domains (7 and summarised in Table 1.2). It is expressed on multiple cell types including fibroblasts (106), keratinocytes (107), monocytes and mature activated human T and B cells (108,109). CD95 shares part of its cytoplasmic death domain (ie a domain which is necessary and sufficient for transduction of the apoptotic signal) with TNF-R1 (7,110).The apoptotic signal transduced by activation of CD95 first requires the binding of its cytoplasmic death domain, to cytoplasmic death-domain containing proteins such as the Fas-associated death domain protein (FADD), to form a death-inducing signalling complex (DISC) (103,111). FLICE [(FADD)-like interleukin-1β-converting enzyme-like protease], also known as caspase-8, is the first in a cascade of interleukin-1β-converting enzyme (ICE)-like proteases which execute the death signal (112, and Fig 1.1)). The ability of the DISC to recruit and process FLICE to

its active form, determines whether the above cascade is triggered and, by implication, the cells' susceptibility to CD95-mediated death (113). A second, similar pathway also exists, and involves binding of receptor interacting protein (RIP) and RAIDD (RIP-associated Ich-1/CED-3 homologous protein with a death domain), and activation of caspase-2 (103,111).

1.2.2.2 CD95-L

CD95 ligand (CD95-L/Fas-L), a 40 kDa protein which exists in membrane bound and soluble forms, was first identified in 1993, in a T cell hybridoma with strong cytotoxic activity against CD95 expressing cells (114,115). Although initially found on activated T cells (115,116), it is now known to be expressed on a variety of other cell types, including activated macrophages (117), dendritic cells (118), B cells (119), epithelial cells (107,120) and in immune-privileged sites such as the eye and the testis (reviewed in 121). It shares significant homology with the ligands of other members of the TNF-R family (Table 1.2), and like TNF, membrane bound CD95-L (mCD95-L) is cleaved by metalloproteinases at the cell surface to yield the soluble form (sCD95-L) (122). sCD95-L circulates as a homotrimer composed of 27kDa monomers and its functional significance in humans is controversial (111). Although initially thought to be fully functional (123), recent work has suggested that sCD95-L not only induces apoptosis less potently, but can also antagonise the effects of mCD95-L (111,124). It seems that CD95-sCD95-L complexes are rapidly internalised which may not allow enough time for the efficient recruitment and activation of caspase 8 via the DISC. Instead , signalling may occur through a slower, less efficient, and as yet poorly defined Daxx-Jun kinase apoptotic pathway (Fig 1.1), or through the nuclear transcription factor NF-κB, causing inflammation rather than apoptosis (111,124). The regulated expression of soluble and membranous forms of CD95-L may therefore be used by different cell types, at different stages of differentiation to alter their sensitivity to CD95-mediated death.

TABLE 1.2 Members of the TNF receptor superfamily and their ligands

Receptor	Soluble form identified	Ligand	Soluble form identified	Function	inhibitor
CD95/ Fas/ APO-1	yes	CD95-L	trimer	apoptosis, but also proliferation of recently activated T cells (see text)	?sCD95-L FLIP
TNFR1/ p55	yes	TNF-α TNF-β/ LT-a	trimer trimer-no membranous form	apoptosis, monocyte/macrophage &T cell activation (see text) uncertain	sTNFR-1
TNFR2/ p75/ CD120b	yes	TNF-α & TNF-β		unclear may interact with membranous TNF-α→apoptosis. TNF-β/TNFR2 complex is non-signalling	
LT-βR	no	LT-β	no	uncertain. forms trimeric complexes with TNF-β on T cell surface. Activates NFκB, and genes for IL-8 and RANTES. Can also induce apoptosis	
DR4 & TRICK-2	no	TRAIL	trimer	apoptosis (see text)	LIT
CD30	yes	CD30-L	yes	proliferation and apoptosis	
CD40	no	CD40-L	trimer	upregulation of CD95 causing apoptosis, but also NFκB. Promotes B cell differentiation into memory cells	
CD27	yes	CD27-L	no	promotes cytolytic activity in gd T cells & B cell antibody production. Can transduce an apoptotic signal	
LNGFR	yes	NGF	dimer	apoptosis, interacts with TrkA	
CD134/ OX 40	no	CD134L/ OX40L	trimer	T cell activation and survival	
CD137/ 4-1BB/ ILA	yes	CD137L/ 4-1BBL	no	inhibits AICD	
DR3	potential splice variants identified	unknown		analogous to TNFR1 can induce both NFκB and apoptosis	

Receptors are all type I transmembrane proteins with 1-6 extracellular ligand binding cysteine rich motifs. Usually form tri- or multimeric complexes. Ligands are all type II transmembrane proteins except NGF and TNF-β. Most form trimeric structures suggesting clustering of ligands and their receptors is required for signal transduction
NGF: nerve growth factor, LNGFR: low affinity NGF receptor, TrkA: high affinity NGF receptor, TNF tumour necrosis factor, LT: lymphotoxin, TRAIL: TNF-related apoptosis-inducing ligand

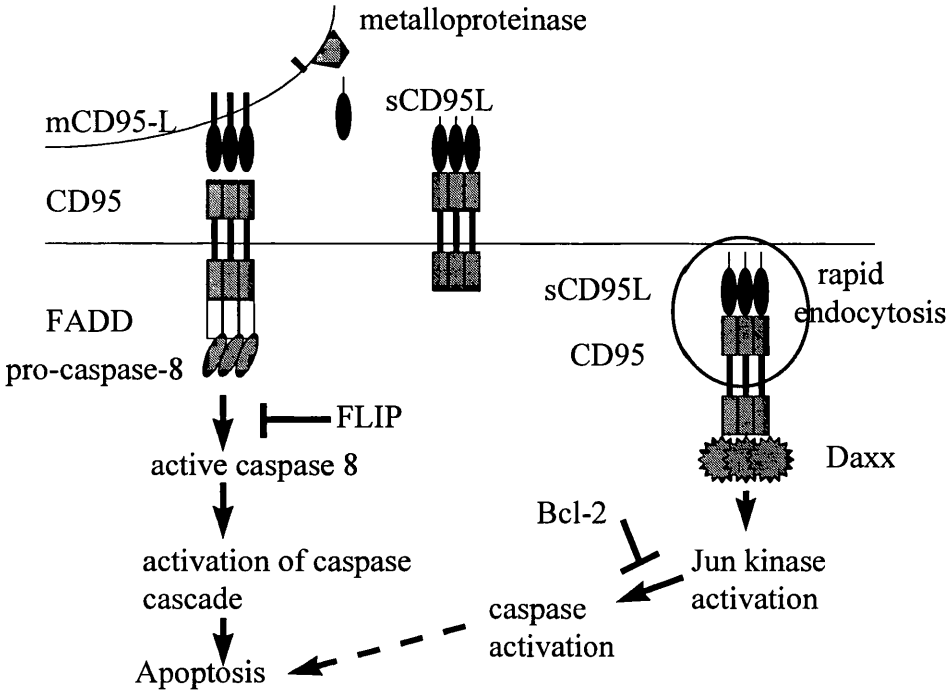


Figure 1.1 Schematic representation of the CD95 death pathway

Following activation of CD95 by membrane bound CD95-L (mCD95-L), the DISC is formed by binding of the cytoplasmic death domain of CD95 to cytoplasmic proteins such as FADD. The DISC can then recruit and activate caspase 8 (FLICE). If the soluble form of CD95-L (sCD95-L) interacts with CD95, the complexes are rapidly internalised and signalling may occur through a slower Daxx-Jun kinase pathway.

1.2.2.3 CD95/ CD95-L interactions

CD95 is highly expressed on resting CD45RO+, but not CD45RA+ T cells, whereas neither subset constitutively express CD95-L (8,108). Following activation, CD95 and CD95-L expression are rapidly induced (8,108,116). Interestingly, however, in the early stages of activation *in vitro*, human T cells are resistant to CD95-mediated, activation induced cell death (AICD), despite high levels of expression of both proteins (113,125 and reviewed in 8). This transient resistance correlates with the expression of FLIP (FLICE inhibitory protein). FLIP binds to the death domain of FADD and interferes with FADD-FLICE interactions, preventing recruitment and activation of FLICE (caspase 8), and thereby inhibiting transduction of the apoptotic signal (125 and Fig 1.1). In contrast, in chronically activated T cells (by day 4 of culture with PHA or anti-CD3 plus IL-2), FLIP expression is reduced and ligation of the CD3-TCR complex of activated cycling T cells leads to AICD (7,8,116). The susceptibility of T cells to AICD may also depend on cell-cycle progression caused by IL-2 or other growth factors and is escalated with increasing quantities of antigen (126,127). Thus, early in an immune response CD95 signalling may facilitate clonal expansion. Later, at the height of the response and during the early part of the resolution phase, cells become sensitive to AICD which contributes to a reduction in cell numbers, and the prevention of excessive immune activation which might damage the host (7,8). T cell CD95-L expression is tightly regulated and requires continued stimulation (8,108,116). As antigenic targets are eliminated at the end of the response, less stimulus for CD95-L expression is present, which leads to a decrease in CD95-mediated death. However, low ambient levels of IL-2R-γ chain cytokines (and therefore Bcl-2 and Bcl-x_L) would ensure that cells continue to be cleared by apoptosis in the late resolution phase (see below).

1.2.2.4 Other functions of the CD95 pathway

In addition to its role in lymphocyte homeostasis, the CD95 pathway has been implicated in mediating cytotoxicity, both in cytotoxic CD8 and in IFN-γ

producing, cytotoxic CD4 cells (114,128-130). Thus, activation by antigen induces CD95-L expression on cytotoxic T cells, which can then kill CD95-expressing target cells. Interestingly, differential susceptibility of CD4 cells to CD95-mediated death has been reported according to their dominant cytokine production profile. Activated IFN-γ producing CD4 cells which express CD95 and its ligand are more sensitive than IL-4 producing cells which predominantly only express CD95 (131). This may be of relevance in conditions such as atopic eczema, where T cells with an IL-4 producing phenotype are thought to predominate.

The importance of CD95 and CD95-L in the maintenance of self-tolerance and regulation of immune responses is further supported by studies in *lpr* and *gld* mice which have spontaneous loss of function mutations in CD95 and CD95-L respectively. These studies suggest that thymic selection operates relatively normally, but that peripheral clonal deletion of auto-reactive cells (peripheral tolerance) and elimination of activated T cells responding to foreign antigen are impaired as a result of defective CD95/CD95-L interactions (reviewed in 7,8,102).

1.2.2.5 CD95 and human disease

In man the CD95 system has been implicated in disease progression in HIV (8,117,132), in evasion of immune surveillance both in immune privileged sites (121,133) and malignancies (134,135), and in auto-immune diseases such Hashimoto's thyroiditis, in which thyrocyte apoptosis is thought to contribute to the eventual development of hypothyroidism (120) (summarised in Table 1.3).

Table 1.3 THE CD95 PATHWAY IN HUMAN DISEASE

	MECHANISM	REFERENCE
Human Immunodeficiency Virus infection	chronically activated CD4 cells more susceptible to AICD	(8)
	AICD mediated via cross-linking of CD4 by gp120/anti-gp120 immune complexes	(8)
	CD95-L expressing HIV infected macrophages kill CD95 expressing cytotoxic T cells (immune evasion)	(117)
Lack of inflammation in immune privileged sites- Immune evasion:		
Anterior chamber of the eye Testicular Sertoli cells	constitutive expression of CD95-L results in death of infiltrating CD95 expressing T cells	(121,133)
Evasion of host tumour-specific immune responses		
Colonic carcinoma cell lines	Tumour cells express CD95-L but are resistant to their own CD95	(134)
Melanoma	CD95, but not CD95-L expression is downregulated early in tumour progression	(136)
	FLIP is expressed in metastatic tumour cells but not normal melanocytes	(125)
Basal cell carcinoma	Tumour cells express CD95-L but not CD95	(137,138)
Chemotherapeutic agents e.g. Doxorubicin, Methotrexate	Up-regulate CD95-L expression in, and cause CD95-mediated death of leukaemia cells	(135)
Tissue destruction in auto-immune disease		
Hashimoto's Thyroiditis	IL-1-β mediated induction of CD95 on thyrocytes, which constitutively express CD95-L results in their apoptotic suicide &/or fratricide	(120)

In the skin the available data suggest that the CD95 system may mediate keratinocyte apoptosis in a variety of conditions, from sunburn to erythema multiforme (107,138,139). It is even possible, since both CD95 and its ligand are constitutively expressed in keratinocytes (KC), that it may play a role in the normal terminal differentiation of KC, which has long been thought to be apoptotic in nature (140). There is very little data, however, investigating the role of CD95/CD95-L expressing infiltrating T cells in inflammatory skin disease. Its exact role in the skin, under normal physiological and disease conditions, is, therefore, far from being fully elucidated.

In summary, several roles for the CD95/CD95-L system have been proposed. T-to-T cell apoptosis (both "suicide" and "fratricide") involved in maintenance of peripheral tolerance and removal of cells both at the height and during early resolution of an immune response; T-to-target apoptosis involved in T cell mediated cytolysis of non-lymphoid cells; and target-to-target apoptosis whereby target cells expressing both CD95 and CD95-L may induce apoptosis in themselves.

1.2.2.6 Other death receptors

Signalling via TNF-R1 (p55), which is expressed on numerous cell types, including monocytes, neutrophils and endothelial cells, has long been known to transmit an apoptotic signal (92,93). However, as with CD95, TNF-R1 signalling can not only promote apoptosis, but also activate other signalling pathways, including NF-κB, and promote survival (103). Its major ligand, TNF-α is synthesised by macrophages and T cells (141), dendritic cells (142), keratinocytes (143) and a wide range of epithelial, glandular and neural tissues. It was the first of the TNF superfamily to be identified, and like CD95-L exists in membrane bound and soluble forms, however, in this instance the soluble form appears to be more potent (144,145). In humans, soluble TNF-α has been shown to cause widespread tissue damage and function as a cachectin in cancer patients (144). It has also been implicated

in the pathophysiology of endotoxic shock and graft versus host disease (GVHD) (144). In the skin, KC constitutively express both TNFR1 and TNF, and stimulation by UV irradiation causes up-regulation of both with resultant apoptosis (146,147).

TRAIL (TNF-related apoptosis-inducing ligand) is another, more recently identified, widely expressed "death factor" to which T cells become sensitive after activation with IL-2 (148,149). Like TNF-α and CD95-L it occurs in membrane bound and soluble forms and binding to its two receptors, DR4 (Death Receptor-4) and TRICK-2 (TNF-related apoptosis-inducing ligand receptor inducer of cell killing-2) can be competitively inhibited by LIT (lymphocyte inhibitor of TRAIL), which lacks a death domain (150-152). Like FLIP, LIT is down-regulated following lymphocyte activation increasing T cell susceptibility to TRAIL-mediated death (152).

Active antigen-driven cell death may thus be induced by the interactions of CD95, TNF-R1 or TRICK-2/DR4 with their ligands. However, surface receptors are not the only means of regulating T cell apoptosis, other intracellular and exogenous factors are also involved.

1.2.3 APOPTOSIS REGULATORY PROTEINS: THE Bcl-2 GENE FAMILY

The Bcl-2 gene was originally identified in 1984, at the chromosomal breakpoint of the t(14;18)-bearing follicular B cell lymphomas. This translocation brings the protein encoding part of the Bcl-2 gene, on chromosome 18, into juxtaposition with the immunoglobulin heavy chain gene locus on chromosome 14. The result was that cells producing immunoglobulin heavy chains also over-expressed a 26 kDa protein, Bcl-2, giving them a survival advantage and oncogenic potential (153). Subsequent studies in transgenic mice over-expressing Bcl-2 revealed that they had prolonged antibody responses and an expanded population of T cells

displaying intermediate levels of TCR. They developed autoimmunity and a variety of malignancies. These findings suggested that Bcl-2 plays an important role in the regulation of both B and T cell survival (5).

Bcl-2 is now known to be part of a large family of apoptosis regulatory proteins which may either antagonise or promote cell death (154-157 and reviewed in 98,158,159).

In man, the best characterised members of this family are Bcl-2, Bcl-x and Bax, and a variety of transgenic studies have investigated their effects on the promotion or inhibition of cell death. Bax, a 21kDa protein was the first with Bcl-2 homology to be isolated and shown to counteract Bcl-2 activity (160). Bcl-x also shares significant homology with Bcl-2, but has two splice variants, a longer variant Bcl-x_L which is a potent inhibitor of cell death, and a shorter Bcl-x_S variant which inhibits the anti-apoptotic effects of both Bcl-2 and Bcl-x_L (161). These proteins differ in their tissue and activation dependent expression patterns, but are all thought to act by stabilising (Bcl-2, Bcl-x_L) or disrupting (Bax, Bcl-x_S) mitochondrial membrane function (98,162,163).

During lymphoid development, downregulation of Bcl-2 is a common feature of lymphoid populations undergoing selection and death, whereas up-regulation of Bcl-2 is part of the positive selection mechanism (164). Bcl-2 deficient mice initially generate lymphocytes, but subsequently develop thymic hypoplasia. They become lymphopenic over time as a result of increased cell death, which is dependent on the presence of Bax (165). In contrast, overexpressed Bcl-2 can prevent cell death induced either by DNA damage, growth factor withdrawal, glucocorticoids, chemotherapeutic agents, Ca2+ mobilising drugs or ceramide (162,166,167) and Bcl-x_L is at least as effective as Bcl-2 at preventing cytokine withdrawal mediated death

(161). Its splice variant Bcl-x-$_S$, and Bax can counter the death repressor activity of Bcl-2. However, neither Bax nor Bcl-x-$_S$ alter viability in themselves, rather they render cells more susceptible to death following an apoptotic signal (160,161).Interestingly, the increased rate of cell death parallels the ratio of Bax to Bcl-2 within cells (160,166).

Accordingly, a number of studies have shown that a dynamic equilibrium exists whereby the ratio of pro- to anti-apoptotic proteins determines whether a cell will respond to an apoptotic signal. Bcl-2 related proteins selectively and competitively dimerize with each other both *in vitro* and *in vivo*, thereby altering their pro-or anti-apoptotic effects (154,156,160,161, and reviewed in 98). Thus, in situations where Bcl-2 expression is low, Bax homodimerizes, favouring apoptosis, whereas in situations of Bcl-2 excess, Bcl-2 competes with Bax to form heterodimers (160,165).This is complicated, however, by the results of studies on gain- and loss-of-function models of Bcl-2 and Bax in transgenic mice which show that each is also capable of regulating cell death independently of the other (165).

In summary, Bcl-2 family proteins appear to exert their effects by altering mitochondrial function, producing either a stabilisation or disruption of the mitochondrial membrane $\Delta\Psi$m. Apoptosis appears to be exquisitely tuned by the balanced expression of these proteins at the cellular level. The biological response can be altered by imbalanced or ectopic expression which modify dimerization, post-translational modifications of the proteins, or alterations in their subcellular localisation (98,168). In physiological terms, one of the crucial features of cells over-expressing Bcl-2 and Bcl-x-$_L$ is their resistance to a number of apoptotic stimuli (3,6,9). Similarly, recently activated T cells express high levels of Bcl-2, and are relatively resistant to apoptosis (167,169). However, such cells need to be cleared at the end of the immune response, suggesting that mechanisms exist which can alter their balanced expression of Bcl-2 and related proteins and consequently render them more

susceptible to apoptosis at that time.

1.2.4 THE ROLE OF IL-2R γ-CHAIN SIGNALLING CYTOKINES

In *in vitro* studies using IL-2 dependant activated T cell lines, apoptotic cells are observed as early as 8 hours after withdrawal of IL-2. By 48 hours, the majority (80-95%) of the cultured cells are apoptotic (9,170). Whereas >95% of such cells co-express Bcl-2 and Bax prior to IL-2 withdrawal, the increased proportion of apoptotic cells observed at 48 hours correlates with a marked reduction in T cell Bcl-2 and Bcl-x-$_L$ expression, whilst Bax levels remain unchanged (9,166,171). Whereas withdrawal of IL-2 induces T cell apoptosis, subsequent re-addition of IL-2 to these IL-2-deprived T cells not only induces proliferation, but independently up-regulates Bcl-2 and Bcl-x-$_L$ relative to Bax and rescues them from apoptosis (9,167,171-173). Thus, cell death can be prevented by cytokines which induce genes such as Bcl-2 and Bcl-x-$_L$ which retard apoptosis, relative to those such as Bax and Bcl-x-$_S$ which promote it (6,9,170,171,174). Other IL-2Rγ chain signalling cytokines such as IL-4, IL-7 and IL-15, as well as IL-10, which signals via a different receptor complex, have subsequently been found to rescue IL-2 deprived T cell lines from apoptosis in a similar manner (9,175). However, a large panel of other cytokines, including IL-1, IL-6, IL-8, TNF-α, TGF-β, IFNγ, and chemokines such as MIP-1, MCP-1 and RANTES do not (9,171).

1.2.5 T CELL DIFFERENTIATION AND SUSCEPTIBILITY TO APOPTOSIS

The degree of T cell differentiation also appears to alter apoptotic potential. Following *in vitro* stimulation with PHA or anti-CD3, naïve T cells move progressively from a CD45RA+RB^{hi}RO- phenotype, through a CD45RA-RB^{hi}RO+ to a CD45RA-RB^{lo}RO+ phenotype which parallels successive cycles of division and reflects increasing differentiation (51). This progressive differentiation of CD45RA+ to CD45RO+RB^{lo} T cells is associated with a

reduction in Bcl-2 and an increase in CD95 (Fas/APO-1) expression (51). Such cells predominate in the *in vivo* activated T cell populations of patients with Human Immunodeficiency Virus (HIV) and acute EBV and Varicella-Zoster Virus (VZV) infections, and after 48 hours of culture, a majority of these T cells exhibit characteristic apoptotic features (169,172,173). Interestingly, it is the CD45RO+ population, and not the CD45RA+ population which is apoptotic (169,172). The apoptosis-prone T cell phenotype thus arises from progressive differentiation (9,51,169,172,174), presumably to allow efficient removal after antigenic clearance. This is pertinent since T cells which preferentially enter sites of cutaneous inflammation also belong to the CD45RO+ subset (176).

Thus, apoptotic potential may be determined both by the degree of T cell differentiation and the cytokine milieu in inflammatory lesions. In particular, the presence or absence of IL-2Rγ chain signalling cytokines may contribute not only to T cell infiltration and proliferation, but also to the resolution of inflammation by promoting cell death.

1.3 FIBROBLASTS, T CELL MEMORY AND CHRONIC INFLAMMATION

The above data all suggest that apoptosis is the mechanism whereby the extent and resolution of T cell mediated immune responses are controlled. However, if clonal deletion rather than "down-sizing" were to occur at the end of such a response, we would have no immunological memory, since all the antigen-reactive cells would be eliminated. A mechanism must therefore exist whereby some of the antigen-specific cells are not only rescued from immediate death, but also maintained in a state in which they will survive and be able to respond to recall antigen in the future. The first clues as to how this might occur came from the observation that co-culturing *in vivo* activated, apoptosis-prone T cells with fibroblast monolayers could rescue them from apoptotic death (11). Under these conditions the T cells survive in a primed but resting (non-cycling) state. They do not proliferate and their Bcl-2 levels are low (12,169). However, they maintain high levels of Bcl-x_L expression (12). If removed from co-culture a majority of these cells die by apoptosis unless they are given IL-2 or re-activated by anti-CD3 antibody in the presence of CD28 co-stimulation (12,13). Both these stimuli result in proliferation and upregulation of Bcl-2 and Bcl-x_L. A fibroblast factor has been identified which is responsible for promoting survival in this population of T cells which would otherwise die by apoptosis (11-13) and recent data from our laboratory suggest that this fibroblast derived survival factor is Interferon-β (10 submitted). Thus two groups of anti-apoptotic cytokines have now been identified. Firstly those of the IL-2R γ chain family and IL-10 which induce the proliferation of activated T cells (9,169), and secondly type I interferons (α and β) which enable retention of the cells in a quiescent state (12,13 and 10 submitted). This second mechanism for rescue from apoptosis also maintains T cells in a state in which they may subsequently be reactivated without dying and could therefore be a mechanism whereby memory T cells are retained at the end of an immune response (12).

Furthermore, an excess of Interferon-β (IFN-β) production might provide a microenvironment in which T cells fail to die appropriately, therefore promoting chronicity of inflammation. The degree of expression of cytokines which prevent apoptosis may therefore be pivotal in determining the difference between the generation of immune memory and inflammation.

In vivo evidence to support this model comes from patients with rheumatoid arthritis, whose inflamed joints contain large numbers of almost exclusively CD45RO+ RB^{lo} CD4 cells (49,177). It is thought that these are preferentially recruited into the joint, by virtue of their phenotypic characteristics (45,49,52), in a non-specific manner, as a result of successive systemic infections (178). This subset of cells should be highly sensitive to apoptosis due to their high CD95 and low Bcl-2 expression(169), however, despite the presence of numerous apoptotic neutrophils, there is no evidence of T cell apoptosis within rheumatoid joints (13). As in the *in vitro* experiments (described above) the cells have low levels of Bcl-2 but maintain Bcl-x_L expression and if they are removed from the environment of the joint and cultured *in vitro* (alone) they die rapidly (13).

The relevance of increased fibroblast numbers and proliferation in RA joints was confirmed by comparing these results to those obtained in T cells from the joints of patients with acute uric acid-induced crystal arthritis (gout) in which negligible increase in fibroblast numbers or proliferation occurs. These T cells express low levels of both Bcl-2 and Bcl-x_L and exhibit high levels of apoptosis *in vivo* (13).

Collectively, these observations suggest that fibroblasts may have an important physiological role in the maintenance of T cell memory, but that if excessive fibroblast numbers are present, they may contribute, via their synthesis and release of IFN-β, to chronicity of inflammation. The

accumulation of T cells in rheumatoid joints may therefore not only be due to increased recruitment and/or proliferation in situ, but also to a failure of cell death occurring as a result of excess production of β interferon by fibroblasts. The relevance of this model to other chronic inflammatory conditions remains to be evaluated.

1.4 CHRONIC ATOPIC ECZEMA-AN EXAMPLE OF PERSISTENT CUTANEOUS INFLAMMATION

One inflammatory condition, in which the persistence of an infiltrate composed of CD4+CD45RO+ cells also occurs, is atopic eczema (AE) (176). Although it is rarely fatal, this disease has a considerable impact on quality of life (179) and accounts for significant morbidity and cost, both in terms of health service expenditure and days off school or work (180). Its incidence has risen steadily in the UK over the last 30 years (181), and it currently affects 8-14% of children under 12, and 0.2% of adults over 40 (182-185).

The possibility that a dysregulation of normal T cell apoptosis, as a result of the cytokine microenvironment generated by infiltrating T cells, macrophages and/or fibroblasts, may contribute to the pathogenesis and chronicity of AE has not been studied, but is clearly of relevance to the development of successful therapies.

Atopy is defined as a genetic predisposition to develop allergic diseases such as AE, asthma, allergic rhinitis and conjunctivitis, associated with raised serum IgE levels and exaggerated production of IgE antibodies to a wide range of common environmental allergens (186-189). Eczema is derived from the Greek term "eczeo", which means "a boiling over". This is an apt description for a chronic, relapsing and remitting disease, characterised by dry skin, intense pruritus (itching), and an erythematous, papulo-vesicular eruption, which may progress to become scaly or lichenified (190). Although patterns vary, in adults lesions are most commonly found in the limb flexures, and not infrequently on the face.

The cause of AE is likely to be multifactorial, with genetic, environmental and immunological components.

1.4.1 GENETIC, ENVIRONMENTAL AND TRIGGERING FACTORS

A genetic basis for this condition is supported by the fact that 50-70% of affected children (and a majority of adults) have a positive family history of atopy; by twin studies showing concordance rates of 77% and 15%, in mono- and dizygotic twins respectively; and by a number of family studies suggesting an autosomal dominant pattern of inheritance and linkage to chromosome 11q13 (184,185 and reviewed in 191). The genetics of AE have been investigated using IgE hyper-responsiveness as a marker, and several candidate genes have been identified. These include the gene encoding FcεRI-β, the β subunit of the high affinity IgE receptor on 11q13; a gene involved in the control of total, but not specific, serum IgE situated near the IL-4 locus on chromosome 5q31; and a gene in the TCR-α region on chromosome 14, found to modify specific IgE responses (reviewed in 191).

AE therefore appears to results from multifactorial inheritance, but the expression of disease is due to interactions between genetic and environmental factors. Indeed, high birth order and gestational age, prolonged breast feeding, reduced incidence of childhood infections, early exposure to animals and lower social class have variably been reported to increase the risk of developing AE (185,192,193).

A number of triggers have been identified in patients susceptible to developing AE. These include contact with irritants (such as wool and soaps), stress, infection, certain foods and climatic change (reviewed in 194).

1.4.1.1 Pathogenicity of *Staphylococcus aureus*

Several lines of evidence suggest a pathogenic role for *Staphylococcus aureus*, known to colonise both normal and lesional skin in over 90% of patients (195,196). Initial clues came from the clinical observation that eczema severity could be reduced by treatment with anti-staphylococcal

antibiotics, and from studies revealing the synergistic effects of combination therapy with topical corticosteroids and antibiotics (197). Subsequently, specific IgE both to staphylococcal antigens and staphylococcal exotoxins was detected in patients serum (198,199), suggesting that both type of antigen may have the capacity to bind to IgE receptor bearing LC in the skin and generate specific T cell responses (see below). A number of recent studies have focused on the role of staphylococcal superantigens which can simultaneously activate large numbers of different T cell clones by binding to MHC class II of antigen presenting cells (APC) outside the peptide binding groove and directly cross-linking it to the Vβ chain of the TCR. One such toxin, enterotoxin B can induce eczema when applied to intact skin *in vivo* (200) and IL-4, IL-5 and IgE secretion from PBMCs *in vitro* (201). Furthermore, *in vitro* studies have shown that KC, known to express MHC class II in AE, can activate T cells in the presence of superantigens, and that stimulation of PBMCs with both staphylococcal and streptococcal superantigens results in the expansion of a population of CLA+, skin homing T cells (62,63). These findings all suggest that continued exposure to staphylococcal antigens and superantigens on the skin has a role in generating or perpetuating T cell mediated inflammation in patients with AE.

1.4.1.2 Pathogenicity of aeroallergens

A major role for aeroallergens has also been suggested. In this respect, the importance of two species of house dust mite (HDM), *Dermatophagoides pteronyssimus* and *D. farinae*, and their allergens Der *p* 1 and Der *f* 1 is well documented (reviewed in 202), and has led to the suggestion that IgE mediated allergic contact sensitivity to HDM and other airborne allergens plays an important part in the pathogenesis of AE.

1.4.2 ATOPIC ECZEMA: A FORM OF DELAYED TYPE HYPERSENSITIVITY

Evidence to support the concept that AE represents an IgE-mediated DTH

response comes from a variety of sources.

Histologically, acute AE is characterised by an infiltrate of activated CD4+ CD45RO+ T cells, macrophages and CD1+ dermal and epidermal DC, with variable numbers of basophils and eosinophils. The epidermis is acanthotic (thickened), with marked intercellular oedema (spongiosis), sometimes resulting in vesicle formation (176,203-206). These histological features are all akin to those of DTH responses induced experimentally (19,20,23). Furthermore, delayed-type (positive after 24-48 hours) eczematous skin reactions can be induced in sensitised AE patients (with high serum allergen-specific IgE levels) by the epicutaneous application of aeroallergens such as Der *p* or grass pollen (patch tests) (207,208).

Both IgE and its high (FcεRI) and low (FcεRII, CD23) affinity receptors are thought to play a pivotal role in the pathophysiology of AE. High levels of specific IgE to aeroallergens, as well as staphylococcal antigens (see above) have been detected in the serum of affected patients (198,199,209). FcεRI is now known to be expressed not only on mast cells and basophils, but also on professional APCs such as monocytes, LC and dermal and peripheral blood DCs, and FcεRII has been identified on LC as well as macrophages and lymphocytes (187). Their expression is upregulated on epidermal and dermal DC in aeroallergen patch tests and in lesional AE (210,211). In addition, FcεRI+ DC which have been shown to bind HDM allergen are required to induce an allergen-specific T cell response *in vitro* and to provoke eczema after cutaneous aeroallergen application *in vivo* (210,212-214). Furthermore, effective therapy for AE has been shown to downregulate IL-4-induced FcεRII expression on peripheral blood mononuclear cells (215) and FcεRI expression on cutaneous DCs (216,217).

On APCs, allergen bound to IgE-IgE receptor complexes is internalised,

processed and presented efficiently to T cells in context of MHC class II, resulting in increased T cell recruitment and activation (210). When these T cells encounter the same MHC-allergen complex on B cells, upregulation of IL-4 and CD40-L expression provide the necessary stimuli for B cell IgE production (209,218) thereby increasing the potential for allergen mediated T cell recruitment by DCs in the skin.

These data suggest that aeroallergens, and staphylococcal antigens and superantigens behaving as "allergens", may penetrate the skin in AE, and induce allergen-specific T cell (DTH-type) responses by binding to IgE/IgE receptor bearing epidermal and dermal DC. This concept is further supported by the fact that, in sensitive patients, CLA+ aeroallergen-specific T cell clones can be extracted from AE and patch test lesions (61,208,219,220).

1.4.3 CYTOKINE MICROENVIRONMENTS IN AEROALLERGEN PATCH TESTS AND LESIONAL ATOPIC ECZEMA

In view of these findings, aeroallergen patch test studies have been used as an approach for studying the cytokine microenvironment involved in the initiation phase of AE. Both immunohistochemical studies and studies employing *in situ* hybridisation techniques to measure lesional cytokine mRNA have shown that in the initiation phase IL-4 is the predominant cytokine produced, whereas later IFN-γ expression is upregulated (207,221). The importance of IFN-γ in chronicity is supported by studies in chronic lesional AE, where 85% of samples were found to contain increased IFN-γ mRNA, whereas increased IL-4 mRNA was identified in only 25%. Furthermore, this excess IFN-γ was down-regulated in patients following successful therapy (222). In contrast, others have found that IL-5 and IL-4, but not IFN-γ mRNA are upregulated in chronic AE lesions (223), and that IFN-γ production is impaired in PBMC of atopic patients (224,225). Furthermore, allergen-specific (HDM and grass pollen) T cell clones extracted from patch tests (208,219,220) and from peripheral blood

(226,227) in AE patients, predominantly produce IL-4 and are capable of enhancing IgE production (reviewed in 228). A majority of these studies employ measurements of cytokine mRNA *in situ* and protein in T cell culture supernatants. Such estimations may not accurately reflect the levels of cytokine protein production *in vivo*, or the production of cytokines by other cell types within the cutaneous microenvironment. Although allergen-specific, predominantly IL-4 secreting cells which promote B cell IgE production, appear to be involved during the induction phase, the predominant cytokines produced in more chronic lesions, remain unclear.

The histology of chronic AE, differs from that in the acute phases of disease. The epidermis becomes more acanthotic and hyperkeratotic (lichenified), the inflammatory infiltrate becomes more patchy, contains greater numbers of mast cells, and the upper dermis becomes fibrosed (189,206,229). This implies that a large number of infiltrating fibroblasts are present. Although fibroblasts have traditionally been considered merely as a source of "scaffolding" on which other cells function and migrate, more recent evidence suggests that they are not only actively involved in recruiting leucocytes into injured tissue (reviewed in 230), but may also promote survival of infiltrating T cells by secreting IFN-β (11-13, and Pilling, D. et al (10) submitted). The possibility that fibroblasts may contribute to chronicity of inflammation in AE has not been studied, and their distribution and cytokine production profiles, clearly merit further investigation.

1.5 CONCLUSIONS

Although a lot of *in vitro* data exists to support the role of apoptosis in immune resolution, there is little *in vivo* data in humans to confirm it. In this study we have used the Mantoux reaction, which is a well recognised, T cell mediated, cutaneous delayed-type hypersensitivity reaction, to study the kinetics of the local response from onset to resolution. The majority of previous studies in humans have not followed the reaction beyond 96 hours and the processes by which activated T cells are removed and inflammation is resolved have not been characterised.

In an effort to understand how the processes responsible for normal resolution may be altered in a situation of chronic inflammation, we have compared the resolving DTH response with chronic lesional AE, which is characterised by the persistence of an infiltrate of activated CD4+ CD45RO+ T cells. Such investigations are of particular importance for understanding persistent inflammatory conditions where disease chronicity may be perpetuated by the inability to terminate the ongoing response.

In addition, since the numbers and relevance of infiltrating fibroblasts have not been established in situations of resolving or persistent cutaneous inflammation, we have investigated both their kinetics of infiltration and proliferation, and levels of lesional IFN-β expression during the DTH response, and compared these with chronic lesional atopic eczema.

1.6 SPECIFIC AIMS OF THIS STUDY

1. To investigate the kinetics and phenotype of infiltrating T cells during induction and resolution of the Mantoux reaction in relation to the cytokine microenvironment.
2. To establish the role of apoptosis in resolution of the Mantoux reaction by investigating the expression of apoptosis regulatory proteins (Bcl-2 and Bax), pro-apoptotic CD95-L and TNF-α, and anti-apoptotic IL-2R-γ chain signalling cytokines and correlate these with T cell numbers and percentages of apoptotic T cells.
3. To investigate whether fibroblasts play a role in the resolution of acute inflammation by investigating fibroblast numbers, proliferation and IFN-β expression in Mantoux reactions
4. To investigate whether a dysregulation of apoptosis occurs in chronic atopic eczema by comparing it with resolving Mantoux reactions using the parameters described in 1) and 2) above.
5. To investigate a possible role for fibroblasts in the maintenance of inflammation by comparing fibroblast numbers, proliferation and IFN-β expression within chronic lesional atopic eczema and resolving Mantoux reactions

Specific aims 1, 2 and 3 are covered in the work presented in Chapter 3, and investigations relating to specific aims 4 and 5 are covered in Chapter 4.

2. MATERIALS AND METHODS

2.1 PATIENTS AND CONTROL SAMPLES

2.1.1 DEMOGRAPHIC DETAILS

Normal skin was obtained from surgical specimens in five subjects: all female, age range 17-39 median 25 years.

Twenty healthy volunteers, previously immunised with BCG (15 male, age range 23-59, median 30 years) were recruited from laboratory and hospital personnel for Mantoux tests. Subjects had no personal or family history of allergic rhinitis (hay fever), asthma or eczema.

Nine patients with atopic eczema were recruited from a specialist eczema clinic: 7 male, age range 21-49, median 28 years. All had long-standing disease (range 11-48 years, median 27 years), involving over 60% of the body surface area. None had been treated with topical steroids, systemic or UVR therapy for at least one month prior to biopsy. Patient details are summarised in Table 2.1

The criteria for the diagnosis of AE were based on those of Hanifin and Rajka (190 and Table 2.2). The diagnosis was made when, in the presence of a rash of typical morphology and distribution, at least two other basic features were present. Severity was assessed using the Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score (231). This score involves the assessment of six signs: erythema, exudation, dryness, cracking and lichenification; at six different sites: arms, hands, legs, feet, head and neck, trunk. Each is scored on a scale of 0 (absent),1 (mild), 2 (moderate), 3 (severe), focusing on the worst affected site within each area. The maximum score is 108. An example of the chart used to record scores is given on p 55. The mean SASSAD severity score for patients in the study was 57.8 +/-7.2.

Table 2.1 ATOPIC ECZEMA SUBJECTS: CLINICAL DETAILS

Subject	Initials	Sex	Age (years)	Age at diagnosis (years)	SASSAD severity score	Other Atopic Disease[2]	Family History of atopy	Biopsy site
1	RG	M	37	3	67	A	No	forearm
2	LT	M	23	12	46	HF	Yes	thigh
3	JS	M	49	<1	66	A, HF	Yes	forearm
4	DC	M	35	<1	57	A, HF	Yes	forearm
5	KK	M	33	<1	60	No	Yes	upper arm
6	CB	M	28	<1	48	No	Yes	thigh
7	CR	F	21	3	56	A	Yes	forearm
8	JM	F	28	<1	58	A, HF	Yes	thigh
9	VM	M	26	13	62	A	Yes	abdomen

[2] A= asthma, HF= hay fever

Table 2.2 DIAGNOSTIC CRITERIA FOR ATOPIC ECZEMA (modified from 190)

Major

- Pruritus
- Flexural involvement and lichenification
- Chronic or relapsing course
- Personal or family history of AE or asthma/hay fever

Minor

- Xerosis (dry skin)
- Early age of onset
- Increased number of skin infections particularly with *Staphylococcus aureus*
- Multiple immediate (type I hypersensitivity) positive skin prick tests
- Elevated serum IgE
- Course influenced by environmental/emotional factors

example of score chart

SIX AREA, SIX SIGN SCORE

Head and neck	
Erythema	
Exudation	
Excoriation	
Dryness	
Cracking	
Lichenification	
Total	

Subject No.

Subject Initials

Visit

Date

Trunk	
Erythema	
Exudation	
Excoriation	
Dryness	
Cracking	
Lichenification	
Total	

Hands	
Erythema	
Exudation	
Excoriation	
Dryness	
Cracking	
Lichenification	
Total	

Score

0 = absent

1 = mild

2 = moderate

3 = severe

Feet	
Erythema	
Exudation	
Excoriation	
Dryness	
Cracking	
Lichenification	
Total	

Arms	
Erythema	
Exudation	
Excoriation	
Dryness	
Cracking	
Lichenification	
Total	

Legs	
Erythema	
Exudation	
Excoriation	
Dryness	
Cracking	
Lichenification	
Total	

Total body score =

2.1.2 MANTOUX REACTIONS

Mantoux tests were performed on the volar surface of the non-dominant forearm. Testing was first with 0.1ml of a 1:10 000 solution of Tuberculin PPD (Evans Medical Ltd, Leatherhead, UK) and then, if negative at 48-72 hours, the test was repeated later with 1:1000 strengths. Subject details and dose of tuberculin PPD received are summarised in Table 2.3.

Erythema and induration were measured at 72 hours and on the day of the biopsy as follows. Erythema was scored using a DermaSpectrometer (Cortex Technology, Hadsund, Denmark), a handheld system designed for measuring the erythema-index (EI) of the skin by measuring light absorption coefficients (232). Erythema indices obtained were grouped and scored as 1 = EI < 5, 2 = EI 5-10, 3 = EI 10-15, 4 = EI >15. Induration was scored as: 1= none detected, 2= just palpable, 3= easily palpable, 4= marked, 5= very marked. The maximum diameter was measured at 72 hours in millimetres and scored as 1= 4-9mm or 2= >10mm. The sum of the erythema, induration and diameter scores was then used to give each subject an overall score, both at the time of biopsy and at 72 hours.

We examined initiation and resolution of the reaction by biopsying early and late time points after PPD injection. The skin was infiltrated with 2% lignocaine and each volunteer had one 4 mm punch biopsy taken from the intradermal injection site. Wounds were closed with one 5.0 ethilon suture (Ethicon ltd, Edinburgh, UK). Biopsies were performed at either 12 hours, 72 hours, 7 or 14 days after PPD injection (5 subjects per time point). Ethics committee approval and subjects' informed consent was obtained.

In addition, a senior staff member (LWP) consented to intradermal injection with 1:10 000 PPD at 6 different sites on the volar aspect of the forearm and

had 4mm punch biopsies performed at 6,12, 24, 72 hours and 7 and 14 days in order to ensure that the kinetics of the reaction could be observed in a single individual.

2.1.3 ATOPIC ECZEMA

Unexcoriated and clinically uninfected lesional AE, present for at least 2 weeks, was biopsied as above. Lesions were biopsied at this time point so that they might theoretically be compared to day 14 (resolution) of the Mantoux reaction. Ethics committee approval and subjects' informed consent was obtained before performing the biopsies.

Table 2.3 CLINICAL DETAILS AND DOSE PPD

INITIALS	SEX[2]	AGE	DOSE INTRADERMAL PPD	CODE
NORMAL SKIN				
LC	F	21		KO27
AK	F	17		KO22
SH	F	25		KO23
AB	F	39		KO26
VM	F	25		KO25
12 HOURS				
LP	M	52	1/10000	KO1
PW	M	52	1/10000	KO16
MT	M	30	1/10000	KO2
TM	M	30	1/10000	KO20
FB	F	30	1/10000	KO19
72 HOURS				
ML	M	23	1/10000	KO5
JF	M	59	1/10000	KO6
SH	F	23	1/10000	KO9
MW	M	31	1/10000	KO8
HB	M	36	1/1000	KO10
7 DAYS				
MG	M	26	1/10000	KO7
SY	M	26	1/10000	KO12
BM	M	37	1/1000	KO13
HS	F	23	1/1000	KO14
BF	F	30	1/1000	KO21
14 DAYS				
PA	M	51	1/10000	KO4
LP	M	52	1/10000	KO3
BB	M	30	1/10000	KO11
JC	M	30	1/10000	KO17
KO	F	32	1/10000	KO18

[2] M= male, F= female

2.2 MOUNTING AND STORAGE

All biopsies were mounted in "Cryo-M-Bed" (Bright's instrument Company Ltd, Huntingdon, Cambs, UK) and snap frozen in isopentane cooled in a bath of liquid nitrogen. Samples were stored in liquid nitrogen until sectioned. 6μm cryostat sections were cut onto poly-L-lysine coated slides to increase section adherence.

Sections were then air-dried for 2 hours and fixed for 10 minutes in chloroform:acetone 1:1. This process of fixation has several functions: preservation of morphology and antigen immunoreactivity, and prevention of diffusion of the antigen during subsequent staining procedures. The chloroform:acetone mixture used has been found in our laboratory to be effective in this regard and not to interfere with subsequent antigen-antibody reactions employed in localising antigens.

Sections were either used immediately or stored wrapped in clingfilm at -20^{0}C prior to immunohistologic staining.

2.3 IMMUNOHISTOLOGY

2.3.1 ANTIBODIES USED IN THE STUDY

The characteristics of the monoclonal antibodies (MoAbs) /polyclonal anti-sera used in this study are documented in Tables 2.4 ,2.5 & 2.6. Antibodies were mouse anti-human monoclonal antibodies unless otherwise stated.

The study employed indirect immunoperoxidase, immunofluorescence, biotin/ streptavidin alkaline phosphatase and TUNEL methods.

2.3.2 INDIRECT IMMUNOPEROXIDASE TECHNIQUE

In this technique, the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A second horseradish-peroxidase-conjugated antibody, raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is applied to the section and allowed to bind to the primary antibody. The complex which forms is then visualised by incubation with an appropriate chromogen/substrate. In this study 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used immediately after preparation in a fume cupboard.

With this method, background staining may be caused by non-immunological binding of specific antibodies by hydrophobic and electrostatic forces to certain sites, in particular connective tissue elements within tissue sections. To minimise such non specific staining, sites which might show a non-specific affinity for immunoglobulin were blocked both by pre-incubation of the sections with normal rabbit serum (NRS) and by the addition of 4% normal human serum (NHS) to the second layer antibody. Such sera provide a source of IgG which will not interfere or react with the specific antibodies used.

Table 2.4 FIRST LAYER ANTIBODIES USED IN THE STUDY[1]

Antibody	Subclass	mW	Code	Source	Specificity	Ref
Tmix = CD2,CD7, CD8,CD4,CD3	IgG1 (CD4,CD8) & IgG2a (CD3, CD2, CD7)		RFTmix	RFH	T cells	(233)
CD45RO	IgG2a	180 kDa	UCHL-1	UCH[2]	Primed T cells	(28)
CD5	IgM	67 kDa	RFT1	RFH	T cells and some B cells	(233)
CD8	IgM	32-34 kDa	RFT8	RFH	Class I MHC restricted T cells	(233)
CD4	IgG1κ	56 kDa	MO716	Dako Ltd, High Wycombe, Bucks, UK	Class II MHC restricted T cells	
CD4	IgG2a	56 kDa	MHCD 0400	Caltag Laboratories, San Francisco, CA	Class II MHC restricted T cells	
CD3	IgG2a	16,20,25-28 kDa	UCHT1	UCH	T cells	(233)
HLA DR	IgG1		HLA DR-2	RFH	specific for DR subregion of MHC class II antigen	(233)
Ki67	IgG1κ	345 & 395 kDa	MO722	Dako Ltd	proliferating cells (outside G_0)	
Ki67	rabbit anti-human polyclonal IgG	345 & 395 kDa	A0047	Dako ltd	proliferating cells (outside G_0)	
Bcl-2	IgG1	24-26 kDa	MO887	Dako Ltd	anti-apoptotic protein	
Bax	rabbit anti-human polyclonal IgG	AA 11-30 at amino terminus of Bax p21	N-20 sc-493	Santa Cruz Biotechnology Inc, Santa Cruz, CA	pro-apoptotic protein	
CD95-Ligand	IgG1k	40 kDa mCD95-L & 26 kDa sCD95-L	NOK-1	PharMingen, San Diego, CA[3]	membrane bound & soluble CD95-L. TNF family protein that binds to CD95	
RFD1	IgM		RFD1	RFH	stimulatory, "dendritic" macrophages	(233, 234)
CD34, QBEnd 10,	IgG1		M87030	Bionostics[4]	vascular endothelial cells, haemopoietic progenitor cells, fibrocytes	
Factor XIIIa	rabbit anti-human polyclonal IgG		233489	Calbiochem-Novabiochem, La Jolla, CA[4]	dermal dendritic cells	

[1] References are provided for antibodies not available commercially. (RFH, Royal Free Hospital).

[2] Kindly provided by Professor PCL Beverley, University College and Middlesex School of Medicine (UCH), London, UK

[3] Kindly provided by Professor H Yagita, Juntendo University School of Medicine, Tokyo, Japan

[4] Kindly provided by Mr Ed Browning, Dept of Histopathology, Royal London Hospital, London, UK

Table 2.5 ANTI-FIBROBLAST ANTIBODIES USED IN THE STUDY

Antibody	Subclass	mW	Code	Source	Specificity	Ref
Fibroblasts	IgG1	30-35 kDa	dia 110 AS02	Dianova, Hamburg, Germany	human adult fibroblasts (and in our hands endothelial cells, and some RFD1+ and FXIIIa+ dermal dendritic cells)	(235)
Fibroblasts	IgG1	unknown	LHF5-C16	RLH[1]	human adult and post-mitotic fibroblasts and basal epidermal keratinocytes[2]	
Fibroblasts	IgG1	unknown	LHF4-G4	RLH[1]	human foetal, adult and to a lesser degree post-mitotic fibroblasts and weak positivity in keratinocytes and endothelial cells	

[1] Kindly provided by Mr Nick Tidman, Senior Scientific Officer, ICRF, Skin Tumour Laboratory, 2 Newark Street, Whitechapel, London E1 2AT. (RLH = Royal London Hospital). These antibodies were raised against mitomycin C treated human fibroblasts. Both produce cytoplasmic staining. Their staining characteristics have not previously been published.

[2] LHF5 may recognise an epitope related to terminin an 84kDa protein present in growing and quiescent but not senescent fibroblasts (281)

1

Table 2.6 ANTI-CYTOKINE ANTIBODIES USED IN THE STUDY

Antibody	Sub-class	Immunogen	mW	Code	Source	Function
anti-IL-15	IgG1	Yeast derived simian IL-15, boosted with human IL-15	13 kDa	M112	Genzyme Diagnostics, Cambridge, MA	T cell chemotaxis and proliferation, anti-apoptotic cytokine
anti-IL-2	IgG1	Natural human IL-2 bound to liposomes	15 kDa	MCA 745	Serotec Ltd, Oxford, UK	T cell activation and proliferation, anti-apoptotic cytokine
anti-IL-4	IgG1	Yeast-expressed recombinant human IL-4	14 kDa	18651D	PharMingen	B, T and mast cell differentiation, anti-apoptotic cytokine
anti-IL-7	IgG1	Purified recombinant human IL-7	17 kDa	1689-01	Genzyme Diagnostics	T cell proliferation and differentiation, anti-apoptotic cytokine
anti-IL-6	IgG1	Purified recombinant human IL-6	26 kDa	1618-01	Genzyme Diagnostics,	T cell activation, proliferation and CTL differentiation
anti-IFN-γ	IgG2a	Purified E. coli derived recombinant human IFN-γ	17 kDa	1598-00	Genzyme Diagnostics	macrophage activation differentiation &TNF-α production; inhibits B cell IgE production
anti-TNF-α	IgG1	Human TNF-α produced in E. coli	17 kDa	80-3399-01	Genzyme Diagnostics,	T cell, endothelial cell, fibroblast and macrophage activation, pro-apoptotic cytokine
anti-TGF-β_1,β_2,β_3	IgG1	Purified natural bovine TGF-β_2 (recognises human TGF-β_1 & TGF-β_2)		80-1835-03	Genzyme Diagnostics	fibroblast proliferation and activation, collagen synthesis, suppression of T cell activation and proliferation
anti-IFN-β	IgG2	Human IFN-β	20 kDa	MAS 291	Harlan Sera-Lab Ltd, Loughborough ,UK	fibroblast-mediated T cell survival

The indirect immunoperoxidase technique was used to detect numbers and distribution of T cells, CD45RO+, Ki67+, CD34+, RFD1+ cells and fibroblasts within tissue sections. Sections were ringed with polysiloxane as a water repellent. Following a 10 minute incubation with NRS (diluted 1:100 in PBS), skin sections were incubated with 50 μl of either a pan anti-T cell IgG MoAb mix (T mix), anti-CD45RO, anti-Ki67 or anti-fibroblast antibodies diluted in phosphate buffered saline (PBS) at pretitrated optimal concentrations for 45 minutes at room temperature. To prevent evaporation of anti-sera, all incubations were carried out in a moist covered chamber. Sections were washed in PBS (twice for 2 minutes) between antibody incubations in order to remove all traces of unbound antisera. This prevents the formation of antigen-antibody complexes which might otherwise precipitate onto the sections and give rise to background staining and problems with interpretation. 50 μl of a secondary peroxidase-conjugated goat anti-mouse IgG antibody (P161; IgG, Dako Ltd, High Wycombe, Bucks, UK) diluted 1:100 in PBS and containing 4% normal human serum (NHS) was then applied. After a further 45 minutes the slides were again washed in PBS and the reaction developed using DAB (10mg of 3,3'-diaminobenzidine tetrahydrochloride dissolved in 16.6ml of PBS, followed by addition of 55.3 μl of 3% hydrogen peroxide). Sections were counterstained in haematoxylin, dehydrated by washing (for 1 minute each) once in 70% ethanol, twice in 90% ethanol, twice in absolute ethanol and cleared twice (for 1 minute each) in Citroclear. Sections were then mounted in DPX (BDH Laboratory Supplies, Poole, England).

2.3.2.1 Control preparations

Three control preparations were employed. Sections of normal human tonsil, in which the distribution and pattern of staining could be tested against tissue architecture, were used as positive controls in each experiment. In addition control incubations to detect background staining were performed on sections of each skin sample, omitting the primary antibody. Thirdly, isotype specificity was confirmed by comparison to staining with irrelevant

monoclonal antibodies of the same isotype as the MoAbs used on tonsil sections.

2.3.3 INDIRECT IMMUNOFLUORESCENCE

In this technique the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A second fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibody , raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is then applied to the section and allowed to bind to the primary antibody. With this technique combinations of antibodies with different isotype specificities which will bind to different fluorochromes can be used on the same section allowing the proportions of single and double stained cells to be counted using a fluorescence microscope.

This method was used to determine CD4:CD8 ratios, proportions of T cells and/or T cell subsets expressing CD45RO, Ki67, HLA DR, Bcl-2 and Bax and proportions of fibroblasts expressing Ki67. It was also used to investigate the proportions of cells staining with the Dianova (Dia 110) anti-fibroblast antibody which were RFD1+ and Factor XIIIa+. Sections were ringed with polysiloxane and incubated for 45 minutes in a moist covered chamber with 50 μl of appropriate combinations of MoAbs diluted in PBS (Table 2.6). After rinsing in PBS, 50 μl of Ig isotype specific FITC and TRITC conjugated affinity purified goat anti-mouse or goat anti-rabbit (Southern Biotechnology Associates, Birmingham, AL) second layer antibodies diluted in PBS, were applied at pretitrated optimal concentrations. Slides were incubated in a moist covered chamber for a further 40 minutes in the dark, then rinsed in PBS, fixed in 4% paraformaldehyde for 5 minutes and mounted in Citifluor, a phosphate-buffered/glycerol "antifadent" (AF1; Citifluor Ltd, London). Three control preparations were performed as above, but using the fluorochrome conjugated second layers alone.

Table 2.6 FIRST LAYER ANTIBODY COMBINATIONS USED FOR DUAL IMMUNOFLUORESCENCE STUDIES

CD4 IgG 1κ	CD8 IgM
CD3 IgG2a	CD8 IgM
CD4 IgG1κ	CD45RO IgG2a
CD8 IgM	CD45RO IgG2a
CD5 + CD8 an IgM antibody mix used to stain T cells	Ki67 IgG 1κ
CD4 IgG2a	Ki67 IgG1κ
CD8 IgM	Ki67 IgG1κ
CD45RO IgG2a	Ki67 IgG1κ
CD4 IgG 1κ	Ki67 rabbit anti-human polyclonal IgG
CD5+CD8 IgM	HLA DR-2 IgG1
CD8 IgM	Bcl-2 IgG1
CD5+CD8 IgM	Bcl-2 IgG1
Tmix IgG1 & IgG2a	Bax rabbit anti-human polyclonal IgG
Fibroblasts (Dia 110) IgG1	Ki67 rabbit anti-human polyclonal IgG
Fibroblasts (Dia 110) IgG1	RFD1 IgM
Fibroblasts (Dia 110) IgG1	Factor XIIIa rabbit anti-human polyclonal IgG
Fibroblasts (LHF5) IgG1	Ki67 rabbit anti-human polyclonal IgG
Fibroblasts (LHF4) IgG1	Ki67 rabbit anti-human polyclonal IgG

2.3.4 BIOTIN/ STREPTAVIDIN/ ALKALINE PHOSPHATASE

This method is more sensitive than the indirect immunoperoxidase or immunofluorescence techniques and was therefore used to identify the distribution of IL-2, IL-15, IL-7, IL-4, IFN-γ, IL-6, TGF-β, IFN-β TNF-α and CD95-L in skin sections. With this technique, the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A second biotin-conjugated antibody, raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is applied to the section and allowed to bind to the primary antibody. A third layer is then applied containing streptavidin conjugated to alkaline phosphatase. Up to 150 biotin molecules can be complexed to one (second layer) antibody and its strong affinity for streptavidin allows amplification of the signal. When the substrate is subsequently added, alkaline phosphatase hydrolyses napthol phosphate esters to phenolic compounds and phosphate and the phenols couple to the chromogen (Fast Red) to produce an insoluble coloured azo dye. Endogenous alkaline phosphatase activity is inhibited by the addition of levamisole to the substrate solution.

Stored frozen sections were removed from -20°C and placed in PBS. Sections were ringed with polysiloxane and then incubated overnight (16-18 hours) with 100μl of th appropriately diluted primary antibody in PBS +0.1% BSA, in a moist covered chamber at +4°C. Sections were washed in Tris buffered saline at pH 7.6 (TBS: Tris 60.55g, NaCl 85.20g, distilled water 500 ml, 1 Molar HCL 370 ml, adjusted to 1000ml with distilled water stored at 4°C and diluted 1/10 before use) and then incubated in a moist covered chamber with 50 μl of affinity purified horse anti-mouse biotinylated second layer (IgG; Vector Laboratories, Peterborough, UK.) diluted 1:100 in PBS-BSA for 1 hour at room temperature. After rinsing in fresh TBS, sections were then incubated for 1 hour with 50 μl of Streptavidin-Alkaline Phosphatase conjugated thir layer (Vector Laboratories) diluted 1:100 in PBS-BSA at room temp in a moist covered chamber. Sections were again rinsed in fresh TBS and the reaction was

developed by 15 minute application of filtered substrate solution, (0.005g Napthol ASBI Phosphate, 10 ml Tris HCL [Tris 1.21g, distilled water 80ml, 1M HCL 4.8ml, adjusted to 100ml with distilled water; pH 8.2], 200μl dimethylformamide, 0.01g Fast Red (TR) and 10 drops Levamisole added last). Sections were then washed in tap water and counterstained with Mayers haematoxylin for 3 minutes before mounting i PBS Glycerol (9:1). Controls were performed on skin sections as above using the steptavidin/biotin second and third layers alone. Isotype specificity was confirmed b comparison to staining with irrelevant mouse anti-human IgG1or IgG2 monoclonal antibodies on skin sections.

2.3.5 IDENTIFICATION OF APOPTOTIC T CELLS

The presence of apoptotic T cells within perivascular infiltrates in PPD reactions was confirmed using a combination of indirect immunofluorescence (described above) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling) methodologies (236). In the TUNEL method DNA breaks in individual apoptotic nuclei are labeled *in situ* in tissue sections. It relies on the specific binding of TdT to exposed 3'-OH ends of DNA followed by the synthesis of a labelled polydeoxynucleotide molecule. TdT is used to incorporate biotinylated deoxyuridine into the sites of DNA strand breaks. The signal is then amplified by avidin-FITC, enabling identification of positive cells under the fluorescence microscope.

Sections were stained with the T mix, using the indirect immunofluorescence technique as above. Sections were then fixed with 4% paraformaldehyde solution for 20 minutes at room temperature and washed in PBS for 30 minutes. Permeabilisation was performed by incubating with 0.1% Triton X-100 (Rohm & Haas, Philadelphia, USA), 0.1% sodium citrate for 2 minutes on ice. Although staining was performed on tissue sections (rather than cell suspensions) the permeabilisation step was found to enhance sensitivity and was therefore included. After rinsing in PBS, sections were incubated with 50

μl of TUNEL reaction mixture (In situ cell death detection kit, fluorescein; Cat No: 1684795, Boehringer Mannheim) for 60 minutes at 37^0C in the dark. Sections were rinsed in PBS and mounted in Citifluor. In each experiment, sections of normal human tonsil, in which apoptotic cells were present predominantly in germinal centres, were used as positive controls. Negative controls were performed as per the manufacturer's instructions, using Label solution (i.e. nucleotide mixture in reaction buffer without terminal deoxynucleotidyl transferase) instead of TUNEL reaction mixture .

2.4 QUANTIFICATION OF IMMUNOHISTOLOGY

All measurements were performed by one observer using coded slides. For immunoperoxidase and biotin/streptavidin/alkaline phosphatase studies, the number and distribution of positive cells was quantified in each section using a computerised image analysis system (Seescan Imaging Ltd, Cambridge, UK Magnification x 320) programmed to measure frame defined areas of the section. This system allows fields seen down the microscope to be visualised on a computer screen in colour. The framed area is also visualised on the computer screen and cells are counted within this area (using a mouse linked to the computer). For example, perivascular inflammatory cell infiltrates were firstly identified and a circular frame was centred on a dermal blood vessel and placed around one representative infiltrate(Plate 1). The size of the area was calculated by the computer. This frame was then repositioned in the same way over other perivascular infiltrates in each section.

In most of the studies, cells were counted per circular frame area (1.5-1.7 $x10^4 \mu m^2$) centred on the largest dermal perivascular inflammatory cell infiltrates, 5 times per section. For quantification of interstitial T cells, fibroblasts and IFN-β expression the frame was centred on interstitial areas, in between perivascular infiltrates and counted in five different areas per section. The frame areas employed were 1.5-1.7 $x10^4 \mu m^2$ for interstitial T cells and 4.5 $x10^4 \mu m^2$ for fibroblasts. For the purposes of statistical analysis and visual display of the data, results were scaled to a frame area of 1 (unit area), except for the fibroblast studies (frame area = $4.5x10^4 \mu m^2$ = unit area). Positive cells infiltrating the epidermis were counted per 100 basal keratinocytes. When assessing the proportion of perivascular cells expressing cytokines or CD95-L expression, the number of cells with either surface or cytoplasmic staining was divided by the total number of cells counted per frame area.

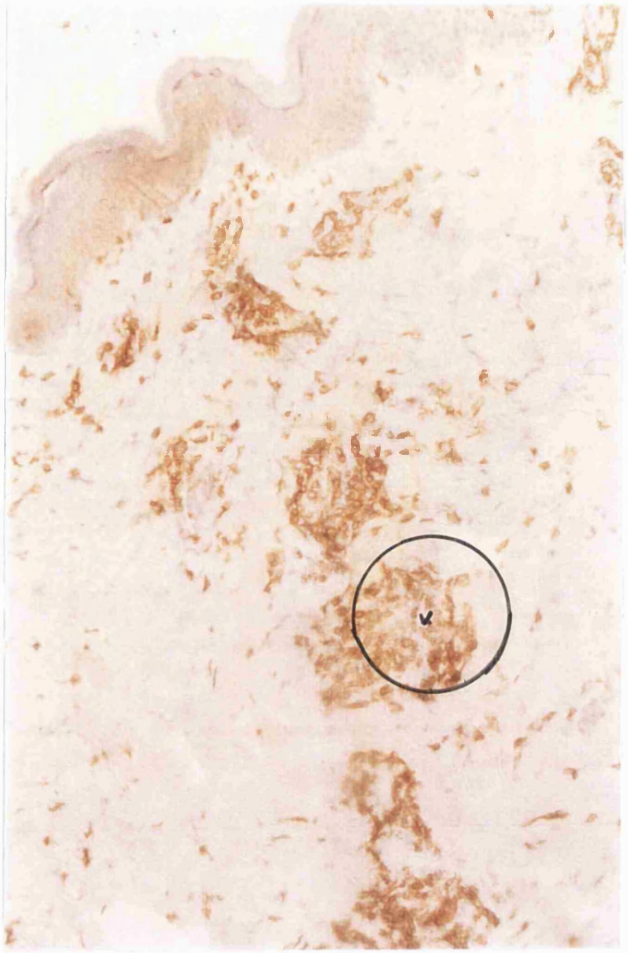


Plate 1 Quantification of immunohistology with an image analysis system

A circular frame was centred on a dermal blood vessel (v) and placed around a representative perivascular T cell infiltrate in the dermis. Cells were counted within the frame area and the frame was then repositioned in the same way over other perivascular infiltrates in each section. Here T cells were stained with Tmix using an indirect immunoperoxidase technique 7 days after intradermal PPD (original magnification x160).

For immunofluorescence and TUNEL studies, the distribution and percentages of T cells were estimated in each section using a Zeiss fluorescence microscope with appropriate selective filters for FITC and TRITC (x 400 magnification). The percentages of positive cells were estimated by dividing the number of dual staining cells (eg CD4+ CD45RO+cells) by the total number of single staining cells being studied (eg total number of cells staining only for CD4) in the five largest dermal perivascular inflammatory cell infiltrates present in the sections, and in five high power fields centred on interstitial areas (in between perivascular infiltrates) where appropriate.

2.5 STATISTICAL ANALYSIS

In this study we were interested to know whether the variables studied (e.g. perivascular T cell number) varied between different groups of individuals (i.e. AE and each of the different time points of the DTH response were considered as different groups). Because we considered repeated measurements (i.e. 5 per subject) on a number of individuals (e.g. 5 per time point in DTH responses) and we were interested in more than two groups of individuals, a two-way Analysis of Variance (ANOVA) was performed. The two-way ANOVA method is a global test which identifies whether there are differences between the groups overall. The total variation in the data is separated into that which can be explained by differences between the individuals in the different groups, that which is explained by differences between the individuals in the same group, and that which is explained by differences within each individual (i.e. the random variation within a single individual). If there are differences between the groups, then the variation between the groups should be greater than both the variation within the groups, and the random variation within a single individual. A significant result indicates that at least one of the groups is different to the others. However, it does not indicate which group this is. Thus, when significant differences were detected by this method, an unpaired t-test was then used to compare specific pairs of groups. A Tukey test was used to control p values so that the overall type I error was set at 5% (i.e. there was no more than a 5% chance that significance was falsely attributed). The calculations involved in the ANOVA are complex and a SAS computer package was used which gave the values directly. An overall p value for the ANOVA is provided and each pairwise comparison is identified as either significant ($p<0.05$) or non-significant ($p>0.05$). The assumptions made when using this method are that the variable studied is Normally distributed and that the variances in each of the groups are the same. It was not possible to be absolutely certain that the data was Normally distributed, due to the relatively small sample sizes, however, the test is relatively robust against moderate departures from

Normality. Visual inspection of the data suggested that the variances of the groups were the same. Expert help was sought from a statistician in the Department of Primary Care and Population Sciences at the Royal Free Hospital School of Medicine, and this approach was felt to be suitable for the data.

Measurements were taken from 5 areas (using the image analysis system or fluorescence microscopy as described above) in each subject and mean values and standard deviations were calculated for each individual. A minimum of 3 subjects were investigated at each time point. Using the ANOVA method described above, differences between the values at the five time points in DTH responses were tested for significance including time and subject as factors in the analysis. The results obtained in AE patients were compared in the same way to those in normal skin (time 0) and the height (day 7) and resolution phases (day 14) of the DTH response. For the purpose of visually displaying the data, the mean values for the different subjects were used to calculate an overall mean value for each time point/group. The standard deviation of these mean values was then used as an estimate of the variability between the subjects at each time point/ in each group.

3. APOPTOSIS IN THE RESOLUTION OF CUTANEOUS INFLAMMATION: DELAYED-TYPE HYPERSENSITIVITY

3.1 INTRODUCTION

In order to investigate why cutaneous inflammation persists in diseases such as AE, it is first necessary to establish the mechanisms involved in "normal" immune resolution. The Mantoux reaction (MR) is a classical DTH reaction which, in humans, peaks clinically at 48-72 hours and resolves within 14 days. This model of cutaneous inflammation enables the study of the kinetics of the local immune response from onset to resolution. Despite this, a majority of studies in humans have concentrated on initiation of the response, and we still know very little about how it comes to an end. A large number of *in vitro* studies, but relatively scarce *in vivo* data, suggest that the process of apoptosis is responsible for clearing T cells at the end of an immune response (3-6). Over-expression of Bcl-2 by T cells has been described in a number of benign and malignant cutaneous inflammatory diseases, including T cell lymphoma (CTCL), bullous pemphigoid, discoid lupus erythematosus and lichen planus, suggesting that prolonged T cell survival may be involved in persistence of cutaneous inflammation (237). Changes in the expression of apoptosis regulatory proteins, and by inference IL-2R γ-chain cytokines may thus also be involved in T cell clearance at the end of the MR. Furthermore, apoptosis mediated by the TNF-α and CD95 pathways is thought to be involved in the induction of KC apoptosis following exposure to UV radiation (107), but their contribution to the resolution of T cell mediated cutaneous inflammation *in vivo* is unknown.

In this study, we have therefore investigated the kinetics of T lymphocyte infiltration, proliferation and apoptosis during a Mantoux reaction in relation to the expression of anti-apoptotic cytokines, apoptosis-inducing CD95-ligand and TNF-α and apoptosis regulatory proteins, in order to characterise features associated with initiation and resolution of inflammation, **(specific aims 1 and 2)**.

In view of the recent suggestion that activated fibroblasts may be involved in the generation both of T cell mediated inflammation and T cell memory via their synthesis and release of pro-inflammatory cytokines and type I interferons (12,13,230 and 10, submitted), we have additionally investigated the kinetics of fibroblast infiltration, proliferation and the expression of TGF-β and IFN-β, during the course of the Mantoux reaction, **(specific aim 3)** .

3.2 RESULTS

3.2.1 RESPONSE TO PPD OF STUDY SUBJECTS

All subjects responded to PPD with maximal reactions (as defined by erythema and induration scores) consistently occurring between 48 and 72 hours. Of the lesions biopsied at 12 hours two showed a marked response and three a minimal response at that time. When reviewed at 48-72 hours all five subjects showed a positive test, even when inflammation induced by the biopsy itself was taken into account. Erythema and induration were scored at 72 hours and at the time of biopsy, the maximum diameter of the reaction was scored at 72 hours (Table 3.1). The sum of these scores for each individual was then used to calculate a mean score and standard deviation for each time point (5 subjects per time point) and are presented in Fig 3.1. There was no significant difference in the mean scores obtained at 72 hours in each of the 4 groups of subjects who received intradermal PPD (ANOVA p = 0.12) confirming that similar responses were obtained regardless of time of biopsy (Fig 3.1a). The mean scores at the time of biopsy paralleled erythema measurements obtained with the DermaSpectrometer, but also took into account induration, which may better reflect the degree of inflammation occurring in the lesions (15). There were significant differences between the scores obtained at different time points (ANOVA p=0.02). These scores were significantly lower at 14 days than at 3 days, confirming that lesions biopsied at that time point were resolving (Fig 3.1b).

The subject who received intradermal PPD at 6 different sites showed markedly positive responses, developing marked erythema and induration within 12 hours. Each site showed >10mm induration and an EI >15 at 72 hours. An example of a 72 hour lesion in this subject is shown in Plate 2.

Table 3.1 CLINICAL RESPONSES TO PPD

SUBJECT	Score at time of biopsy		Score at 72hrs		
	Erythema	induration	Erythema	Induration	Size
12 HOURS					
LP	4	5	4	5	2
PL	4	5	4	5	2
MT	1	1	3	3	1
FB	1	2	3	4	1
TM	1	2	3	5	1
72 HOURS					
ML			2	3	1
JF			4	5	2
SH			2	4	2
MW			2	2	1
HB			2	3	1
7 DAYS					
MG	2	1	3	4	2
SY	3	2	4	4	2
BM	1	2	2	3	1
HS	2	2	3	4	2
BF	2	2	2	4	1
14 DAYS					
PA	1	1	3	4	2
LP	1	2	4	5	2
BB	1	1	4	5	2
JC	1	2	4	5	2
KO	1	1	3	4	2

- ERYTHEMA INDEX

1 = <5, 2 = 5-10, 3 = 10-15, 4 = >15

- INDURATION

1 = none detected, 2 = just palpable, 3 = easily palpable, 4 = marked, 5 = very marked

- DIAMETER/SIZE

1 = 4-9mm , 2 = >10mm

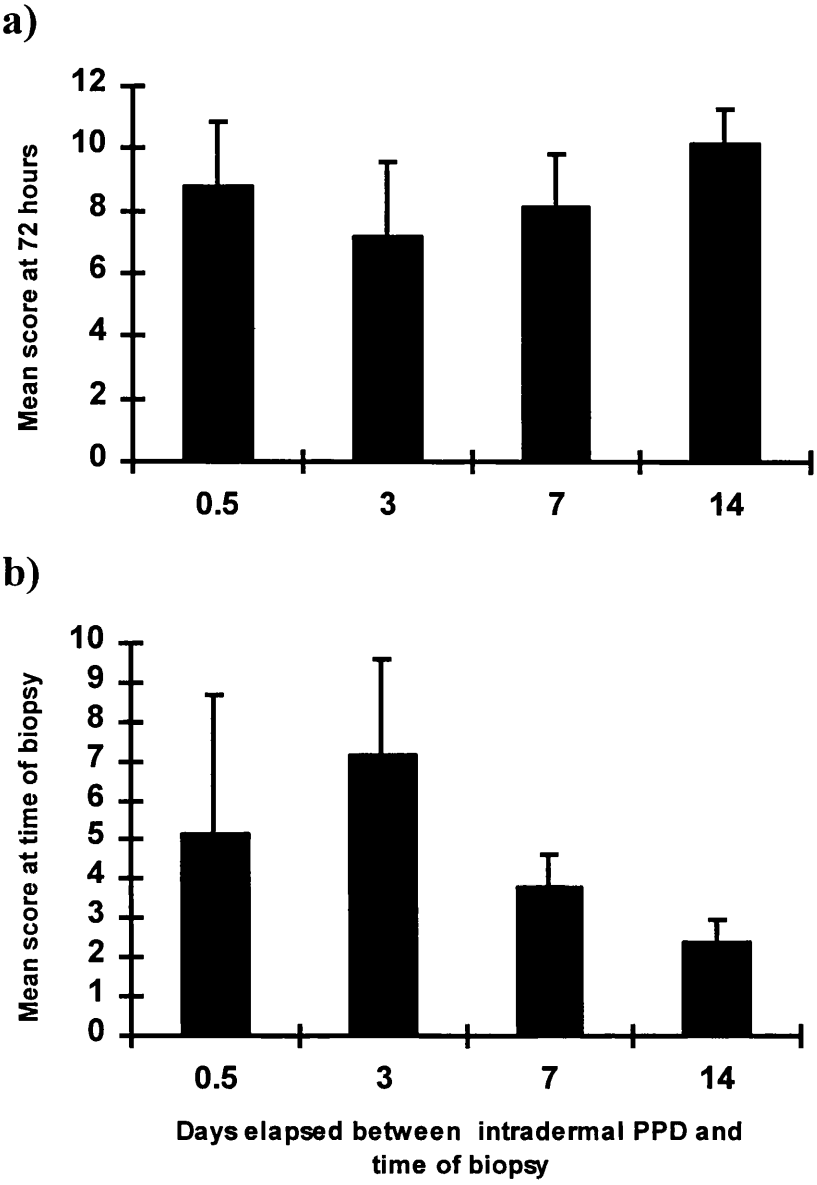


Figure 3.1 Response of study subjects to intradermal PPD.
All subjects responded to PPD with maximal reactions consistently occurring between 48 and 72 hours. Erythema and induration were measured at 72 hours and on the day of the biopsy as follows. Erythema was scored using a DermaSpectrometer as 1 = <5, 2 = 5-10, 3 = 10-15, 4 = >15. Induration was scored as: 1= none detected, 2 = just palpable, 3 = easily palpable, 4 = marked, 5 = very marked. The maximum diameter was measured in millimetres (1 = 4-9mm or 2 = >10mm) at 72 hours. The sum of these scores for each individual was then used to calculate a mean score and standard deviation for each time point (5 subjects per time point) **a)** at 72 hours after intradermal PPD and **b)** at the time of biopsy. Error bars indicate standard deviations.

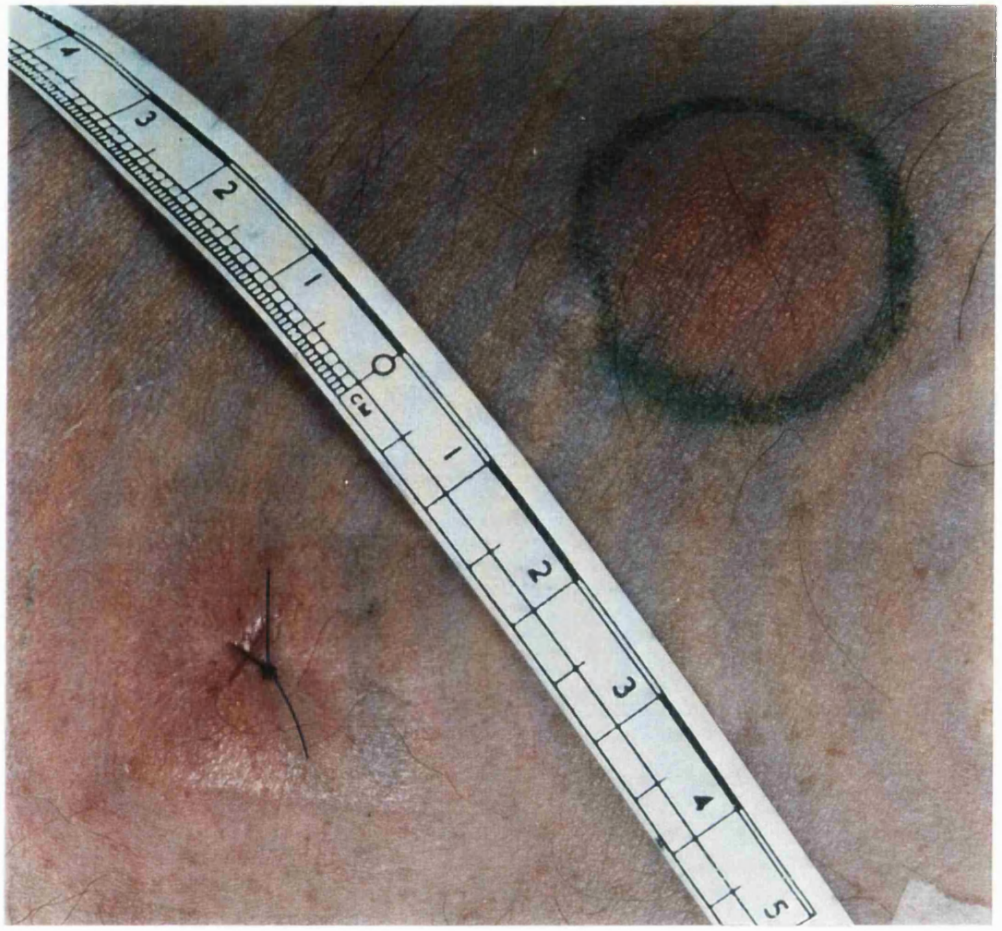


Plate 2 Clinical response to intradermal PPD (1:10 000) at 72 hours in LWP who was studied at multiple time points.

3.2.2 CHARACTERISTICS OF INFILTRATING T CELLS

3.2.2.1 T cell numbers and distribution in a single subject

In order to ensure that the kinetics of the reaction could be observed in a single individual, one subject had Mantoux tests performed at 6 different sites and was biopsied at 6,12, 24, 72 hours and 7 and 14 days. In this subject, T cells began to accumulate perivascularly in the reticular dermis as early as 6 hours after intradermal PPD (16 +/- 6.3 T cells/ unit area [UA]) and continued to increase up to day 3 (47.4 +/- 7.7 cells/UA) (Fig 3.2a). By day 14 perivascular cell numbers had decreased significantly (25.8 +/- 10.2 cells/ UA) compared to day 3. T cells were first seen infiltrating the epidermis at 12 hours. Numbers in this area were also maximal at day 3 (53/ 100 basal cells), and thereafter declined up to day 14 (11 /100 basal cells) (Fig 3.2b). Small numbers of T cells were seen scattered in the upper reticular and papillary dermis in between collagen bundles (we will hereafter refer to these cells as interstitial cells). No cells were seen in this area until 24 hours after challenge. Interstitial T cell numbers were maximal at day 1 (5 +/- 3.5 cells/ UA) and day 3 (4 +/- 3 cells/ UA) but declined progressively thereafter (1.6 +/- 1.5 cells/ UA at day 14, Fig 3.2c). Collections of periadnexal T cells were also noted in the deeper portions of the reticular dermis throughout the course of the reaction but were not counted. In addition, cells with marked endogenous peroxidase activity resembling neutrophils were scattered throughout the papillary and upper reticular dermis. Although they were not counted, few such cells were present at 6 hours, numbers increased markedly at 12 hours, were reduced slightly at 24 hours and markedly by day 3.

These results are entirely consistent with previous reports (19,20,23-25). It is of interest, however, that on day 7 when perivascular T cell numbers were reduced, there were still large perivascular collections of cells

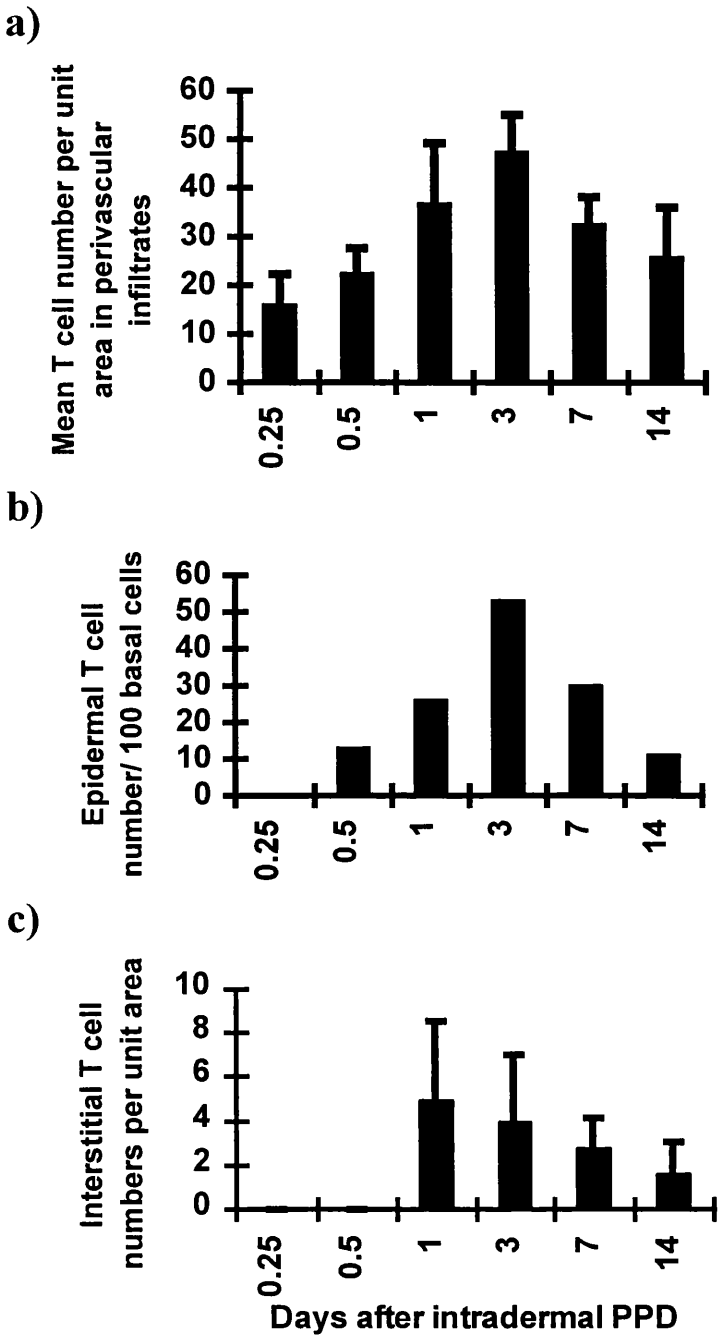


Figure 3.2 Mean numbers of infiltrating T cells in Mantoux reactions in a single individual.

T cells were stained by an indirect immunoperoxidase method and quantified in each section, per circular frame area centred **(a)**on the largest dermal perivascular inflammatory cell infiltrates, and **(c)** on areas of the upper reticular and papillary dermis in between perivascular infiltrates using an image analysis system, 5 times per section. In **(b)** the number of epidermal T cells was counted per 100 basal keratinocytes.

in the mid reticular dermis which were not T cells. In addition, whereas at day 14 there were few perivascular cells in the upper and mid dermis, periadnexal collections were still quite prominent in the deep dermis, sometimes extending down to subcutaneous fat.

3.2.2.2 T cell numbers- Pooled data from different subjects

Normal skin contained small numbers of T cells (4.5 +/-1.9/ unit area [UA]). After intradermal PPD T cells accumulated perivascularly within the dermis and numbers rose significantly by 12 hours (15.0+/- 6.9/UA); and further by 72 hours (39.6 +/- 7.8/ UA,), there was also a further increase from this figure up to day 7 (54.2 +/- 4.7/UA). T cell numbers then fell significantly by day 14, although they remained in excess of numbers in normal skin (23.7 +/- 6.3/UA) (Fig 3.3a). Overall analysis revealed that there were significant differences between T cell numbers at the five time points studied ($p<0.0001$). Further, T cell numbers at each time point were significantly different to those at the preceding and subsequent time points.

Although a majority of infiltrating cells were present within perivascular areas, smaller numbers of T cells were seen infiltrating the interstitium and epidermis. The numbers of cells in these areas showed much greater inter-subject variability than in perivascular infiltrates, confirming previous observations (23). However, numbers were maximal on day 3, and subsequently declined up to day 14 after challenge (Fig 3.3 b & c).

The time course of the reaction described above in a single individual was accelerated when compared to that obtained from the pooled data. This may be explained in part by inter-subject variability, but also by the fact that the individual studied at multiple time points had not only had multiple antigenic challenges (with PPD), but also showed a very marked response.

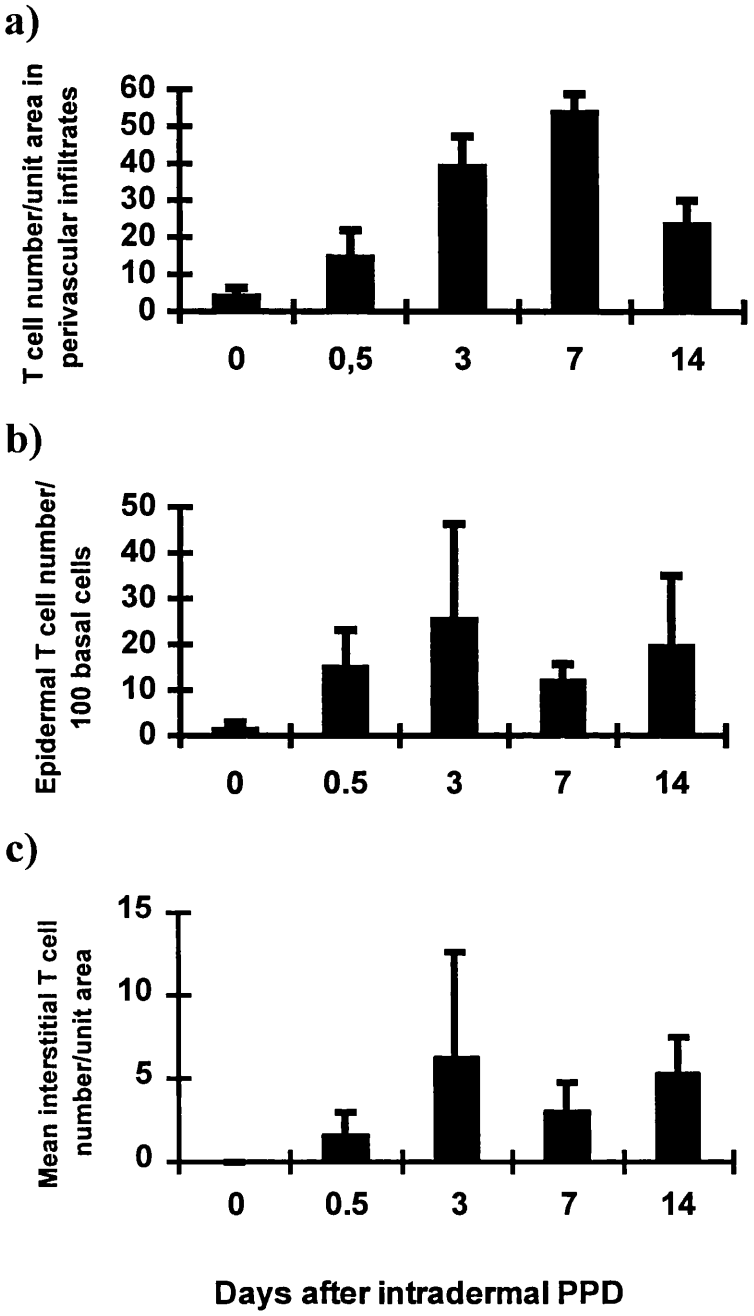


Figure 3.3 Mean T cell numbers were determined in pooled data from Mantoux reactions.

T cells were stained by an indirect immunoperoxidase method and quantified in each section, per circular frame area centred **(a)**on the largest dermal perivascular inflammatory cell infiltrates, and **(c)** on areas of the upper reticular and papillary dermis in between perivascular infiltrates using an image analysis system, 5 times per section. In **(b)** the number of epidermal T cells was counted per 100 basal keratinocytes, 3 times per section. (n=5 at each time point.)

In the pooled data, although the clinical and overall histologic responses to intradermal PPD showed different kinetics, peaking at 3 and 7 days respectively, by day 14 both were resolving. Since the majority of infiltrating cells were present perivascularly and inter-subject variability was less marked in these areas, it was decided to concentrate on perivascular cells in the remainder of the study.

3.2.2.3 CD4:CD8 Ratios

In normal skin CD4+ cells predominated, but numbers were too small to calculate meaningful ratios. Throughout the course of the Mantoux reaction the number of CD4+ T cells exceeded the CD8+ cells within the perivascular infiltrates. The ratio of CD4 to CD8 cells was lowest at 12 hours (2.23 +/- 0.85) and reflected proportions of CD4 and CD8 cells found within the peripheral circulation. Thereafter the proportion of CD4 cells increased (Fig 3.4 & Plate 3A). This suggests that either active recruitment or proliferation of CD4 cells occurred.

In view of the previous observation that greater proportions of CD8 cells were present in the interstitium (23), we compared the CD4:CD8 ratios in perivascular infiltrates with those in the interstitial cells in one representative subject per time point. Although results did not reach significance, there did appear to be relatively more CD8 cells in interstitial areas at earlier time points (Fig 3.5 and Plate 3B). Dual immunofluorescence was performed on one representative section per time point with CD3 and CD8. No CD3 - CD8 + cells were identified in any of the sections examined, indicating that the CD8 + cells identified were T rather than NK cells. In addition, no $CD4^+$,$CD8^+$ double positive T cells were observed.

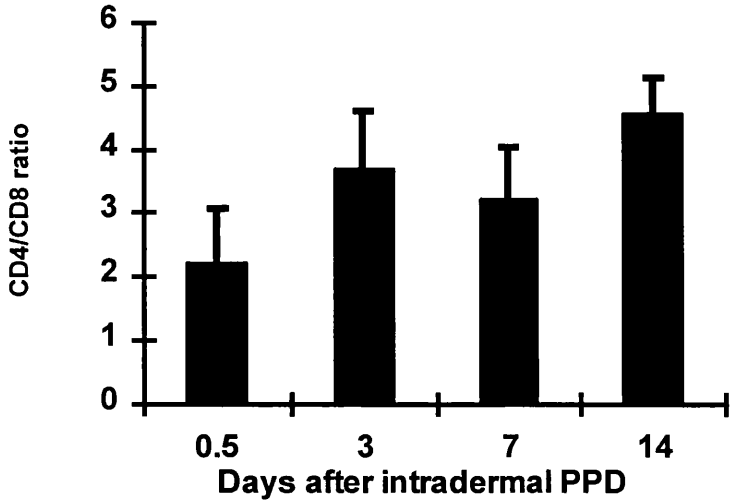


Figure 3.4 Mean CD4/ CD8 ratios

CD4 and CD8 cells were enumerated by dual immunofluorescence and proportions of positive cells in the five largest dermal perivascular inflammatory cell infiltrates were quantified in each section using a Zeiss fluorescence microscope (n=4 at 72 hours, n=3 at other time points). CD4 cells predominated in normal skin but the numbers of cells were too small to give meaningful ratios. Error bars indicate standard deviations.

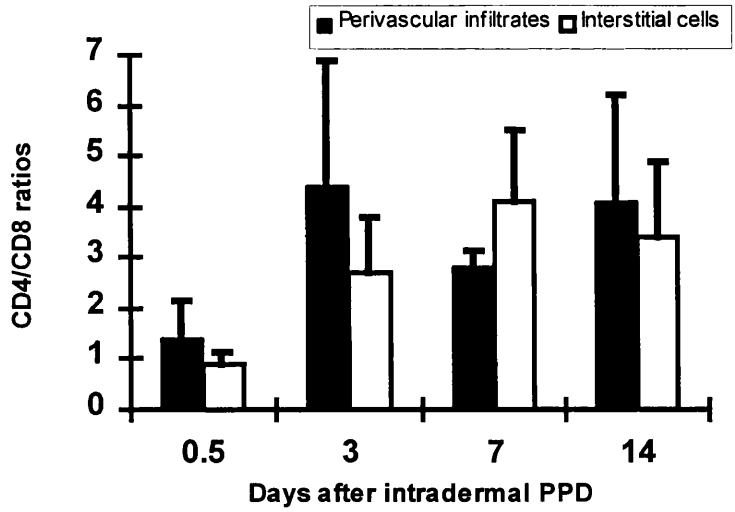


Figure 3.5 Mean CD4/ CD8 ratios in perivascular areas and the upper dermal interstitium in one representative subject per time point.

Cells were enumerated by dual immunofluorescence and proportions of positive cells in the five largest dermal perivascular inflammatory cell infiltrates, and per high power field in 5 interstitial areas, were quantified in each section using a Zeiss fluorescence microscope. Error bars indicate standard deviations.

3.2.2.4 Characteristics of CD45RO+ cells

There were small numbers of CD45RO+ cells in normal skin and at 12hours after intradermal PPD (3.54 +/-1.2/UA and 6.0 +/- 1.8/UA respectively). The number of CD45RO positive cells within the perivascular infiltrates increased between 12 and 72 hours (6.0 +/- 1.8 to 17.1 +/- 6.3/UA) and again between day 7 and day 14 (19.8 +/- 4.0 to 27.0 +/- 6.6/UA). Overall analysis revealed that there were significant differences between CD45RO+ cell numbers at the five time points studied (ANOVA $p<0.0001$). Further analysis showed significant differences between values at 12 and 72 hours and between those at 7 and 14 days.

Double immunofluorescence studies revealed that at 12 hours 68.7 +/- 4.6 % of CD8 cells within perivascular infiltrates were CD45RO+. This figure rose to 83.4 +/- 0.6% at 7 days after intradermal PPD (Fig 3.6).This suggests that even from early time points during the reaction, CD8 cells recruited into the lesions were already primed.

In contrast, the proportion of primed (CD45RO+) CD4 cells was lowest at 12 hours (44.9 +/- 22.2%), increased significantly by 72 hours (74.4 +/- 7.9%, Plate 3C) and thereafter continued to rise gradually up to 14 days (86.9 +/- 4.2%; Fig 3.6) Overall ANOVA $p<0.0001$.

3.2.3 T CELL PROLIFERATION IN MANTOUX REACTIONS

In order to determine whether in-situ proliferation could account for increasing cell numbers within Mantoux reactions we measured numbers of Ki67+ cells within perivascular infiltrates. Overall analysis revealed that there were significant differences between Ki67+ cell numbers at the five time points studied (ANOVA $p<0.0001$, Fig 3.7).

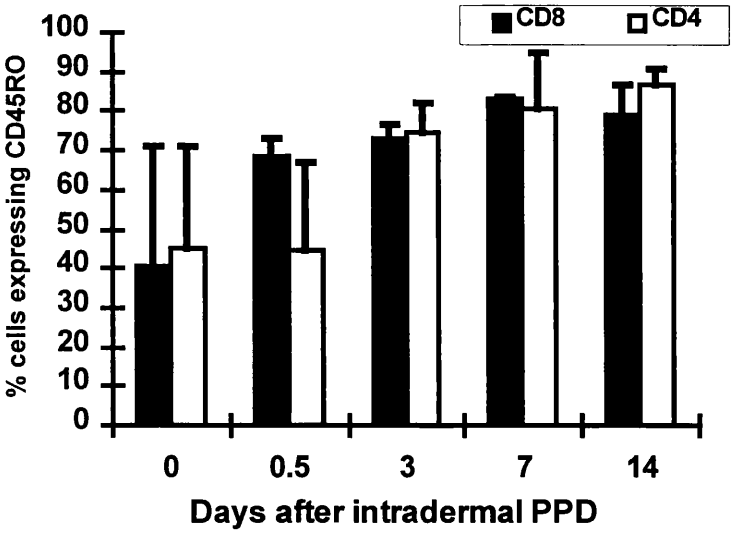


Figure 3.6 Proportions of CD4+ and CD8+ cells expressing CD45RO
Within perivascular infiltrates the proportions of CD4+ and CD8+ cells expressing CD45RO were measured using dual immunofluorescence techniques and quantified in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope. Error bars indicate standard deviations (mean of n=3 at each time point).

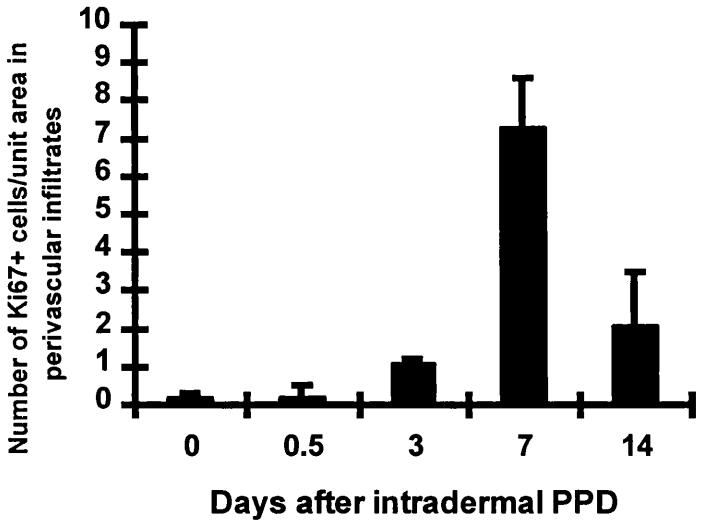


Figure 3.7 Mean numbers of proliferating cells
Cells were counted within perivascular infiltrates. An indirect immunoperoxidase technique and an image analysis system were used, and cell numbers per circular frame area centred on the 5 largest dermal perivascular inflammatory cell infiltrates were found to be significantly increased at 7 days (overall ANOVA $p<0.0001$, n=3 at each time point).

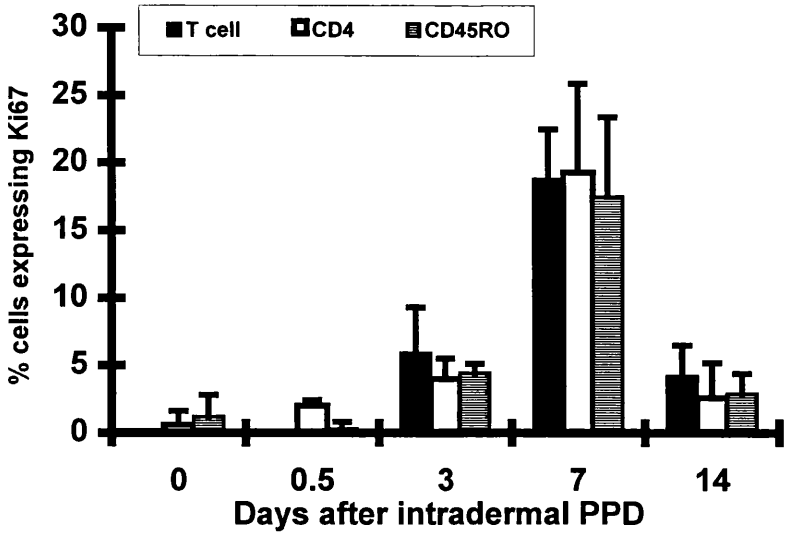


Figure 3.8 Proportions of proliferating T, CD4+ and CD45RO+ cells
Dual IMF studies were performed using antibodies to either CD5+CD8 (T cells), CD4 or CD45RO in combination with anti-Ki67. Proportions of double positive cells were quantified in each section, in the five largest dermal perivascular inflammatory cell infiltrates using a Zeiss fluorescence microscope. Error bars indicate standard deviations (mean of n=3 at each time point).

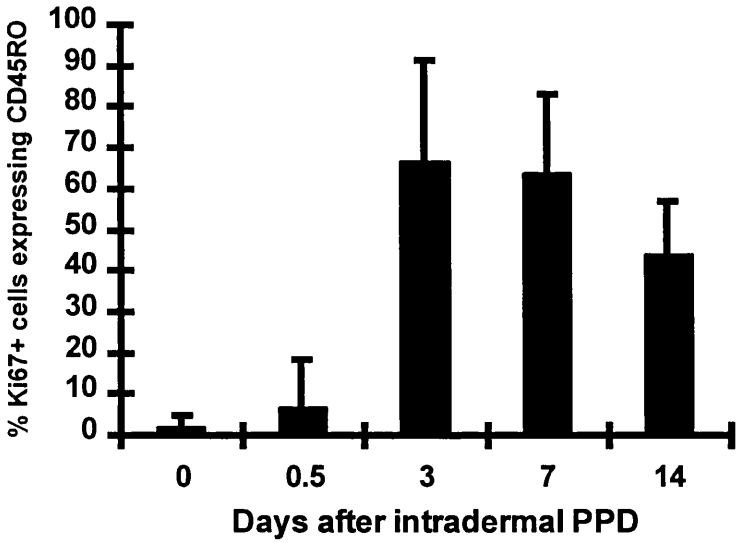


Figure 3.9 Proportions of Ki67+ cells expressing CD45RO
Dual immunofluorescence was employed to measure the proportion of proliferating cells which were CD45RO+ in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope. Error bars indicate standard deviations (mean of n=3 at each time point).

Numbers of Ki67+ cells rose significantly from 0.2 +/- 0.2/UA at 12 hours to 7.3 +/-0.9/UA at 7 days, and then fell to 1.5 +/- 1.4 at 14 days (Fig 3.7). To investigate which cells were induced to proliferate, dual immunofluorescence studies were performed (Fig 3.8). At 12 hours very few T cells expressed Ki67. 72 hours after intradermal PPD 5.8 +/- 3.3% of T cells were Ki67+, and by 7 days the percentage of proliferating T cells increased significantly to 18.8 +/- 3.7%. However, at 14 days this percentage fell to 4.2 +/- 2.3% (overall ANOVA $p < 0.0001$). The proliferating cells were CD4+, CD45RO+ (Fig 3.8 & 3.9 and Plate 3D). At 72 hours 3.9 +/- 1.5% of CD4+ cells were actively proliferating, by day 7 this percentage had increased significantly to 19.3 +/- 6.6 %, and by day 14 it had fallen to 2.63 +/- 2.65% (overall ANOVA $p<0.0001$). A similar trend was observed in the CD45RO+ subset (Fig 3.8). No Ki67+ CD8 cells were identified in any of the sections examined. These results suggest that the increase in CD4+ CD45RO+ T cells during the course of the Mantoux reaction was likely to be due, in large part, to the induction of proliferation within this subset.

3.2.4 DISTRIBUTION AND CHARACTERISTICS OF HLA DR POSITIVE CELLS

T cell HLA DR expression is known to be upregulated after activation (238). The consequence of such expression is that T cells become able to present antigen to other T cells. However, in the absence of appropriate costimulatory signals such interactions result either in the induction of anergy or CD95-mediated apoptosis (239,240). In addition, it has been suggested that anergic T cells may suppress proliferation in other T cells with the same antigen specificity (241). Increasing T cell HLA DR expression may therefore provide a means of down-regulating T cell-mediated immune responses, and we were interested to examine T cell HLA DR expression to determine whether this mechanism might be involved in the resolution of the DTH response.

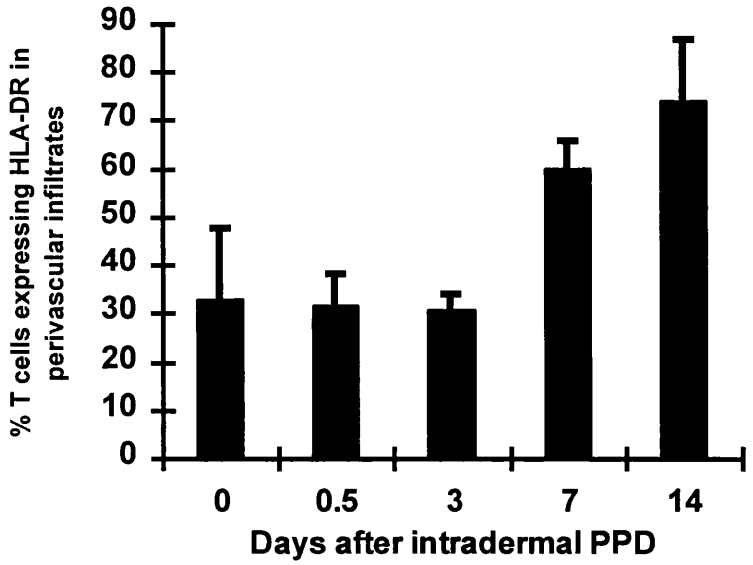


Figure 3.10 Mean T cell HLA DR expression

Dual IMF studies were performed using CD5+CD8 (T cells) and HLA DR, and proportions of double positive cells were quantified in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope, in 3 subjects per time point. Error bars indicate standard deviations.

Plate 3 Characteristics of infiltrating T cells in DTH responses

Dual immunofluorescence studies were used in A-F (x400 magnification).

A Proportions of CD4+ cells (green) and CD8+ cells (red) in a dermal perivascular inflammatory cell infiltrate 7 days after intradermal PPD.

B Proportions of CD4+ cells (green) and CD8+ cells (red) in the interstitium 3 days after intradermal PPD.

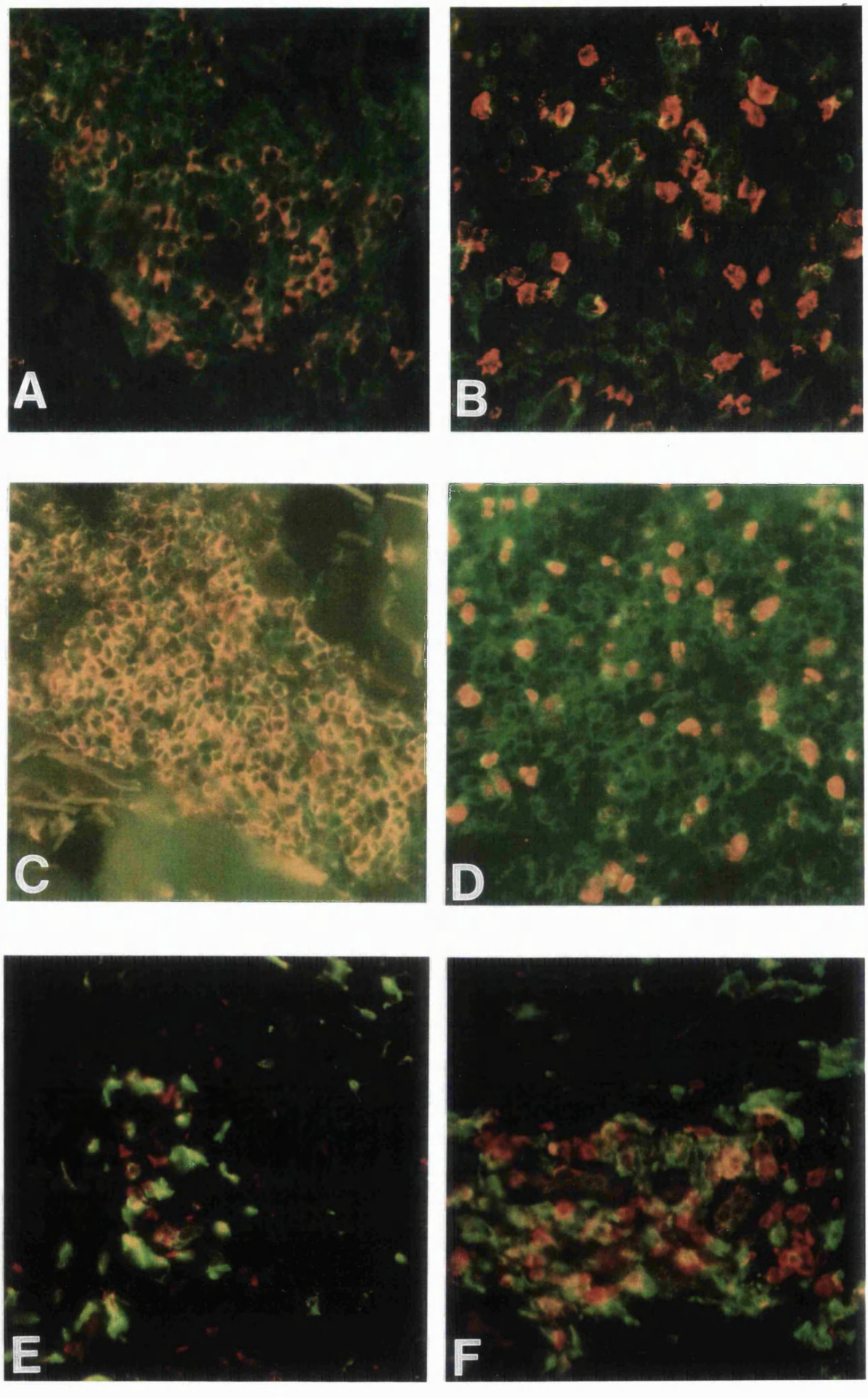
C Proportions of CD4 cells (red) expressing CD45RO (green) 3 days after intradermal PPD.

D Ki67 expression (red) within CD45RO+ cells (green) in a dermal perivascular inflammatory cell infiltrate 7 days after intradermal PPD.

E Proportions of T cells (red) expressing HLA DR (green) 12 hours after intradermal PPD.

F Proportions of T cells (red) expressing HLA DR (green) in a perivascular infiltrate 7 days after intradermal PPD.

PLATE 3



HLA DR was not expressed on KC in normal skin or 12 hour samples. It was patchily up-regulated on day 3 but strongly positive on a majority of KC at 7 and 14 days. Positive epidermal LC were present in all sections examined. In perivascular areas the numbers of HLA DR+ cells greatly exceeded the numbers of infiltrating T cells at all time points (Plate 3E).

Positive cells included endothelial cells and dendritic and spindle shaped cells. Such positive cells were also visible in the interstitium in increasing numbers from 12 hours after challenge.

Dual immunofluorescence studies revealed that there were significant differences in perivascular T cell HLA DR expression at the different time points (overall ANOVA $p<0.0001$, Fig 3.10). The proportions of perivascular T cells expressing HLA DR was 33.2 +/- 14.6% in normal skin, and remained relatively stable up to day 3 (31.0 +/- 3.3%). Thereafter significant increases in expression occurred to 60.3 +/- 5.8% on day 7 and 74.3 +/- 12.9% on day 14 after intradermal PPD (Plate 3F).

3.2.5 RESOLUTION OF THE MANTOUX REACTION-MARKERS OF APOPTOSIS

3.2.5.1 TUNEL+ T cells

To investigate whether the reduction in T cell numbers and resolution of the DTH reaction occurred as a result of T cell apoptosis, we employed a combination of indirect immunofluorescence and TUNEL methodologies. Overall analysis revealed significant differences in the proportions of TUNEL+ T cells at the five time points studied (ANOVA $p< 0.0001$). No TUNEL positive T cells were seen within perivascular areas in normal skin. At 12 hours after intradermal PPD, 0.05 +/- 0.1% (range 0-0.3%) of perivascular T cells were TUNEL positive. 72 hours after challenge the

percentage of TUNEL positive perivascular T cells had increased (0.5 +/- 0.4%), but not significantly. However, there was a significant increase at day 7 after intradermal PPD (compared to normal skin and the 12 and 72 hour time points) to 1.83 +/-0.74% (range 0.81-2.48%) and a further rise to 2.5 +/- 0.93% (range 1.5-3.75%) at day 14 (Fig 3.11 and Plate 4A). This percentage increase at 7 and 14 days remained significant when the variation in T cell numbers between time points was taken into account.

TUNEL positive T cells were present in all the 7 and 14 day specimens examined and were located predominantly at the periphery of perivascular infiltrates. Additional investigations revealed that PI (propidium iodide) positive fragmented apoptotic nuclei at these time points were located within macrophages (Plate 4B), suggesting that the numbers of apoptotic T cells detected represented an underestimate of the total amount of apoptosis occurring.

3.2.5.2 T cell Bcl-2 and Bax expression

Previous studies have shown that the propensity for T cells to die by apoptosis due to cytokine deprivation correlates with a reduction in their Bcl-2 expression relative to Bax (9,167,171-173). We thus investigated the percentages of T cells expressing Bcl-2 and Bax within perivascular infiltrates to assess the extent to which the apoptosis observed could be due to lack of cytokines. Dual immunofluorescence studies revealed significant differences in T cell Bcl-2 expression at the five different time points (Overall ANOVA $p<0.0001$). The proportions of T cells expressing Bcl-2 perivascularly rose significantly between 12 hours after intradermal PPD (43.4% +/- 5.8%) and 3 days (76.3% +/- 7.5%). This percentage remained relatively stable (70.9% +/- 6.1%, Plate 4C) to day 7 and then fell significantly by day 14 (23.5% +/-3.3%; Fig 3.12 and Plate 4D).

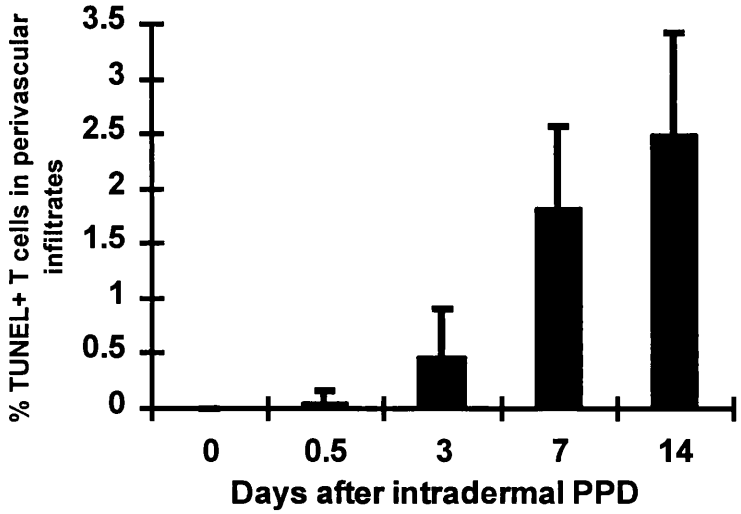


Figure 3.11 Mean percentage of TUNEL+ perivascular T cells

A combination of indirect IMF and TUNEL methodologies were employed to quantify mean percentages of TUNEL+ perivascular T cells. Proportions of double positive cells in the five largest dermal perivascular infiltrates were counted in each section using a Zeiss fluorescence microscope (n= 3 at day 0, n=5 at day0.5, n=4 at other time points). Error bars indicate standard deviations.

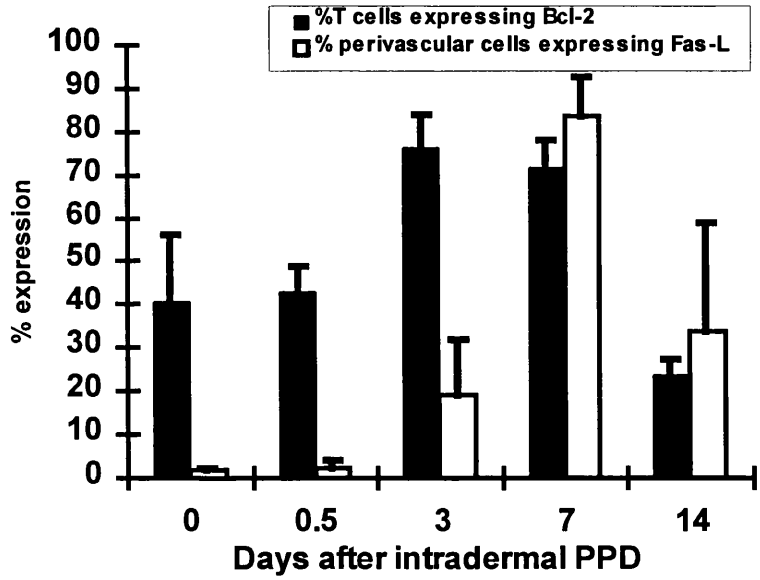


Figure 3.12 Mean perivascular Bcl-2 and CD-95-L expression.

Dual IMF studies were performed using CD5+CD8 (T cells) and Bcl-2 and proportions of double positive cells were counted in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope, in 5 subjects per time point. A biotin/streptavidin method and an image analysis system were used to determine CD95-ligand expression in the five largest dermal perivascular infiltrates. The percentage of cells with cytoplasmic or membrane positivity were counted (n = 5 at day 14, n = 4 at other time points). Error bars indicate standard deviations.

Plate 4 Markers of apoptosis in DTH responses

Dual immunofluorescence studies were used in A-E. (x400 magnification).
A TUNEL positive T cells in a dermal perivascular inflammatory cell infiltrate 7 days after intradermal PPD. T cells were labelled with TRITC (red) and then apoptotic cells were stained using TUNEL reaction mixture (green).

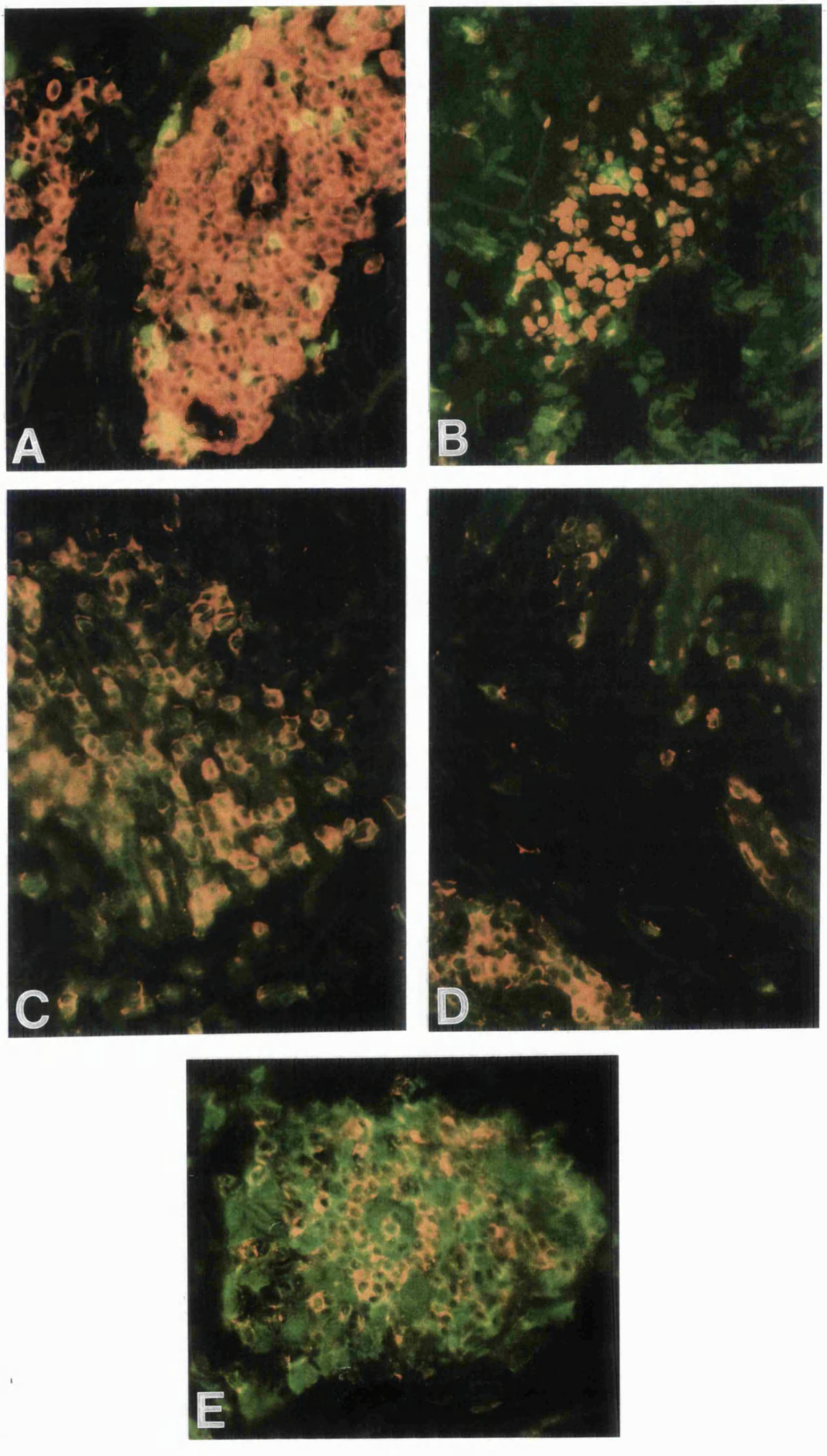
B PI positive apoptotic nuclei within a perivascular macrophage 14 days after intradermal PPD. Macrophages were stained with CD68 and FITC (green) and pteridium iodide was used to stain cell nuclei (red).

C Proportions of T cells (red) expressing Bcl-2 (green) 7 days after intradermal PPD.

D Proportions of T cells (red) expressing Bcl-2 (green) 14 days after intradermal PPD.

E Proportions of T cells (red) expressing Bax (green) 3 days after intradermal PPD.

PLATE 4



In contrast, the proportions of T cells expressing Bax within these areas remained constant at >98% at all the time points studied (Plate 4E), and although T lymphocyte numbers were much smaller, high Bax expression was also found in normal skin.

3.2.5.3 CD95 ligand expression

The high level of Bcl-2 expression on day 7 suggested that lack of this molecule, and by inference cytokines, was not responsible for the T cell apoptosis observed at this time. We therefore investigated the expression of CD95-ligand in perivascular infiltrates during the Mantoux reaction to determine if this alternative pathway to apoptosis may contribute to death.

From the basal to granular cell layers, keratinocytes (KC) in normal skin (n=4) expressed weak to moderate CD95-ligand. Weak positive cytoplasmic staining was present in occasional (1.3 +/- 0.5%) perivascular cells, while interstitial cells showed no expression (Plate 5A). 12 hours after intradermal PPD (n=4) levels and distribution of CD95-ligand in KC and interstitial cells were similar to those in normal skin (Plate 5B). However, in perivascular areas within the papillary dermis, endothelial cells were weakly positive and occasional (2.5 +/- 1.8%) large, oval, macrophage-like cells with strong cytoplasmic staining were present. At 72 hours (n=4), KC CD95-ligand expression was upregulated, and 19.2 +/- 12.7% of perivascular cells with moderate/strong cytoplasmic as well as membranous CD95-ligand expression was observed (Fig. 3.12 and Plate 5C). Cytoplasmic staining was present predominantly in large macrophage-like cells and endothelial cells. On day 7 (n=4), expression was significantly upregulated in perivascular areas (83.5 +/- 9.4% and Plate 5D). A majority of perivascular cells showed strong cytoplasmic CD95-ligand expression and a majority of cells not expressing cytoplasmic CD95-ligand showed surface staining. After 14 days however, there was a significant reduction in both proportions of cells (34.2 +/- 25%, n=5)) and the intensity with which they expressed CD95-ligand in

perivascular infiltrates (Fig.3.12 & Plate 5E). Expression was markedly down-regulated both in epidermal KC and perivascular areas in 3/5 subjects. In 2/5 subjects weaker, but predominantly cytoplasmic expression, remained in up to 50-60% of perivascular cells, particularly in the mid and lower reticular dermis. The distribution of positive cells was more patchy than at 7 days with areas of completely negative cells within these infiltrates. Overall analysis revealed that there were significant differences between numbers of perivascular cells expressing cytoplasmic and/or surface CD95-L at the five time points studied (ANOVA $p<0.0001$). Further, numbers at each time point were significantly different to those at the preceding and subsequent time points.

These findings confirm that KC constitutively express CD95-L *in vivo* (107), and show that expression is upregulated by 72 hours and variably reduced at day 14 after intradermal PPD. Macrophages appear to be a major source of CD95-L, since cytoplasmic staining is detectable as early as 12 hours, but maximal 7 days after challenge. In T cells, both cytoplasmic and membranous CD95-L expression were maximal at day 7 and reduced by day 14. The antibody used stains both soluble and membranous forms of CD95-L and the relative contributions of each of these forms to CD95-mediated apoptosis in MR is unclear.

Nevertheless, the apoptosis which was observed on day 7, coincided with peak CD95-ligand expression, suggesting that CD95/CD95-ligand interactions may account for at least a proportion of death observed at the height of the response. However, the significant downregulation of CD95-ligand expression at day 14 suggests that it is unlikely that CD95/CD95-ligand interactions were the main trigger for apoptosis during the resolution phase.

Plate 5 CD95 ligand expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of CD95-L in normal skin and at different time points after intradermal PPD (x400 magnification)

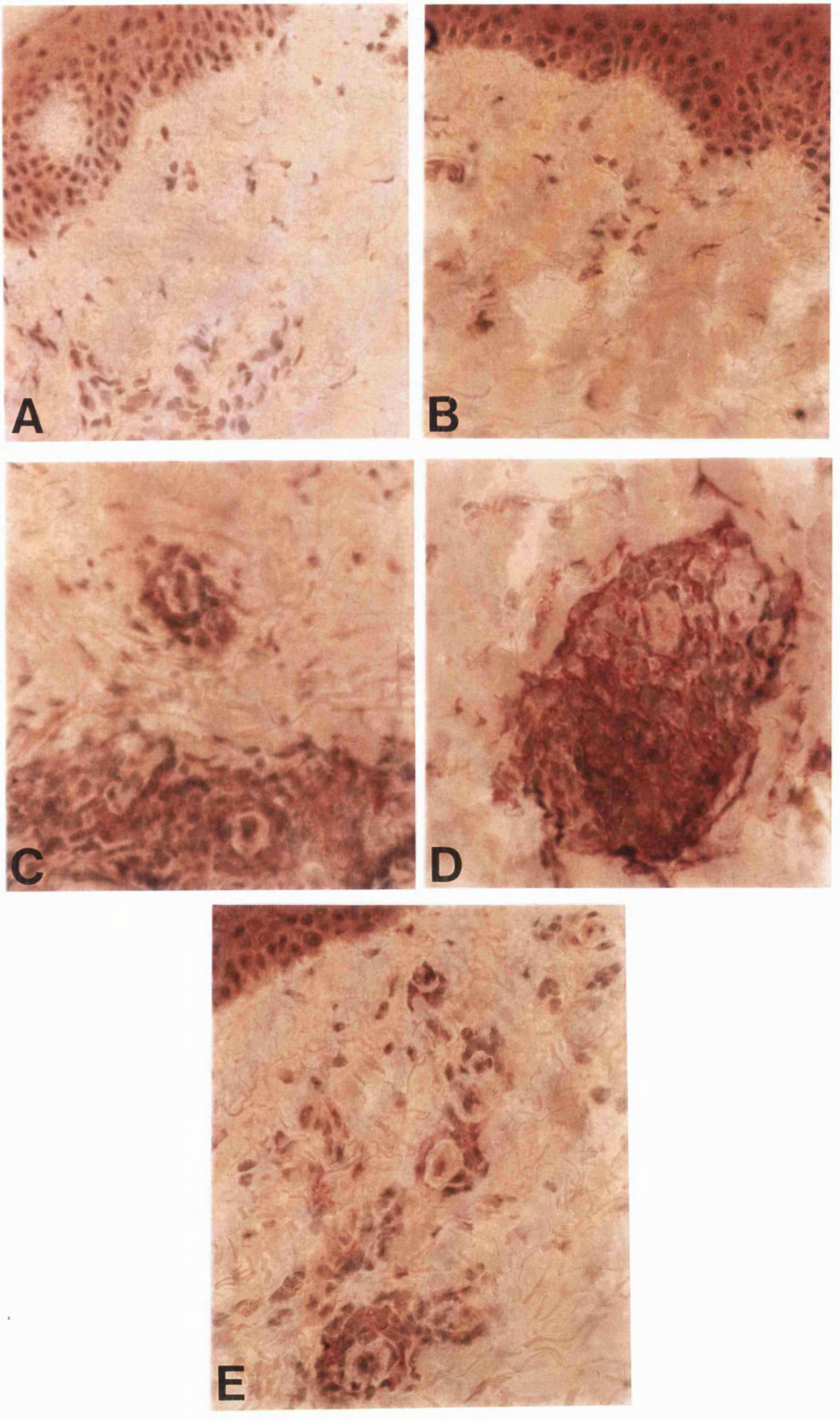
A Weak CD95-L expression in keratinocytes in normal skin. **B** Occasional positive endothelial and interstitial cells and upregulated expression in keratinocytes 12 hours after challenge.

C Up-regulated expression in perivascular areas on day 3.

D Maximal perivascular CD95-L expression on day 7.

E Reduced perivascular expression on day 14..

PLATE 5



3.2.5.4 TNF-α expression

We additionally investigated expression of TNF-α since this cytokine has also been implicated in the induction of apoptosis (6,92,93).

TNF-α was expressed by epidermal KC and dermal vascular endothelial cells at all time points, in all subjects. In normal skin (n=3) moderate-strong cytoplasmic expression was present in occasional DC in the epidermis and the papillary dermis and, although numbers were small, in up to 60% of perivascular cells. 12 hours after intradermal PPD (n=3) distribution and intensity of expression in DC were similar to those in normal skin. In perivascular areas 50-60% of cells expressed TNF-α, and strong endothelial cell positivity was noted (Plate 6A). At 72 hours (n=3) marked up-regulation of TNF-α expression occurred. In the papillary dermis large numbers of DC with strong cytoplasmic expression were present. 80-90% of perivascular cells were positive, a large majority showing strong cytoplasmic staining (Plate 6B). On day 7 (n=3) the intensity and distribution of staining in perivascular areas was similar, with 75-98% of cells showing strong, predominantly cytoplasmic expression (Plate 6C). However, greater numbers of dendritic and spindle-shaped interstitial cells, scattered throughout the superficial and deep dermis, showed strong cytoplasmic TNF-α expression. At 14 days (n=3) considerable inter-subject variability occurred in the intensity and distribution of staining. Reduced TNF-α expression was observed in the papillary and upper reticular dermis, although moderate-strong positivity was still present in 40-95% of perivascular cells (Plate 6D). However, large numbers of positive dendritic and spindle-shaped interstitial cells were present in 2/3 subjects extending from the mid to lower reticular dermis

Plate 6 TNF-α expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of TNF-α at different time points after intradermal PPD (x400 magnification).

A TNF-α expression in keratinocytes, endothelial cells and some perivascular and interstitial cells at 12 hours.

B Up-regulated expression in interstitial dendritic cells and perivascular areas on day 3.

C Intense perivascular TNF-α expression and positive dendritic and spindle shaped cells in the reticular dermis on day 7.

D. Reduced expression in the upper dermis on day 14.

PLATE 6

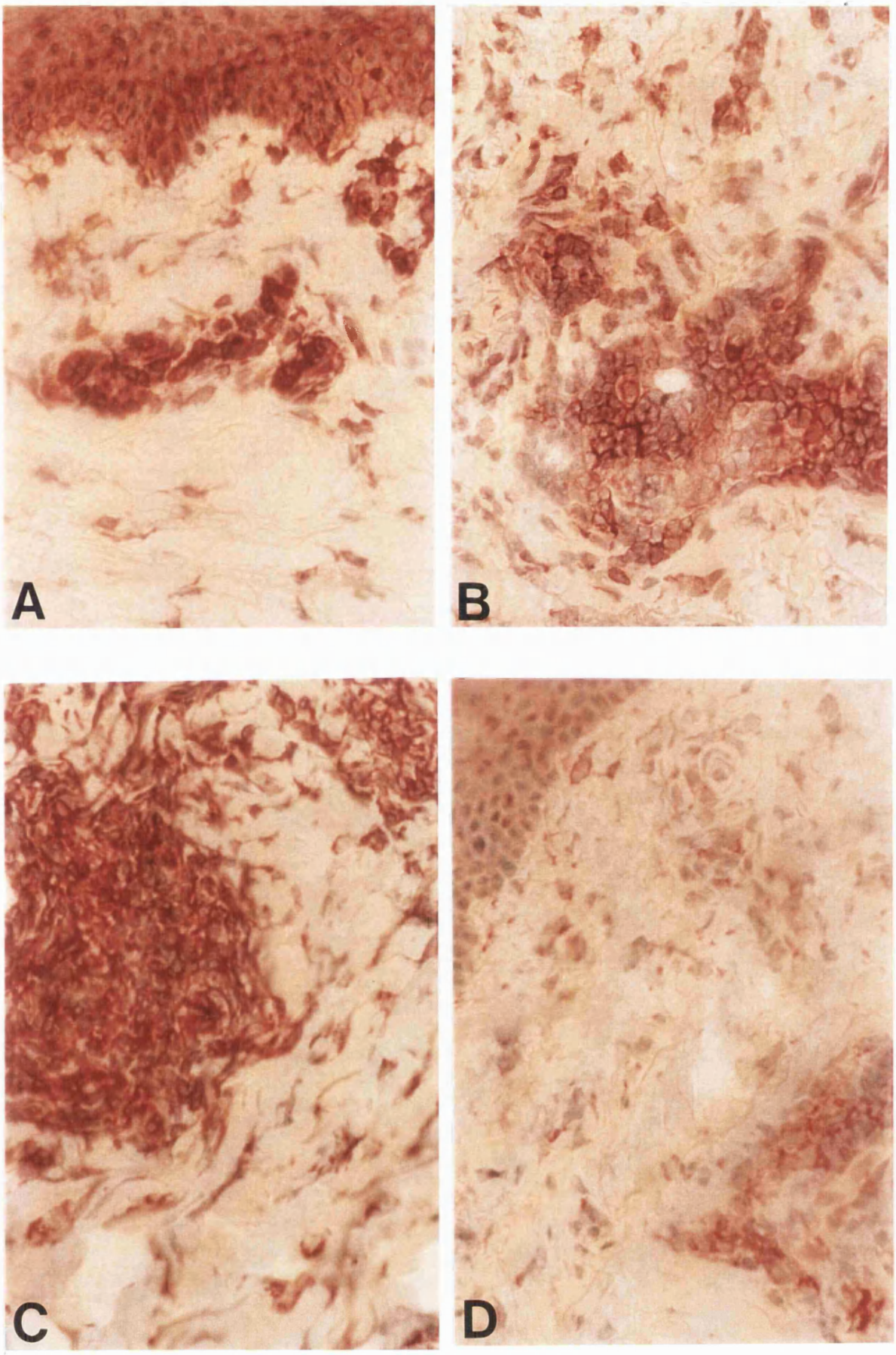


Table 3.2 CYTOKINE EXPRESSION IN PERIVASCULAR CELLS IN MANTOUX REACTIONS

CYTOKINE		DAYS AFTER INTRADERMAL PPD				
		0	**0.5**	**3**	**7**	**14**
TNF-α	% perivascular expression†	57.6%	52.9%	89%	86.3%	69.9%
	Range	50-60%	50-60%	80-90%	75-98%	40-95%
	Intensity of staining*	++	++	+++	+++	+ to ++
IL-15	% perivascular expression	1.9%	28.4%	77.6%	84.6%	7.9%
	Range	0-5%	15-50%	70-85%	75-90%	5-10%
	Intensity of staining	++	++	+++	++	+
IL-2	% perivascular expression	0%	0%	59.5%	65.7%	3.5%
	Range			50-70%	60-75%	0-5%
	Intensity of staining	-	-	++	+++	++
IL-7	% perivascular expression	7.1%	5.4%	33.4%	56.3%	36.4%
	Range	5-10%	2.5-10%	25-50%	50-65%	30-40%
	Intensity of staining	+	++	++	+++	++
IL-4	% perivascular expression	37.6%	38.9%	69.9%	75.2%	85.4%
	Range	25-50%	20-65%	60-80%	60-80%	80-95%
	Intensity of staining	++	++	+++	+++	+++/++
IFN-γ	% perivascular expression	22.2%	17.4%	50.8%	78.8%	61.7%
	Range	5-35%	5-35%	30-65%	70-95%	30-90%
	Intensity of staining	+	+	++	+++	+-+++
IL-6	% perivascular expression	23.3%	44.7%	48.5%	95.1%	93.7%
	Range	5-30%	30-60%	40-60%	90-98%	90-98%
	Intensity of staining	+++	++	++	+++	+++
TGF-β	% perivascular expression	73.8%	49.8%	47.5%	87.4%	88.3%
	Range	60-80%	40-60%	40-60%	85-90%	85-90%
	Intensity of staining	++	+	+	+++	++

† Both cells with membranous and cytoplasmic staining were taken into account when calculating the mean and range percentages of positive perivascular cells. A minimum of 3 subjects were investigated at each time point.

* Intensity of staining was graded as follows: - = none, + = weak, ++ = moderate, +++ = strong

In summary, KC and endothelial cells appear to constitutively express TNF-α, and although KC expression was variably downregulated at day 14, no obvious upregulation occurred in these sites during the course of the MR. In perivascular areas, peak TNF-α expression occurred between days 3-7 and at 14 days, proportions of strongly positive perivascular cells were reduced (summarised in Table 3.2). In contrast an increase in numbers of positive interstitial dendritic and spindle-shaped cells was observed on days 7 and 14. It appears therefore, that two "waves" of TNF-α production occurred during the MR. The first was mediated by perivascular and upper dermal macrophages, DDC and T cells and mirrored the height of the response. The second occurred later and may be mediated by fibroblasts or DDC in the mid and lower dermis.

Thus, although it is possible that this cytokine may contribute to the death observed at the peak of the response on day 7, it may not have a major role on the apoptosis observed on day 14, when perivascular TNF-α expression was reduced.

3.2.5.5 T cell Bcl-2 and Bax expression in a single subject

The results obtained suggested that apoptosis at day 7 was not due to changes in Bcl-2 expression. However, the significant decreased Bcl-2 expression on day 14 was likely to contribute to apoptosis at this time. To further investigate if apoptosis occurring after the peak of the Mantoux reaction was due to the decrease in Bcl-2 relative to Bax, we examined the kinetics of T cell accumulation together with expression of these molecules in six samples taken at different times from a single individual. This individual had a strongly positive response to PPD (>10 mm induration and >15 erythema index at 72 hours, Plate 2), and showed slightly accelerated kinetics of the reaction. Nevertheless, the trends in T cell numbers and Bcl-2 and Bax expression were similar to the pooled data from different individuals

at each time point. In this individual, T cell numbers were maximal 3 days after intradermal PPD and then progressively declined to day 14. In contrast, Bcl-2 expression appeared maximal at day 1 and had declined significantly by day 3, before the numbers of T cells were seen to be reduced (Fig 3.13). Bax expression remained relatively constant throughout the reaction (data not shown).

Thus, the fall in Bcl-2 preceded the fall in T cell numbers suggesting an association between the downregulation of this molecule and apoptosis at the later stages of the Mantoux reaction.

Since the infiltrating T cells in MR are predominantly CD4+ CD45RO+ cells (49,50 and this study), and this subset is known to be particularly sensitive to apoptosis (169,174), we used dual immunofluorescence studies to investigate Bcl-2 expression in CD8+ cells and compared it to that in the T cell population as a whole. Although the pooled data suggested that a majority of infiltrating CD8 cells are also CD45RO+, our studies in a single subject showed that Bcl-2 expression remained higher in this subset at day 14 (63% Vs 23%, Fig 3.14). This suggests that Bcl-2 downregulation, and by inference increased susceptibility to apoptosis, occurs primarily in the expanded CD4+ cell population that needs to be cleared at the end of the response.

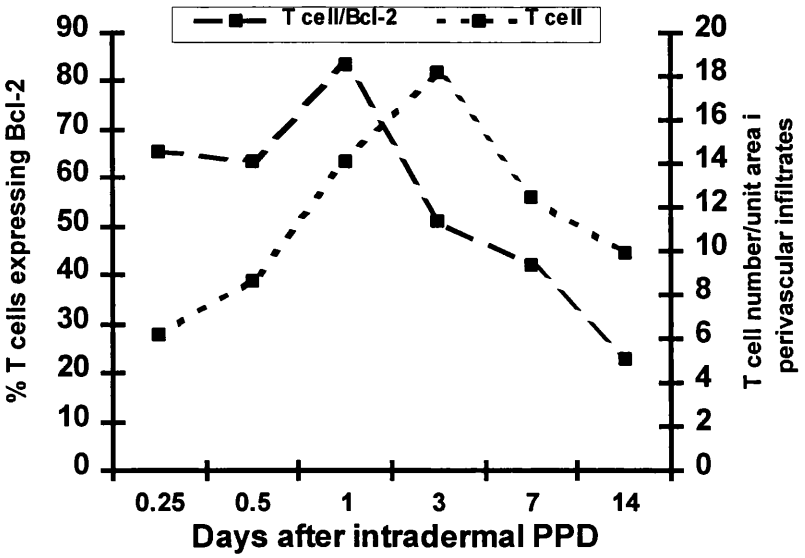


Figure 3.13 Mean T cell numbers and percentage of T cells expressing Bcl-2 in Mantoux reactions at different time points in a single subject.

An indirect immunoperoxidase technique and an image analysis system were used and cell numbers were counted per circular frame area centred on the 5 largest dermal perivascular inflammatory cell infiltrates. In dual IMF studies, the proportions of double positive cells were quantified in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

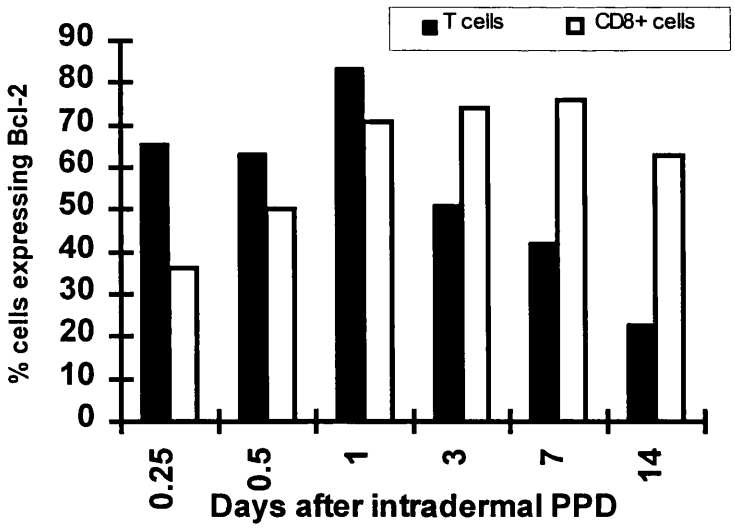


Figure 3.14 The proportions of T cells and CD8+ cells expressing Bcl-2 in perivascular infiltrates were compared in a single subject.

Dual IMF studies were performed using CD5+CD8 (T cells) and Bcl-2 and CD8 and Bcl-2 and proportions of double positive cells were counted in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

3.2.6 IL-2R γ CHAIN SIGNALLING CYTOKINES

Withdrawal of cytokines such as IL-2 and IL-15, which signal via the IL-2R γ chain, can induce T cell apoptosis by down-regulating their Bcl-2 expression relative to Bax (9). These cytokines, especially IL-15, are also involved in the induction of the immune response through promotion of T cell chemotaxis and proliferation (80-83,89,242). Thus IL-2R γ chain signalling cytokines may not only be involved in the induction of the DTH response, but their withdrawal may favour resolution by promoting T cell apoptosis. We therefore investigated whether changes in IL-15, IL-2, IL-7, and IL-4 expression occurred throughout the course of the Mantoux reaction (in a minimum of 3 subjects per time point). Although some variability occurred between individuals, the overall trends in cytokine expression at different time points were the same (summarised in Table 3.2).

3.2.6.1 Distribution of IL-15

In normal skin, epidermal keratinocytes (KC) showed moderate cytoplasmic staining with IL-15 in the prickle and granular cell layers and occasional strongly positive cells were seen in the basal layer (Plate 7A). Within the dermis only occasional cells (<5%) with cytoplasmic staining were seen in close proximity to blood vessels.

12 hours after intradermal PPD, staining intensity was greater in epidermal KC, particularly in the prickle cell layer (Plate 7B). Occasional strongly positive cells with a dendritic morphology resembling Langerhans cells, were also present in this area. In the papillary dermis intracytoplasmic IL-15 was present in numerous large, macrophage-like cells and occasional dendritic cells (DC), both within perivascular infiltrates in close proximity to lymphocytes, and in the interstitium (Plate 7B). In addition, up to 50% of perivascular T cells showed membrane staining with IL-15. At 72 hours, although fewer IL-15 positive KC were present, greater numbers of strongly

positive epidermal DC were seen. In the papillary and upper reticular dermis the distribution of IL-15 was the same as at 12 hours, but the staining intensity and number of positive cells were greater (Plate 7C). Thus, in the papillary dermis, numerous strongly positive, large macrophage-like cells and dendritic cells were seen in perivascular infiltrates, in close proximity to lymphocytes, a majority of which (>75%) showed surface IL-15 expression. In addition, moderate numbers of dermal interstitial cells expressed membrane bound IL-15. In the reticular dermis fewer dendritic or macrophage-like cells were seen, but a majority (>75%) of perivascular cells expressed membranous IL-15. At 7 days when T cell proliferation and cell numbers were maximal, fewer dermal cells with intracytoplasmic staining were seen, but a majority (75-90%) of perivascular cells still expressed membrane bound IL-15 (Plate 7D). Epidermal KC IL-15 expression was reduced although occasional positive DC remained.

By day 14 KC IL-15 expression was markedly down-regulated and absent in a majority of sections, although occasional positive DC remained. Only very occasional perivascular macrophage-like cells were seen and minimal or no membranous IL-15 was present on perivascular lymphocytes (Plate 7E).

In summary, early upregulation of IL-15 expression occurred (12 hours) within macrophages and epidermal KC. The maximal response was seen at 72 hours when numerous LC, perivascular macrophages and DC expressed cytoplasmic IL-15, and maximal intensity of membrane staining on perivascular lymphocytes was present. Lesional IL-15 expression was markedly reduced by day 14 (Table 3.2).

Plate 7 IL-15 expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of IL-15 in normal skin and at different time points after intradermal PPD (x 400 magnification).

A Constitutive IL-15 expression in keratinocytes in normal skin.

B Upregulation of expression in keratinocytes and dendritic cells 12 hours after intradermal PPD.

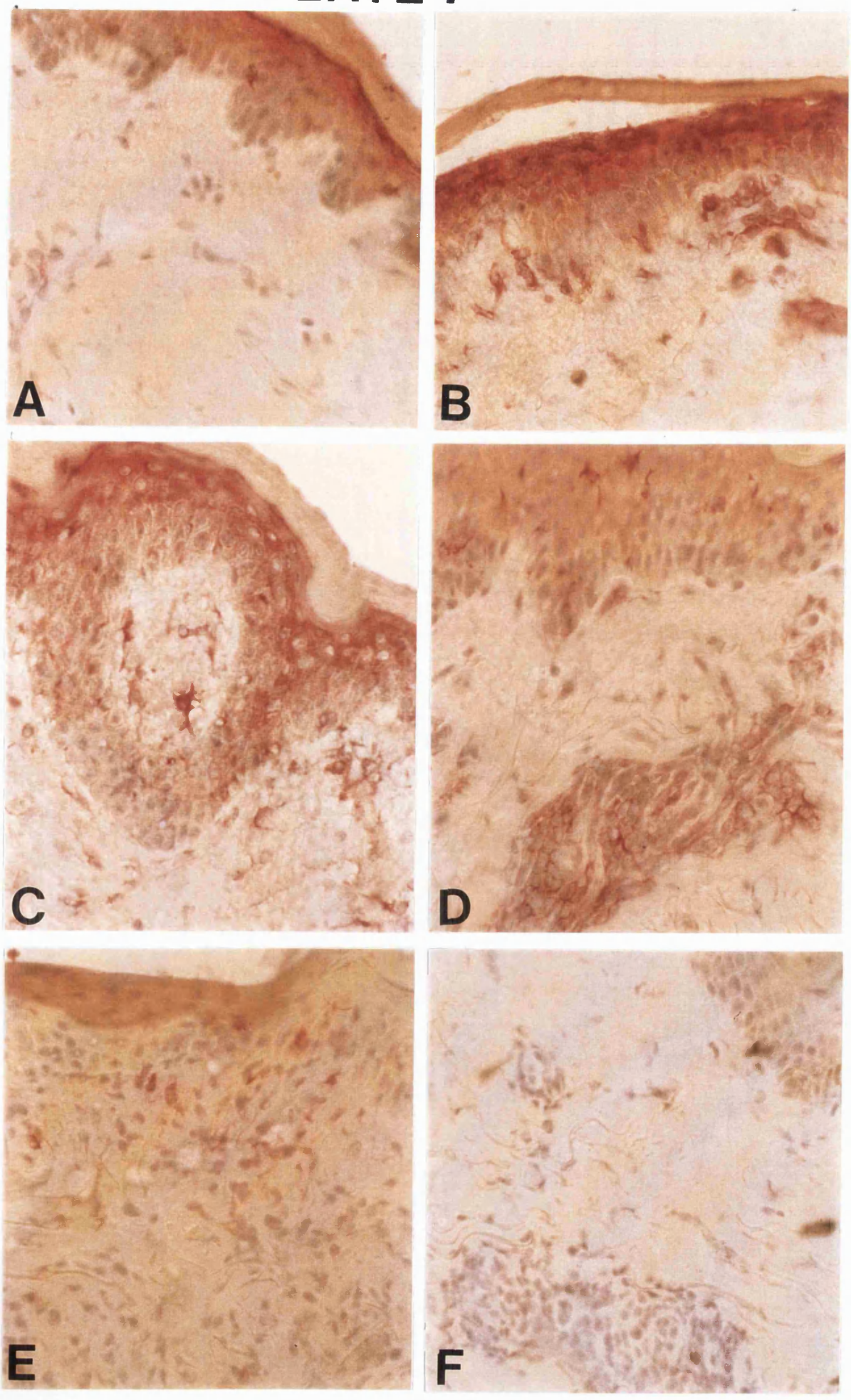
C Maximal IL-15 expression on day 3.

D On day 7 keratinocyte expression was reduced, but a majority of perivascular cells showed surface staining.

E Marked downregulation of expression on day 14.

F Staining with an irrelevant isotype specific control (mouse IgG1)

PLATE 7



3.2.6.2 Distribution of IL-2

Normal skin showed no staining with IL-2. At 12 hours after intradermal PPD, occasional (<5%) dermal interstitial T cells expressed cytoplasmic IL-2, but no positive cells were seen in perivascular infiltrates or the epidermis (Plate 8A). By 72 hours there was marked cytoplasmic expression in a majority (>50%) of interstitial T cells and occasional positive cells were seen penetrating the epidermis (Plate 8B). Within perivascular infiltrates, 50-60% of cells showed membrane staining with IL-2, and a small percentage expressed cytoplasmic IL-2. Expression appeared maximal at 7 days when both the numbers of T cells present and the extent of proliferation were at their highest levels. At that time point >75% of interstitial cells and many perivascular cells expressed cytoplasmic IL-2 (Plate 8C) and the majority of the remaining perivascular T cells expressed membrane bound IL-2. Whereas at 72 hours cytoplasmic staining was predominantly in large cells at 7 days it was also present in numerous small round perivascular cells. By day 14 after intradermal PPD only occasional large oval-round cells (<5%) interstitial and perivascular T cells expressed cytoplasmic or membrane bound IL-2 (Plate 8D).

This decrease in IL-2 at 14 days coincided with the decrease in cell numbers in the resolving Mantoux reactions (Table 3.2).

3.2.6.3 Distribution of IL-7

In all sections, strong staining with IL-7 occurred in the basement membrane zone, both in the epidermis and hair follicle epithelium. This could have represented non-specific staining or positivity in basal keratinocytes.

In normal skin (n=3) weak cytoplasmic IL-7 expression was present in KC, and in 5-10% of perivascular cells in the papillary dermis (Plate 9A). No IL-7 expression was observed in interstitial cells or in the reticular dermis.

Plate 8 IL-2 expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of IL-15 in normal skin and at different time points after intradermal PPD (x400 magnification).

A Low levels of IL-2 expression were detected at 12 hours.

B Expression was up-regulated in interstitial lymphocytes on day 3.

C Maximal IL-2 expression was observed on day 7.

D On day 14 IL-2 expression was downregulated.

PLATE 8

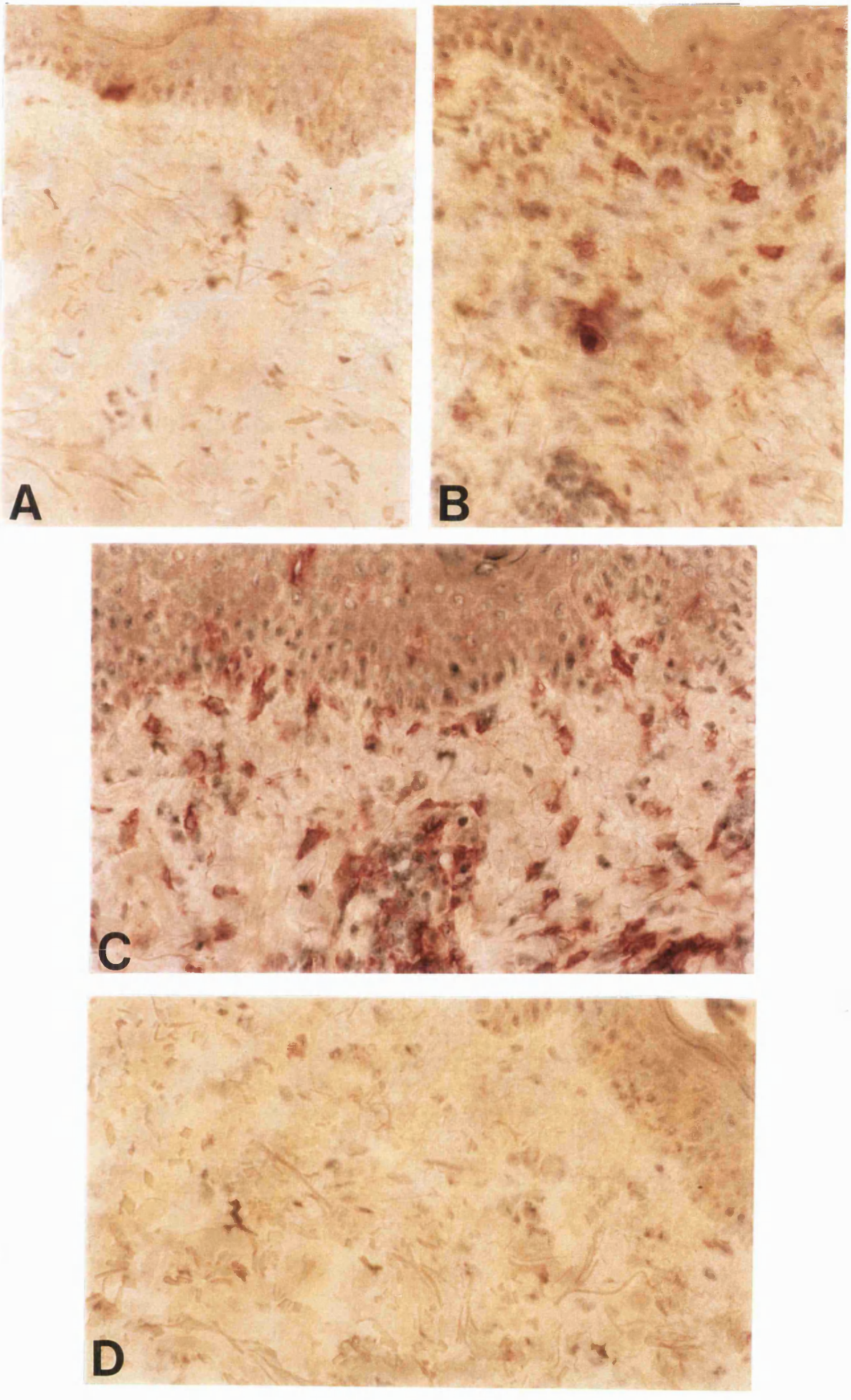


Plate 9 IL-7 expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of IL-7 in normal skin and at different time points after intradermal PPD (x400 magnification).

A IL-7 expressed in keratinocytes in normal skin. The strongly positive band visible at the dermo-epidermal junction may be non-specific or reflect staining in the cytoplasm of basal keratinocytes.

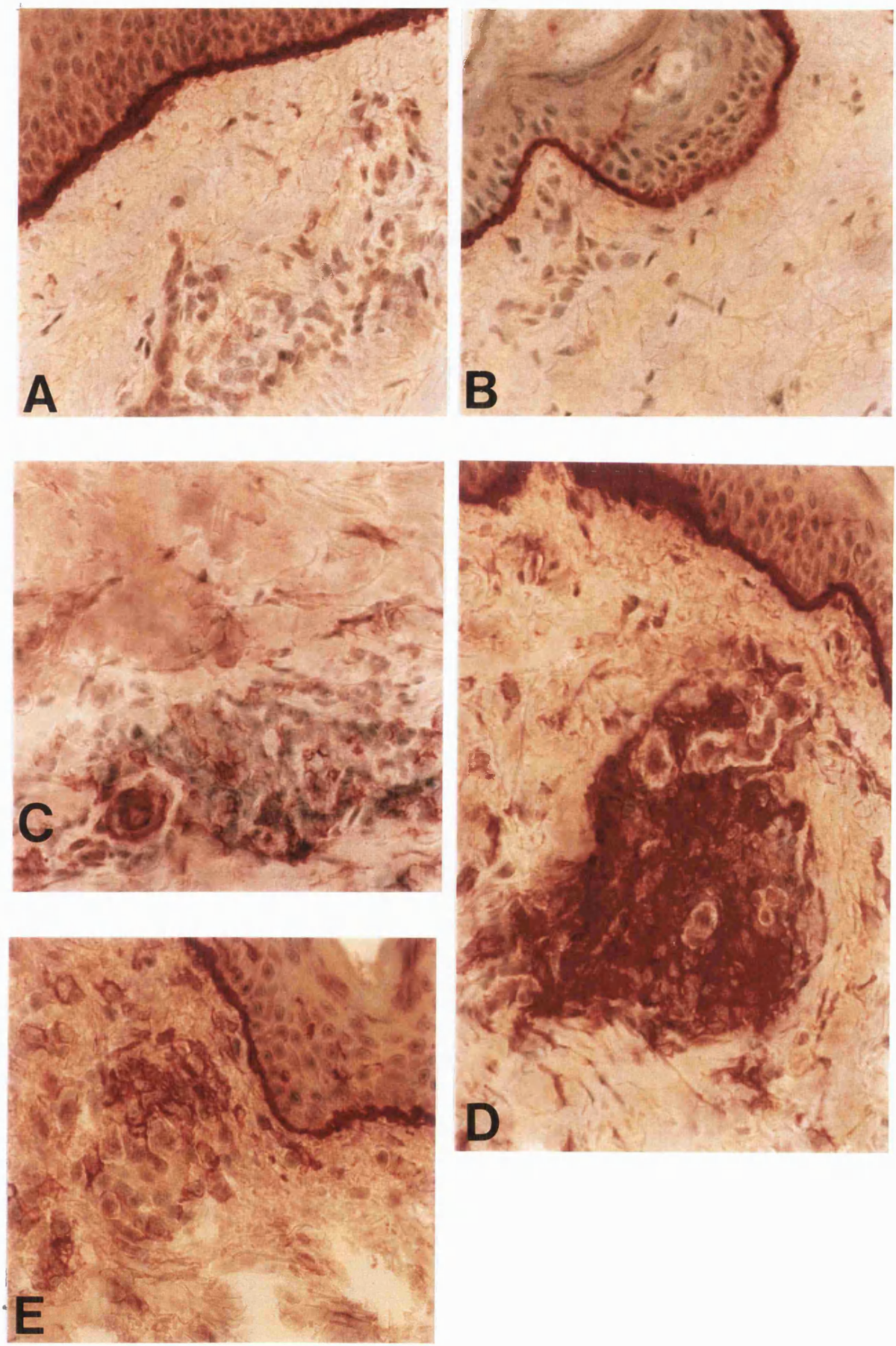
B Downregulated keratinocyte IL-7 expression 12 hours after challenge.

C Increased perivascular expression on day 3.

D. Maximal IL-7 expression on day 7.

E. Reduced IL-7 present on day 14.

PLATE 9



12 hours after intradermal PPD (n=4) epidermal KC IL-7 expression was downregulated (Plate 9B). In perivascular areas 2.5-10% of cells showed moderate-strong cytoplasmic IL-7 expression, whilst the majority of cells showed no staining. Positive cells were large with oval or round nuclei. At 72 hours (n=4) staining intensity in KC was reduced. IL-7 expression varied from one subject to another with 25-50% of perivascular cells showing moderate-strong cytoplasmic staining (Plate 9C). Little or no membrane staining was observed, and interstitial cells were negative. Maximal expression was detected on day 7 (n=4) when a majority of perivascular cells (50-65%) expressed IL-7 (Plate 9D). Although a proportion of lymphocytes showed membrane staining, perivascular IL-7 expression was predominantly cytoplasmic in large cells with oval/round nuclei. 14 days after intradermal PPD (n=4), although 30-40% of perivascular cells still expressed IL-7, overall, the intensity of staining and degree of cytoplasmic expression were reduced compared to 7 day specimens (Plate 9E).

In summary, KC IL-7 expression appeared downregulated after 12 hours, whilst maximal perivascular expression occurred at the time of maximal T cell proliferation on day 7 (Table 3.2). Conversely, as with IL-2 and IL-15, the decrease in IL-7 at 14 days coincided with the decrease in cell numbers in the resolving reactions.

3.2.6.4 Distribution of IL-4

IL-4 was constitutively expressed in KC in normal skin (Plate 10A) and upregulated on days 3 and 7 compared to other time points (n=4). 12 hours after challenge, strong staining was detected in cells with indistinct margins, scattered throughout the upper and mid dermis and in perivascular areas (Plate 10B). This pattern of staining was also observed with IL-6 and TGF-β (see below), and may be occurring in mast cells or neutrophils. However, it may also have been non-specific, and these cells were therefore excluded when estimating proportions of positive perivascular cells. At this time point, perivascular IL-4 expression was variable (20-65% of cells; Plate 10B).

Expression was upregulated in perivascular areas on day 3 with strong cytoplasmic and/or surface staining occurring in a majority of lymphocytes (60-80% of cells; Plate 10C). Intensity and distribution of staining were similar 7 days after challenge. By day 14 the intensity of IL-4 expression was slightly reduced perivascularly, however, in contrast to the results obtained with IL-2, IL-15 and IL-7 a majority (80-95%) of cells remained positive (Plate 10D and Table 3.2).

Early IFN-γ production is thought to be critical in the development of DTH responses (39). We therefore went on to investigate the kinetics of expression of this cytokine with a view to comparing it with that of IL-4.

3.2.7 DISTRIBUTION OF IFN-γ

In normal skin (n=3), epidermal KC showed strong cytoplasmic expression with IFN-γ and, as with IL-7, there was some accentuation of staining in the basement membrane zone. Dermal vascular endothelial cells also showed strong cytoplasmic IFN-γ expression (Plate 10E). A majority of interstitial cells were negative, although occasional cells with dendritic morphology expressing cytoplasmic IFN-γ were present just below the dermo-epidermal junction. Within perivascular areas 5-35% of cells showed membrane staining, but no cytoplasmic IFN-γ was present.

12 hours after intradermal PPD (n=3) the proportions and distribution of positive cells was similar to that in normal skin. At 72 hours (n=3) endothelial cells remained positive, KC expression appeared down-regulated, and a majority of interstitial cells were negative. Greater proportions (30-65%) of perivascular cells expressed IFN-γ, particularly in larger infiltrates. In a majority staining was membranous although increased numbers (compared to normal skin and 12 hour specimens) showed cytoplasmic expression (Plate 10F).

Plate 10 IL-4 and IFN-γ expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of IL-4 and IFN-γ in normal skin and at different time points after intradermal PPD (x400 magnification).

A IL-4 in keratinocytes and occasional perivascular cells in normal skin.

B Strongly positive cells scattered throughout the upper dermis at 12 hours.

C. Upregulated perivascular expression on day 3.

D Slightly reduced IL-4 positivity in a majority of perivascular cells on day 14.

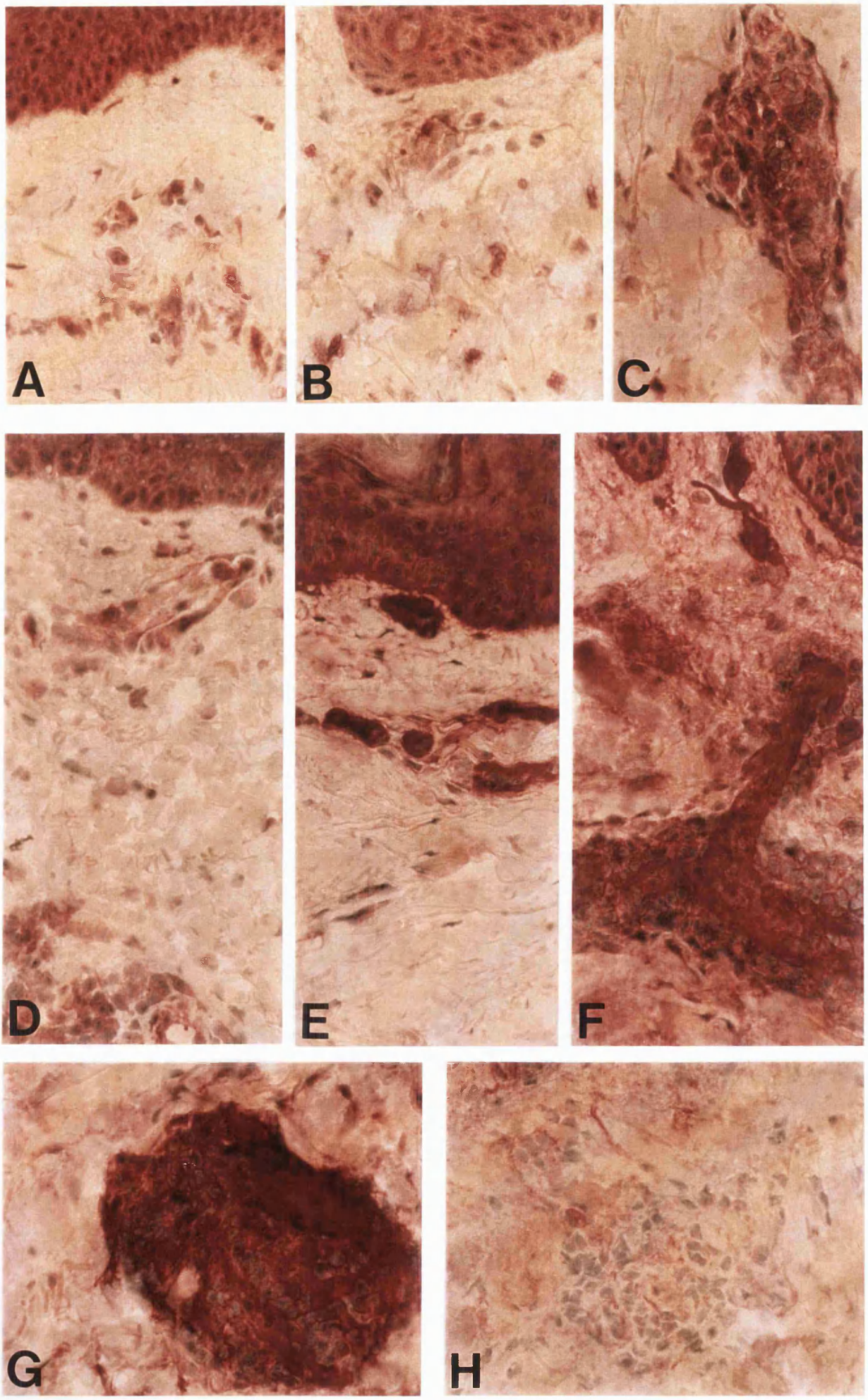
E IFN-γ expression in keratinocytes and endothelial cells in normal skin.

F. Reduced expression in keratinocytes and up-regulated endothelial cell and perivascular expression on day 3.

G. Maximal perivascular expression on day 7.

H. Markedly downregulated expression occurred in 2/4 subjects on day 14.

PLATE 10



On day 7 (n=3) perivascular IFN-γ expression was maximal with a majority of cells (60-95%) showing cytoplasmic staining (Plate 10G). Cytoplasmic staining was particularly marked in cells at the centre of the infiltrates, whereas negative or surface staining cells were present more peripherally.

14 days after intradermal PPD (n=4) greater inter-subject variability occurred with 30-90% of perivascular cells expressing IFN-γ . In 2/4 subjects marked downregulation occurred (Plate 10H). However, although smaller proportions of cells expressing cytoplasmic IFN-γ were present, in 2/4 subjects intensity of staining and proportions of cells with membrane staining remained high (70-90%).

In summary IFN-γ appeared to be constitutively expressed in KC, and downregulated during the course of the MR. In contrast, IFN-γ expression in lymphocytes and monocytes/macrophages was strongly upregulated and maximal on day 7. During the resolution phase, on day 14, expression was variably reduced (Table 3.2).

3.2.8 DISTRIBUTION OF IL-6

IL-6 was used as a control cytokine, to further investigate whether the marked reduction in γ chain cytokine expression on day 14 was specific to IL-2, IL-15 and IL-7. This cytokine has previously been identified in blister fluid from PPD induced DTH reactions (49), and in addition to its T cell co-stimulatory effects (243), may be involved in upregulation of the cutaneous lymphocyte antigen (CLA) on CD4+ CD45RO+ responsible for this subsets' ability to home to skin (50).

In normal skin, IL-6 was weakly expressed in epidermal KC, and showed strong cytoplasmic expression in DC and a minority (5-30%) of perivascular cells in the papillary dermis (Plate 11A).

Plate 11 IL-6 expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of IL-6 in normal skin and at different time points after intradermal PPD (x400 magnification unless otherwise stated).

A. Weak IL-6 expression in keratinocytes and interstitial cells in the papillary dermis in normal skin.

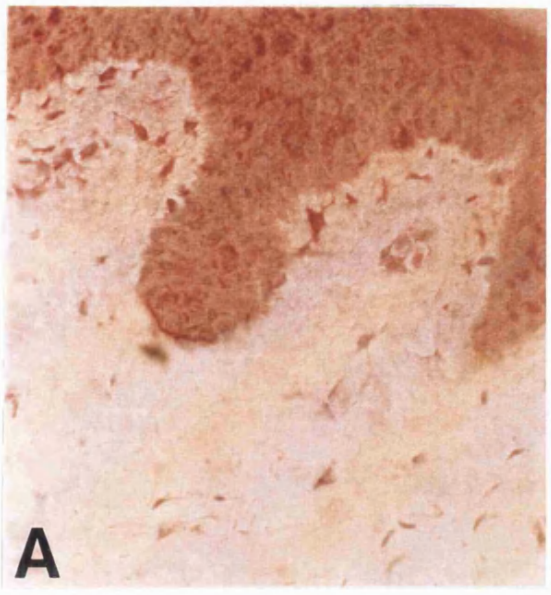
B. Strong cytoplasmic expression in perivascular and interstitial cells at 12 hours,(x250 magnification).

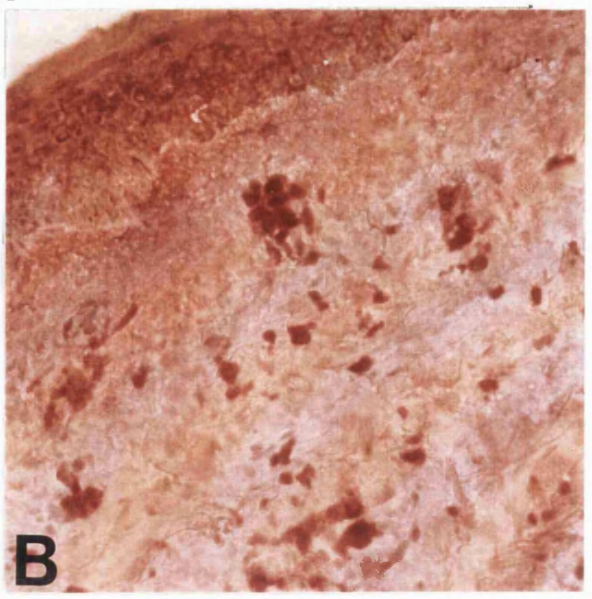
C. Increased perivascular surface staining detected on day 3.

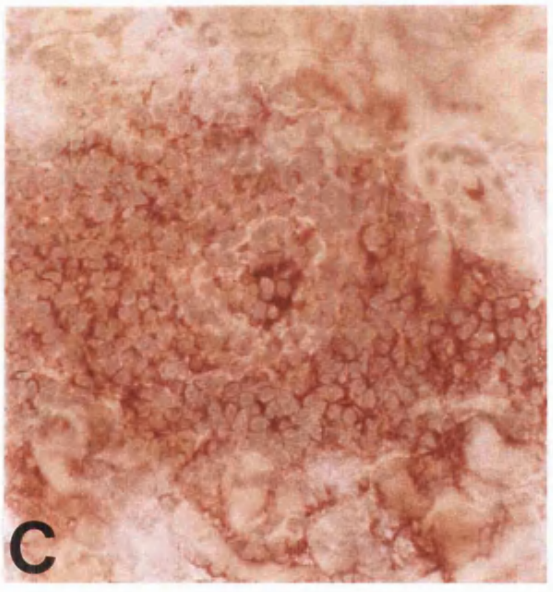
D. Up-regulated perivascular IL-6 expression on day 7.

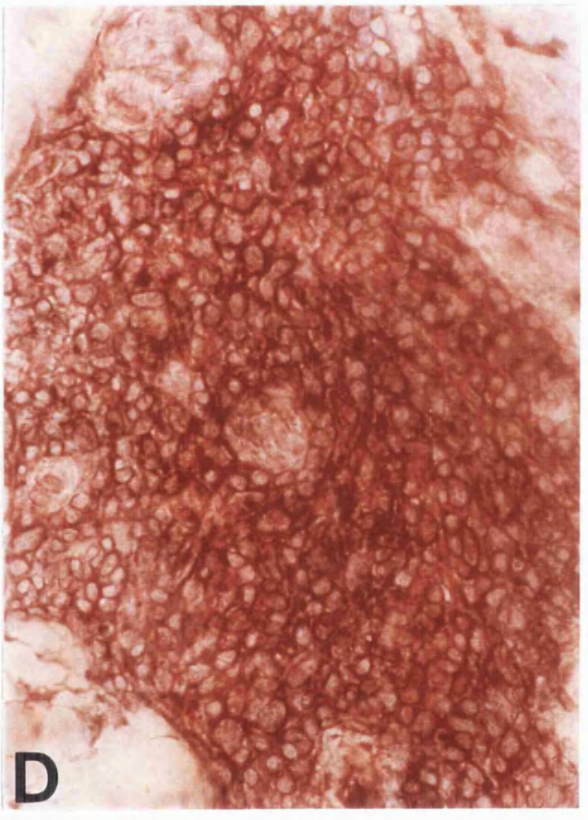
E. High perivascular IL-6 levels and numerous positive interstitial dendritic and spindle cells on day 14.

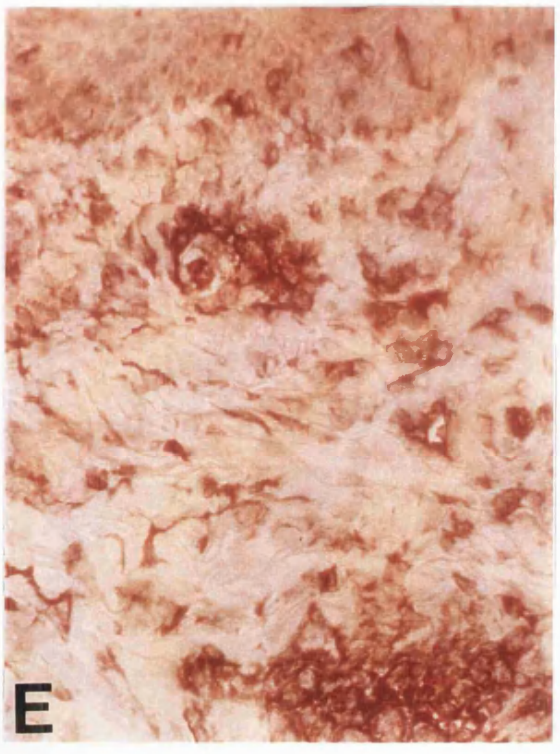
PLATE 11











12 hours after intradermal PPD epidermal KC IL-6 expression was unchanged and fewer positive DC were present in the papillary dermis. However, as with IL-4, staining was observed in numerous large cells with indistinct margins thought to be mast cells, both in and between perivascular areas in the upper dermis (Plate 11B). This pattern of staining may also have been non-specific and these cells were therefore excluded when estimating proportions of positive perivascular cells. At 72 hours occasional epidermal and dermal DC expressed cytoplasmic IL-6. Further, greater proportions of perivascular lymphocytes showed moderate membrane staining and large oval perivascular macrophage or blast-like cells expressed cytoplasmic IL-6 (Plate 11C). 7 days after challenge IL-6 expression was markedly up-regulated in epidermal KC, dermal DC and perivascular areas compared to the 12 and 72 hour time points. Numerous strongly positive DC and spindle-shaped cells were present in the papillary dermis, and in perivascular areas 75-98% of lymphocytes expressed cytoplasmic and/or membranous IL-6 (Plate 11D, Table 3.2).

At day 14 KC IL-6 expression was markedly reduced compared to day 7. However, membranous and cytoplasmic staining remained intense in perivascular lymphocytes, with slightly more cells expressing cytoplasmic IL-6 than at 7 days. In addition, numerous positive dendritic and spindle shaped cells were present throughout the dermis (Plate 11E).

These findings were in stark contrast to the reductions in IL-15, IL-2 and IL-7 expression which occurred at day 14 . Like TNF-α, IL-6 appeared to be expressed in dendritic and spindle-shaped, DDC and fibroblast-like cells both at the height of the response on day 7 and during the resolution phase. These results suggested that fibroblasts might be actively involved in the inflammatory response. Another cytokine which is known both to promote fibroblast growth and proliferation, and to limit the clonal expansion of T cells is TGF-β (244). We therefore went on to investigate the distribution of this

cytokine in the DTH response.

3.2.9 DISTRIBUTION OF TGF-β

In normal skin, TGF-β was expressed in epidermal KC, in upper dermal DC, and in endothelial cells. A majority of perivascular cells showed moderate positivity (60-80%; Plate 12A).

12 hours after intradermal PPD, basal KC no longer expressed TGF-β and the intensity of staining in suprabasal KC was reduced. A number of cells with indistinct margins, exhibiting strong TGF-β expression were present both in the interstitium, and in perivascular areas (Plate 12B). Some appeared to be endothelial cells. The identity of others is uncertain but they could represent mast cells or neutrophils. This pattern of staining may also have been non-specific and these cells were therefore excluded when estimating proportions of positive perivascular cells. Weak-moderate membrane staining was present on 40-60% of cells in perivascular areas. At 72 hours, epidermal KC TGF-β expression appeared further downregulated, but occasional suprabasal KC remained positive. Smaller numbers of the strongly positive cells mentioned above were present (Plate 12C). Although 40-60% perivascular cells showed membrane staining at this time point, levels of expression were very weak (Plate 12C). On day 7, epidermal KC TGF-β expression was markedly upregulated compared to the 12 and 72 hour time points. Expression was also maximal in the interstitium and in perivascular areas at this time point, with strong cytoplasmic and membrane expression on 85-90% of perivascular (Plate 12D) and numerous interstitial cells. 14 days after intradermal PPD epidermal KC expression of TGF-β was again markedly reduced, although occasional positive epidermal DC were present. In perivascular areas 85-90% of cells remained positive (Plate 12E, Table 3.2). In addition numerous spindle shaped cells with cytoplasmic staining were present in the deeper reticular dermis at this time point (Plate 12F).

Plate 12 TGF-β expression in DTH responses

A biotin/streptavidin method was used in to determine the cutaneous distribution of TGF-β in normal skin and at different time points after intradermal PPD(x 400 magnification unless otherwise stated).

A TGF-β in keratinocytes, as well as perivascular and interstitial cells in normal skin (x250 magnification).

B Reduced keratinocyte expression, but strongly positive cells in the dermis at 12 hours (x250 magnification).

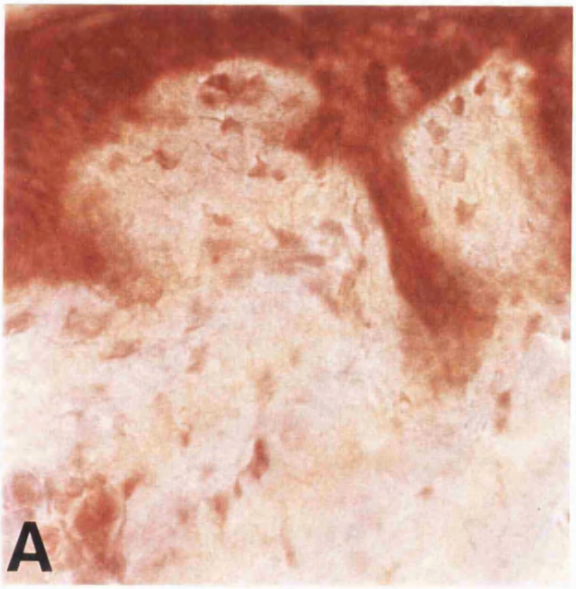
C Cytoplasmic staining in neutrophils or mast cells in perivascular areas on day 3.

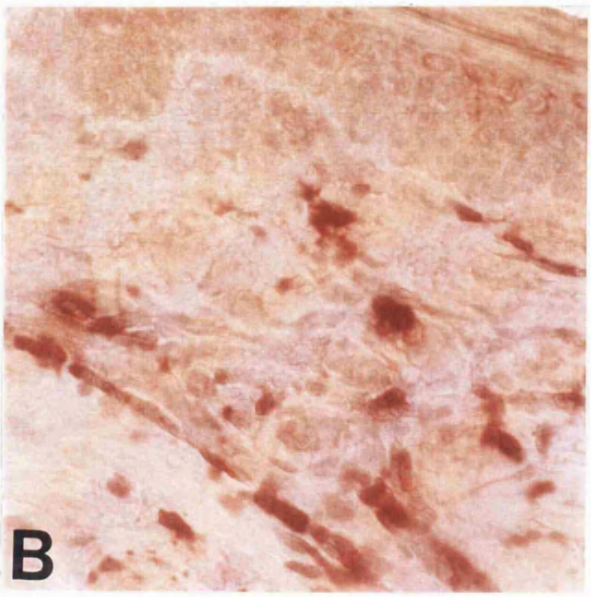
D Upregulated perivascular surface staining on day 7.

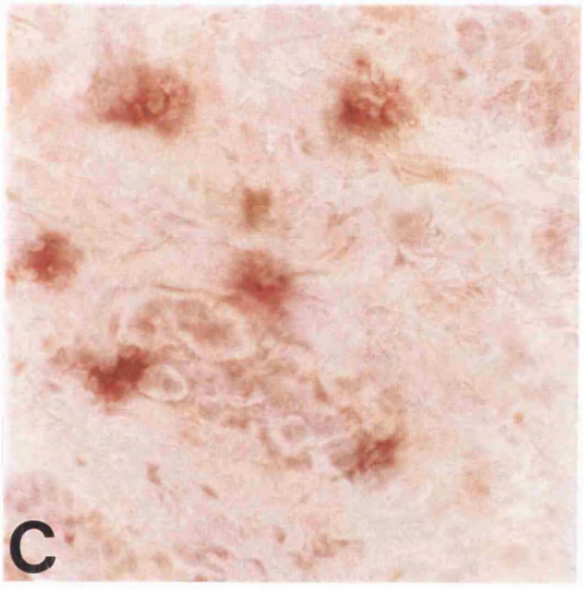
E Moderate reduction in intensity of perivascular expression on day 14.

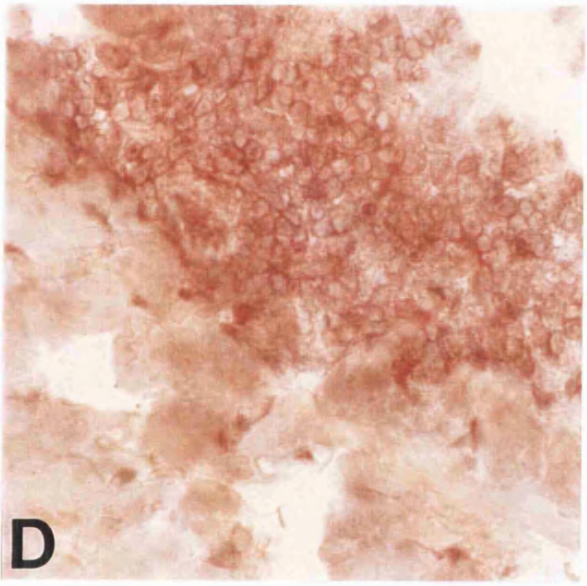
F Positive spindle-shaped and dendritic cells detected deeper in the dermis on day 14.

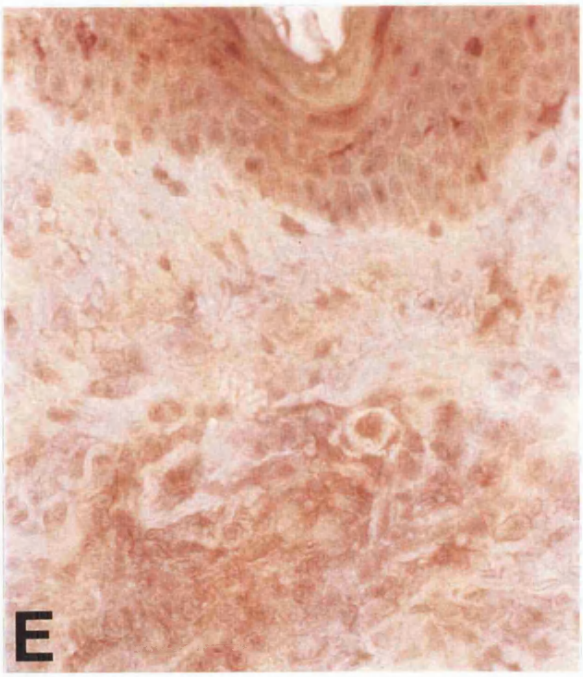
PLATE 12

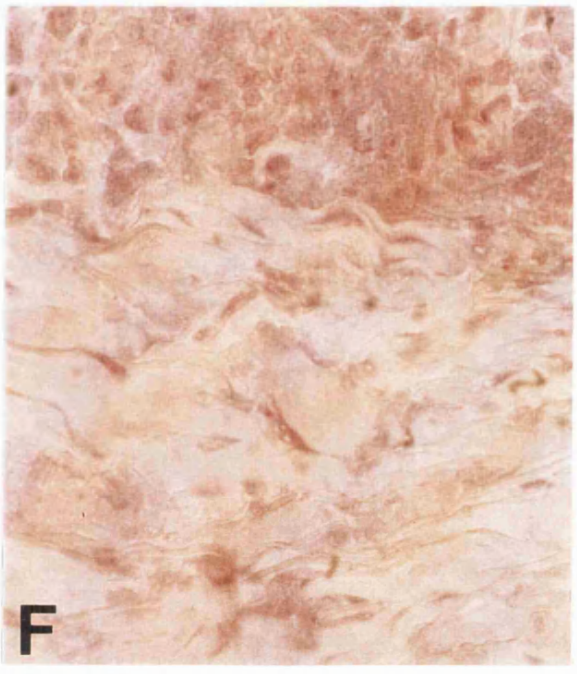












In summary, TGF-β was constitutively expressed by KC and downregulated in this area at all time points except day 7. Surface staining also appeared down regulated on perivascular T cells at 12 and 72 hours, but was markedly upregulated on day 7 and remained high on day 14. As with IL-6 and TNF-α, TGF-β was detected in dermal spindle-shaped cells, which may represent fibroblasts, on day 14.

The above findings suggested not only that increasing numbers of interstitial fibroblasts might be accumulating during the course of the DTH response, but also that their ability to up-regulate IL-6,TNF-α and TGF-β might be contributing to the inflammatory process. This is particularly pertinent since a fibroblast derived survival factor, recently identified in our laboratory as IFN-β, has been implicated both in the generation of T cell memory, and the persistence of T cell-mediated inflammation. We therefore went on to investigate fibroblast numbers, distribution and proliferation, and IFN-β expression in Mantoux reactions.

3.2.10 CHARACTERISTICS OF INFILTRATING FIBROBLASTS

3.2.10.1 Fibroblast numbers

- Dianova antibody

Fibroblasts were stained with the Dianova antibody (Dia 110, AS02), and an indirect immunoperoxidase technique. In perivascular areas positive cell numbers were difficult to assess accurately. This was not only because the antibody also stained endothelial cells, but because a degree of background staining was detected in these areas, reflecting either a lack of specificity of the antibody, or the large numbers of dendritic processes present. Despite this, we observed that the perivascular collections of positive cells were larger, and cells were more loosely arranged around vessels at later time points. In interstitial areas, in addition to positive spindle-shaped cells, this antibody stained large numbers of cells with stellate and dendritic

morphology, particularly at later time points.

In view of the difficulties in clearly identifying perivascular cells, positive interstitial cells in which a cell nucleus was clearly identifiable were counted per unit area in both the upper (papillary and upper reticular) and deep reticular dermis. Overall analysis revealed significant differences between the five different time points studied (ANOVA $p<0.0001$). Positive interstitial cell numbers increased significantly in both areas on day 14 after challenge, and this increase was greatest in the upper dermis (from 4.1 +/-2.7/UA in normal skin to 11.7 +/- 1.9/UA on day 14; Fig 3.15 and Plate 13A-C).

- Cross-reactivity of Dia 110 with Factor XIIIa and RFD1

Although the Dianova antibody has previously been shown not to cross react with CD68+ macrophages, CD1a+ LC, or α-smooth muscle actin in skin, the type of skin employed (i.e. normal or inflamed) in this report was not specified (235). Work on lymph node sections in our laboratory, and in both normal and inflamed skin in this study, showed endothelial cell labeling. Furthermore, in inflamed skin, large numbers of positive interstitial dendritic cells were identified. Such cells resemble the population of bone marrow-derived Factor XIIIa+ (FXIIIa) cells with dendritic morphology previously reported in the dermis of both normal and inflamed skin (26,27). These dermal dendritic cells (DDC) have been shown to function efficiently as antigen presenting cells (27) and may therefore actively participate in the generation of the DTH response. We were therefore interested to know whether the Dia110 antibody was also staining this population of cells. Dual immunofluorescence studies were performed in normal skin and 14 day DTH responses with Dia 110 and FXIIIa. In normal skin few positive interstitial cells were labeled with either antibody, but occasional double positives were observed. On day 14 of the DTH response, however, numerous Dia 110 and FXIIIa positive interstitial cells were present throughout the dermis. The close relationship between cells and dendritic processes made it difficult to

quantify dual staining cells accurately. However, up to 30% of Dia110+ cells in these areas appeared to also be positive for FXIIIa. Approximately 50% of the FXIIIa+ cells appeared to be dual stained (Plate 13D). FXIIIa has previously been shown not to be expressed by Te7+ fibroblasts (26). To investigate whether it might be expressed on fibrocytes, labeling with anti-CD34 was performed in the same subjects. Indirect immunoperoxidase staining showed positivity in spindle cells in the mid and deep dermis both in normal skin and on day 14 of the DTH response (data not shown). In contrast in the papillary and upper reticular dermis, where FXIIIa+ DDC were present in large numbers on day 14, endothelial cell positivity was detected, but only very occasional interstitial or perivascular cells were labeled with CD34 (data not shown). It was therefore felt unlikely that significant co-expression occurred. Although it is possible that other fibroblast subsets may express FXIIIa, the above findings strongly suggest that the Dianova anti-fibroblast antibody also stains a proportion of DDCs.

In view of these findings, we additionally investigated whether there was any cross-reactivity of Dia 110 with RFD1+ cells. RFD1 stains a subpopulation of dermal cells with dendritic morphology, which have been shown to present antigen to, and be stimulatory for T cells, and which are thought to be derived from the macrophage lineage (234). Immunoperoxidase staining was performed in one representative subject per time point and showed that maximal numbers of perivascular RFD1+ cells were present on day 7. In contrast, in the upper dermal interstitium they were more numerous at 12 hours and on day 14 (data not shown). Dual immunofluorescence studies revealed 2 distinct populations of cells staining with RFD1 and Dia110 in normal skin, and 12 and 72 hours after intradermal PPD (Plate 13E). On days 7 and 14, however, a small proportion of double positive cells (5-10% of Dia110 + cells) was observed (Plate13F).

In conclusion, the Dianova "fibroblast specific antibody" was also shown to label a proportion of FXIIIa+ DDCs and RFD1+ cells in inflamed skin.

Plate 13 Fibroblast staining with Dia 110 and specificity of the Dia 110 antibody in DTH responses

A-C Fibroblasts were stained with an indirect immunoperoxidase technique and the Dia 110 anti-fibroblast antibody (x400 magnification).

A Few positive interstitial cells were present in normal skin. A majority of positive cells in perivascular areas were thought to be endothelial cells.

B Increase in positivity in perivascular and interstitial areas on day 3.

CMaximal numbers of positive interstitial cells present in the upper dermis on day 14.

D-E Dual immunofluorescence studies were employed (x400 magnification).

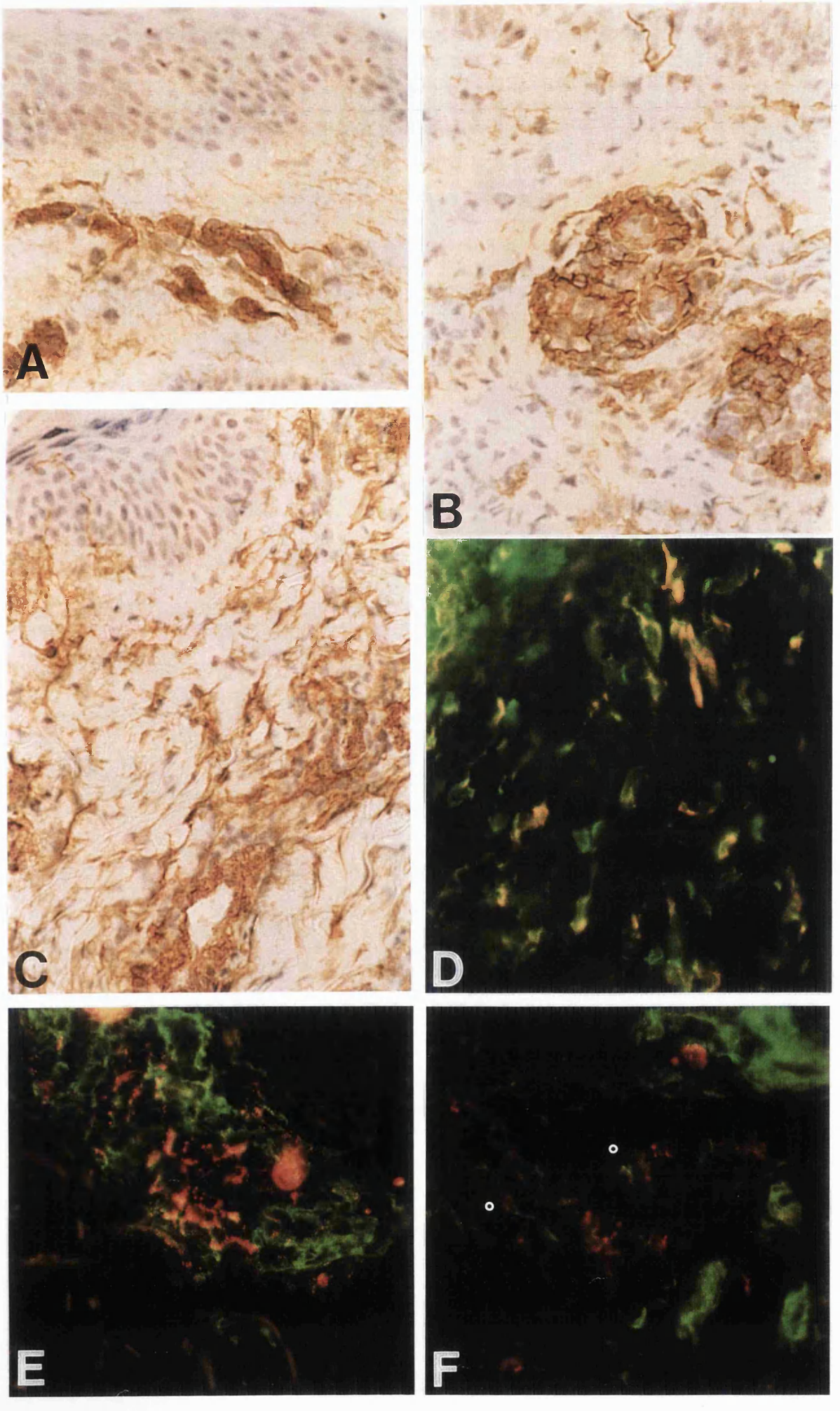
D Dia110+ cells were labeled with TRITC and FXIIIa+ cells with FITC. On day 14 up to 30% of Dia110+ interstitial cells (red) also stained with FXIIIa (green).

E & F RFD1+ cells were labeled with TRITC and Dia110+ cells with FITC.

E Perivascular infiltrate on day 3, showing 2 distinct populations of cells stained with RFD1(red) and Dia110 (green).

F On day 14, 5-10% of Dia110+ interstitial cells (green) were also positive for RFD1 (red).

PLATE 13



- Staining with other anti-fibroblast antibodies

In order to further quantify numbers of infiltrating fibroblasts, immunoperoxidase studies were performed with two other anti-fibroblast antibodies. Sufficient material was available to investigate their expression in one representative section per time point. These antibodies appeared to stain slightly different cell populations. The LHF5 antibody is known to be expressed by human adult and post-mitotic fibroblasts and also stained the basal layer of epidermal KC in all sections examined. In interstitial areas positive spindle shaped and large cells with more granular cytoplasmic staining were observed. The latter were also occasionally observed in perivascular areas. The LHF4 antibody stained predominantly spindle-shaped and dendritic cells in interstitial areas, whilst fewer positive perivascular cells and no cells with granular cytoplasmic staining were detected. Weaker keratinocyte and endothelial cell staining was also detected. Neither of these antibodies stained the large populations of stellate/dendritic cells observed in the interstitium with the Dianova antibody. Numbers of positive interstitial cells were quantified in the upper and deep dermis as before, and, although numbers were smaller, similar trends were observed. With LHF5, numbers of positive upper dermal interstitial cells were low in normal skin, and at 12 and 72 hours, but increased on days 7 and 14 of the DTH response (1+/- 0.7/UA in normal skin, 2.4 +/- 0.5/UA on day 7 and 4 +/- 2.1 on day 14, Fig 3.16 and Plate 14 A-D). Similarly, with LHF4, numbers of labeled cells increased from 1.4+/-0.5/UA in normal skin to 7.2+/-1.3/UA on day 7 and 8.2+/- 2.9/UA on day 14 (Fig 3.17 and Plate 14 E-H). Although insufficient material was available for further studies to be performed with these antibodies, the above data suggest that increasing numbers of fibroblasts were present at later time points during the DTH response.

Plate 14 Fibroblast staining with LHF5 and LHF4 antibodies in DTH responses

Indirect immunoperoxidase studies were used to stain fibroblasts with LHF5 **(A-D)** and LHF4 **(E-H)** in normal skin and at different time points after intradermal PPD (x400 magnification).

A Normal skin showing occasional interstitial LHF5+ cells (positivity was detected in basal keratinocytes).

B Increased numbers of large cells with granular cytoplasmic staining in the upper dermis on day 7.

C On day 14 large numbers of spindle and dendritic cells present in the upper dermis.

D Staining with LHF5 in the deep dermis in the same subject as in **C** above.

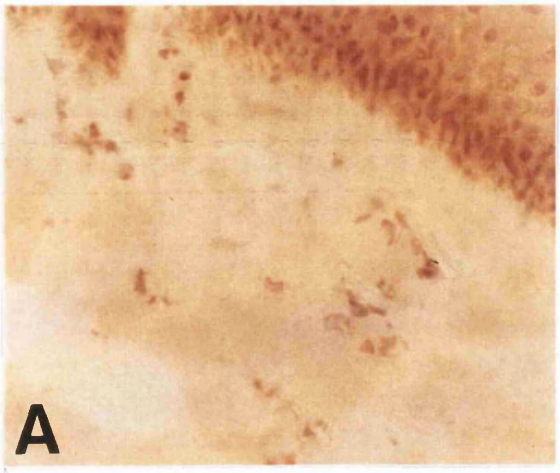
E Occasional LHF4+ interstitial cells were present in normal skin.

F Greater numbers of labeled interstitial cells in the upper dermis, on day 7.

G Day 14, the section is >5μm in thickness, however numerous positive cells are clearly visible just below the dermo-epidermal junction.

H Staining with LHF4 in the deep dermis, in the same subject as in **G** above, on day 14.

PLATE 14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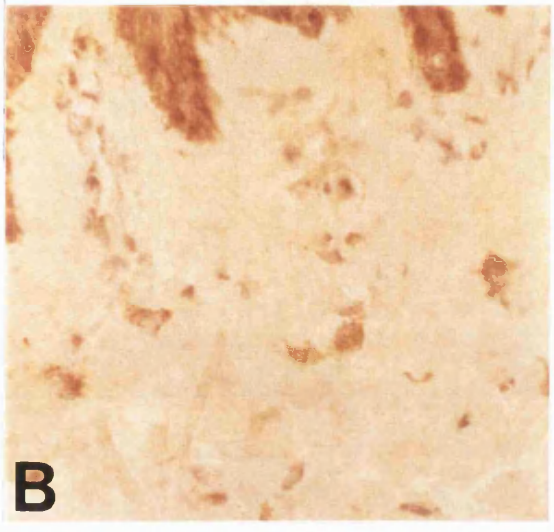
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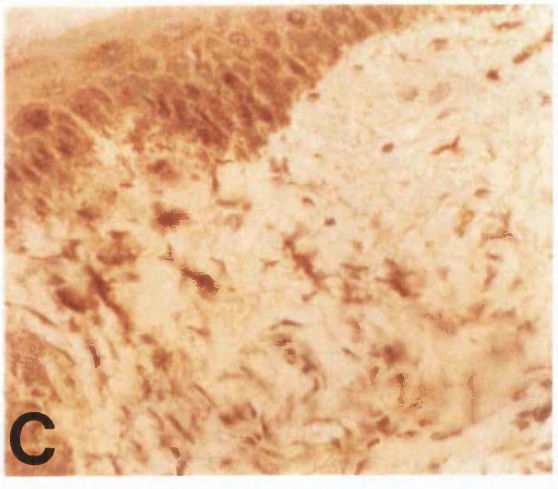
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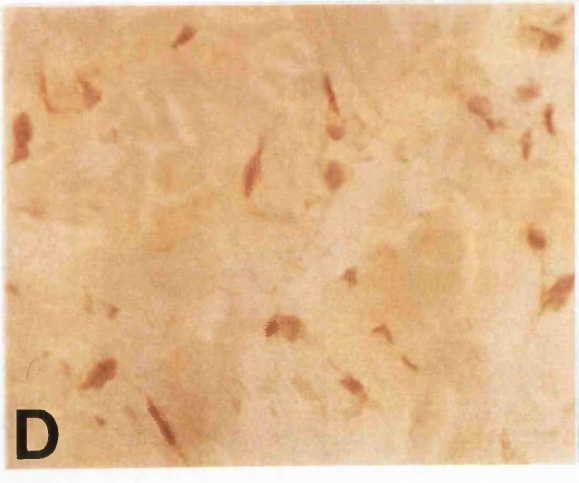
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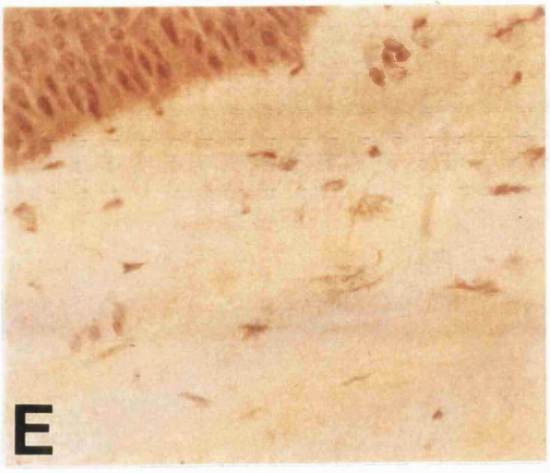
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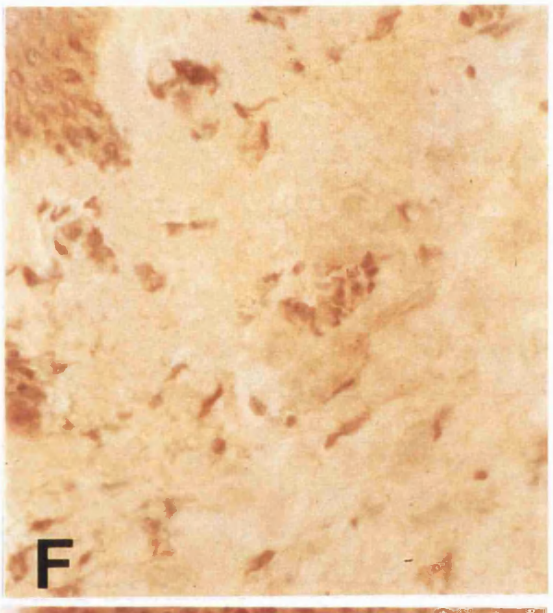
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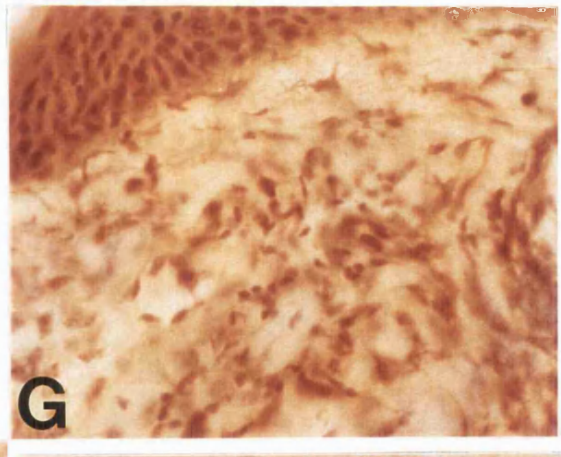

G


H

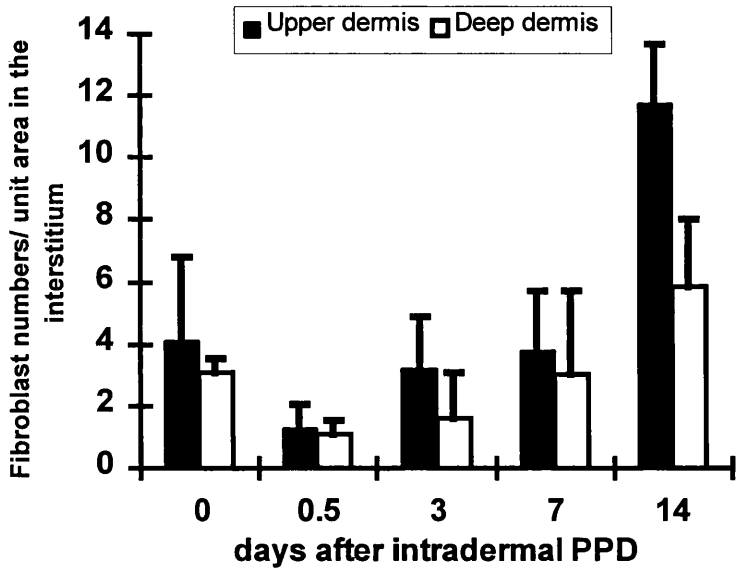


Figure 3.15 Mean Interstitial fibroblast numbers obtained with the Dianova anti-fibroblast antibody in Mantoux reactions.

Fibroblasts were stained by an indirect immunoperoxidase method (using Dia110) and quantified in each section, per rectangular frame area centred on areas in between perivascular infiltrates, both in the upper and in the deep dermis using an image analysis system, 5 times per section. Only cells with clearly identifiable nuclei were counted.(n=3 at each time point except day 14 when n=4.) Error bars indicate standard deviations.

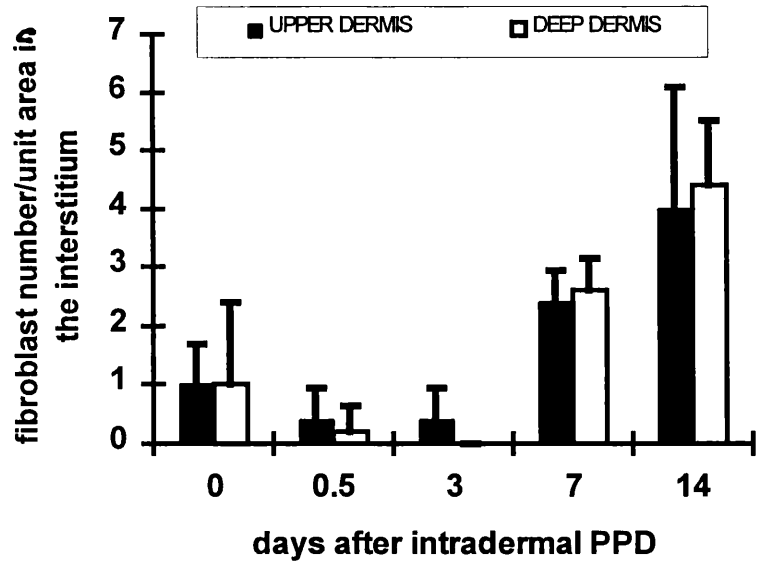


Figure 3.16 Mean Interstitial fibroblast numbers obtained with the LHF5 anti-fibroblast antibody in one representative subject per time point in Mantoux reactions.

Fibroblasts were stained with LHF5 by an indirect immunoperoxidase method and quantified per rectangular frame area, centred between perivascular infiltrates, in the upper and in the deep dermis using an image analysis system, 5 times per section. Only cells with identifiable nuclei were counted (n=1 at each time point). Error bars indicate standard deviations.

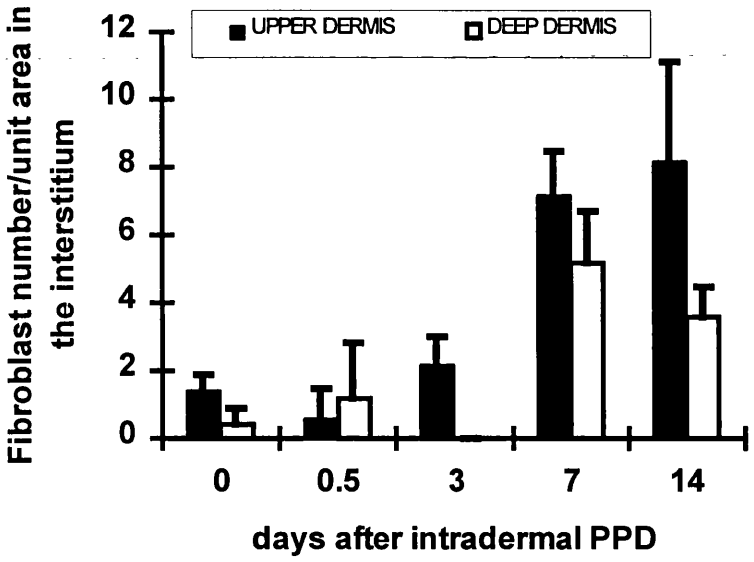


Figure 3.17 Mean Interstitial fibroblast numbers obtained with the LHF4 anti-fibroblast antibody in one representative subject per time point in Mantoux reactions.

Fibroblasts were stained with LHF4 by an indirect immunoperoxidase method and quantified per rectangular frame area, centred between perivascular infiltrates, in the upper and in the deep dermis using an image analysis system, 5 times per section. Only cells with identifiable nuclei were counted (n=1 at each time point, no positive cells were observed in the deep dermis on day 3). Error bars indicate standard deviations.

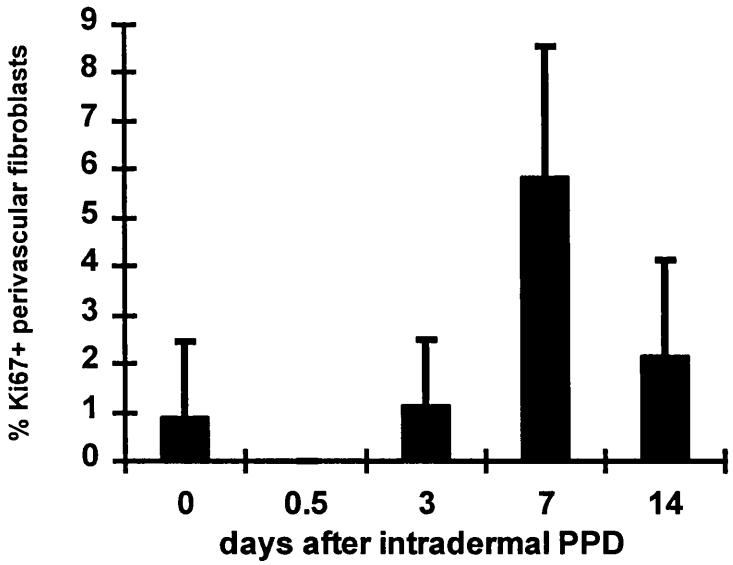


Figure 3.18 Proportions of proliferating Dia 110+ fibroblasts in perivascular infiltrates.

Dual IMF studies were performed using Ki67 and the Dia 110 anti-fibroblast antibody. Proportions of double positive cells were counted in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.(n=4 at 72 hours and 14 days, n=3 at other time points) Error bars indicate standard deviations.

3.2.10.2 Fibroblast proliferation

In order to ascertain whether in situ proliferation contributed to fibroblast accumulation during the course of the DTH response, dual immunofluorescence studies were initially performed with the Dianova antibody and Ki67. With this technique, it was possible to clearly distinguish between endothelial cells and other cells staining with Dia 110. The pattern of endothelial cell staining obtained was weaker and cytoplasmic, and the morphology of the cells was in sharp contrast to the strong membrane staining obtained on other Dia110+ cells, allowing the cell body and dendritic processes to be clearly identified. Proliferating non-endothelial cells were therefore counted in perivascular areas in these experiments. Proliferating basal KC were present in all the sections examined, providing a positive internal control.

Low levels of proliferation were detected in normal skin and at 12 hours (0.9 +/-1.6% and 0% Dia 110+ cells respectively). On day 3 a majority of proliferating perivascular cells were not fibroblasts, although 1.2 +/- 1.3% Ki67 positivity was observed in Dia 110+ cells. This proportion increased to 5.9 +/- 2.7% on day 7 (Fig 3.18 and Plate15A). Again a majority of the proliferating cells in these areas were not fibroblasts. On day 7 small numbers of interstitial Dia 110+ cells were in cycle and Ki67 positivity was also identified in a small proportion of endothelial cells. On day 14 rates of perivascular fibroblast proliferation were reduced to 2.2 +/- 1.9% . However, concomitantly with the increase in interstitial Dia 110+ cell numbers, slightly greater proportions of Ki67+ cells were observed in these areas on day 14 than on day 7. Overall analysis revealed significant differences in the proportion of proliferating perivascular Dia110+ cells between the five different time points (ANOVA p= 0.0001). The rate of proliferation on day 7 was significantly greater than at other time points.

In view of the cross-reactivity observed with this antibody and FXIIIa+ and RFD1+ cells, fibroblast proliferation was additionally investigated by performing dual immunofluorescence studies with Ki67 and each of the other two anti-fibroblast antibodies LHF5 and LHF4. Sufficient material was available to investigate one subject per time point. Although Ki67 positivity was present in basal keratinocytes in all sections and in perivascular areas at later time points, no fibroblast proliferation was detected in any of the sections examined with the LHF5 antibody. This is not surprising since the antibody is has been shown to stain quiescent post-mitotic fibroblasts. With LHF4 no fibroblast proliferation was observed either in normal skin, or at 12 and 72 hours after intradermal PPD. However, Ki67 positivity was detected in 5.75 +/- 4.4% of perivascular fibroblasts on day 7, and 3+/-4.2% of perivascular fibroblasts on day 14 (data not shown). The numbers of proliferating fibroblasts varied considerably from one perivascular area to another, and this is reflected in the standard deviations obtained. Although it was only possible to investigate one subject per time point, these data suggest that *in situ* proliferation does contribute to fibroblast accumulation at later time points during the DTH response.

3.2.11 DISTRIBUTION OF IFN-β

Since work in our laboratory has implicated fibroblast-derived IFN-β both in T cell survival and the generation of T cell memory (10, submitted), its expression was investigated during the course of the DTH response. IFN-β was expressed in epidermal KC in normal skin and downregulated in KC 12 hours after challenge (Plate 15B & C) . Thereafter expression increased and remained high throughout the epidermis up to day 14. Some cytoplasmic IFN-β expression was present in EC in normal skin, and expression was upregulated from day 3 to day 14. In perivascular areas, the intensity of staining was markedly increased from day 3 (Plate 15D). Surface expression of similar intensity was detected in lymphocytes on days 3, 7 and 14, but it was not possible to accurately determine whether perivascular fibroblasts

were positive with this technique. In contrast, cells with spindle and dendritic morphology expressing cytoplasmic IFN-β, were clearly detectable in the interstitium at these time points, and increased in number from 0.8 +/- 1.1 in normal skin to 5.9 +/- 1.8 on day 7 and 12.25 +/- 2.1 on day 14 (Plate 15E & F and Fig 3.19).

Overall analysis revealed significant differences in the numbers of interstitial cells expressing IFN-β between the different time points (ANOVA p=0.0001), and this number was significantly greater on days 7 and 14 compared to other time points. In addition, the intensity of IFN-β expression in interstitial cells appeared up-regulated on day 14 compared to other time points. These IFN-β+ interstitial cells were of similar morphology to those staining with the Dianova anti-fibroblast antibody. Furthermore, there was a close correlation between the number of interstitial IFN-β+ cells and those staining with this antibody at later time points (Fig 3.20). Activated fibroblasts may exhibit stellate or dendritic morphology, however, since we have shown a relative lack of specificity with the Dianova antibody, it is likely that other cells, in particular activated DDC, may additionally provide a source of IFN-β during the resolution phase of the DTH response.

In summary, fibroblast numbers appeared to increase significantly during the resolution phase, on day 14 of the response. Proliferation was greatest in perivascular areas and maximal on day 7. These findings suggest that *in situ* proliferation contributes to intralesional fibroblast accumulation. IFN-β expressing cells were particularly prominent in interstitial areas in the upper dermis and increased significantly on days 7 and 14. Intensity of staining appeared maximal on day 14. IFN-β+ and Dia 110 anti-fibroblast antibody + cells were of similar morphology. Although these cells may represent a different subpopulation of fibroblasts to those staining with the LHF4 and LHF5 antibodies, the relative lack of specificity of the Dianova antibody suggests that IFN-β may also be derived from activated DDC .

Plate 15 Fibroblast proliferation and IFN-β expression in DTH responses

A Using dual immunofluorescence studies, Ki67 was labelled with FITC and Dia110 with TRITC. Ki67 positivity (green) in perivascular Dia110+ cells including endothelial cells on day 7 (x400 magnification).

B-F A biotin/streptavidin method was used in to determine the cutaneous distribution of IFN-β in normal skin and at different time points after intradermal PPD (x400 magnification).

B IFN-β expression in keratinocytes and endothelial cells in normal skin.

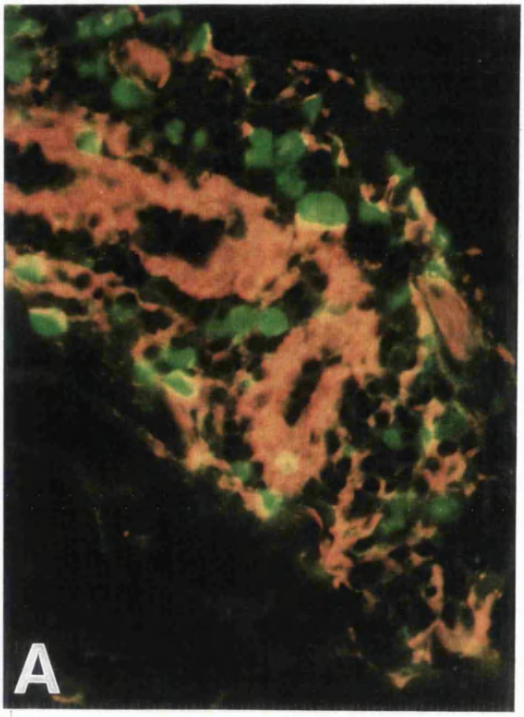
C Downregulation of expression at 12 hours.

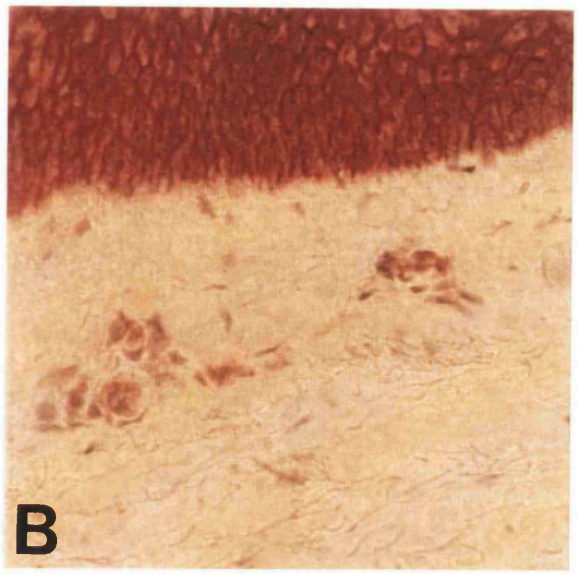
D Increased perivascular and keratinocyte IFN-β expression on day 3, and weak positivity in interstitial dendritic cells.

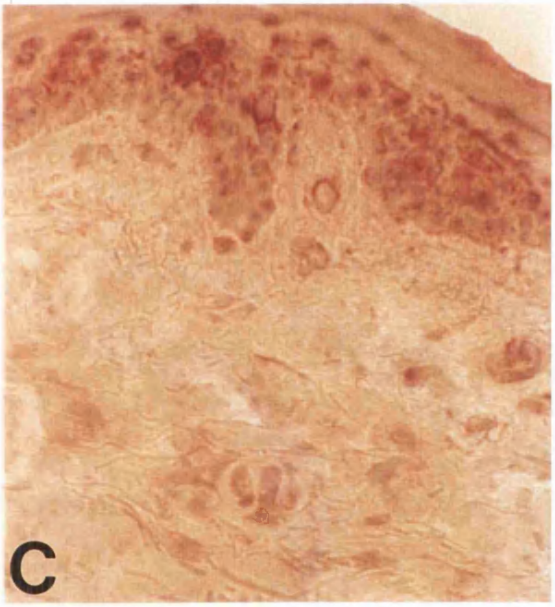
E High levels of expression in perivascular areas and interstitial cells in the mid-reticular dermis on day 7.

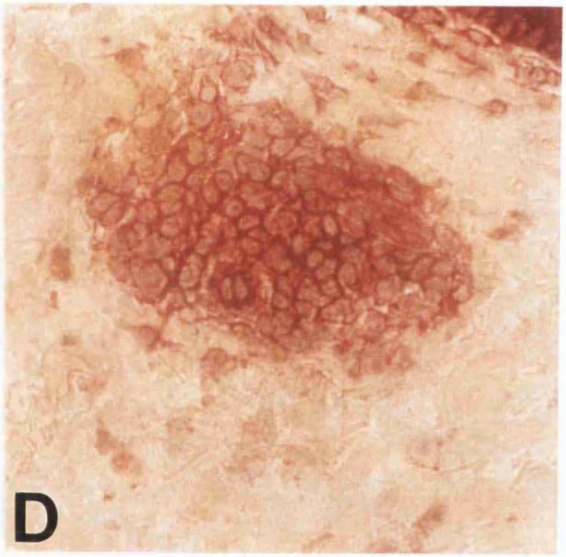
F Marked up-regulation of IFN-β expression in interstitial cells on day 14.

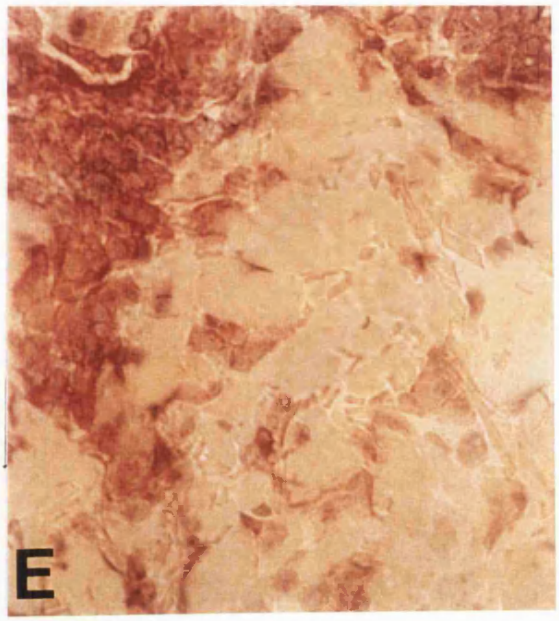
PLATE 15

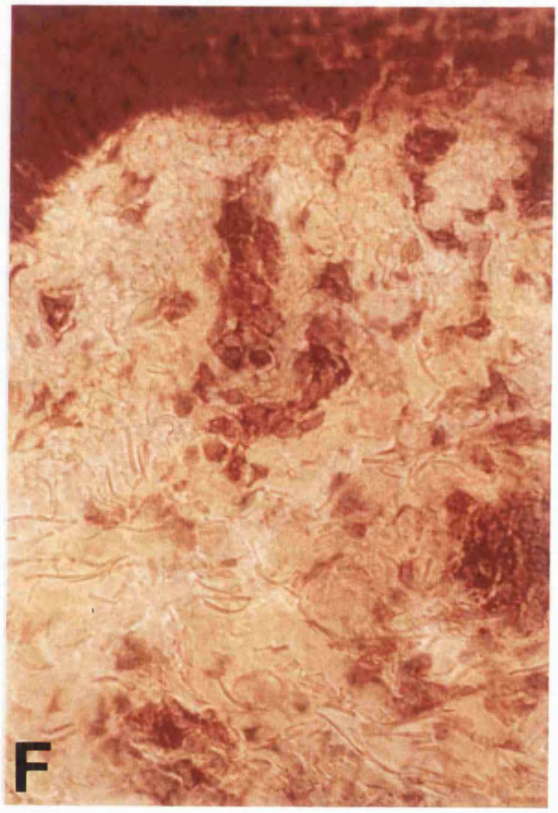












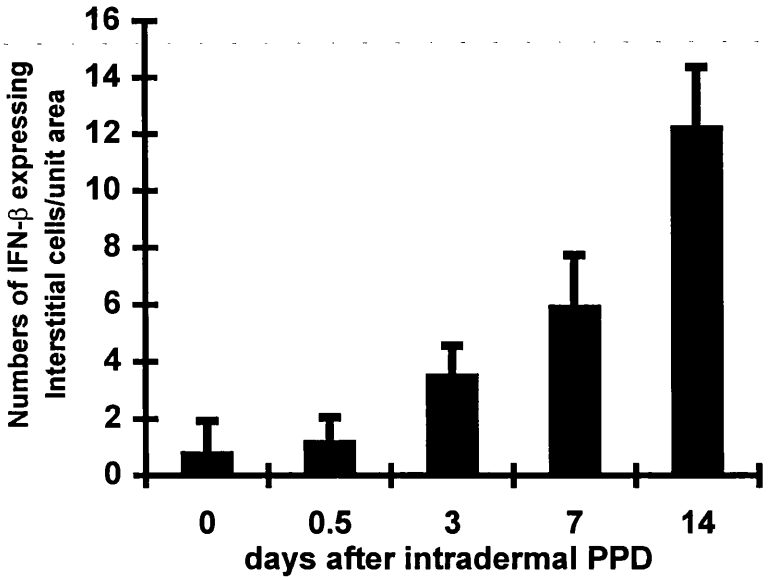


Figure 3.19 Mean numbers of interstitial cells expressing IFN-β in the upper dermis.

A biotin/streptavidin method and an image analysis system were used to determine IFN-β expression in interstitial cells (with stellate and spindle morphology, and identifiable nuclei) per rectangular frame area centred on areas in between perivascular infiltrates in the upper reticular and papillary dermis, 5 times per section. (n=3 at each time point except days 3 and 14 when n=4). Error bars indicate standard deviations.

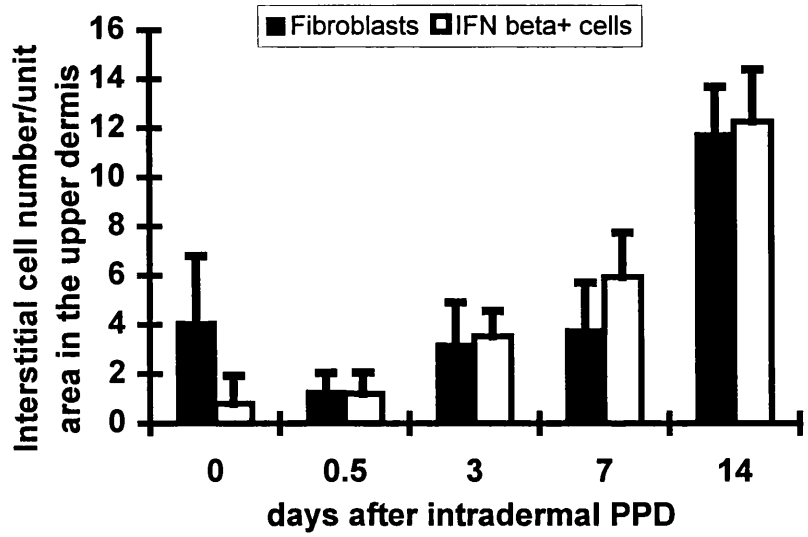


Figure 3.20 Comparison of mean numbers of interstitial Dia110 positive cells and IFN-β expressing cells in the upper dermis.

The results presented in figures 3.15 and 3.19 are presented together. There is a close correlation between the number of interstitial Dia 110+ cells and the number of interstitial cells expressing IFN-β in the upper dermis, from 12 hours after intradermal PPD.

3.3 DISCUSSION

The generation and resolution of a localised immune response is governed by the migration of leukocytes into the site of injury, the proliferation of cells *in situ* and the removal of these cells after antigenic clearance (1,169,245). Abnormal regulation of any of these phenomena may lead to chronic inflammation. We have investigated the kinetics of a Mantoux reaction to clarify factors which influence the generation and resolution of the response in order to better understand why inflammation persists in cutaneous diseases such as atopic eczema (189,205).

Following the intradermal injection of PPD, T cells began to accumulate perivascularly within the dermis by 12 hours, reflecting increased transendothelial migration rather than proliferation, since no Ki67+ T cells were present at this time. As previously discussed, a number of factors, including the expression of homing receptors and adhesion molecules by CD45RO+ T cells (49,50,52,69), and the release of chemotactic factors such as IL-1, IL-8 and RANTES (21,36,38,53) are likely to contribute towards this increased migration. We have demonstrated that there was upregulation of IL-15 as early as 12 hours after PPD challenge. In addition to its effects on the rates of T cell transmigration, this cytokine has been shown to be an important chemoattractant for T cells (80,89,242). IL-15 may therefore also play a role in the early accumulation of T cells after PPD challenge.

There was an initial recruitment of both CD4 and CD8 cells into the lesions, followed later by a selective increase in CD4+,CD45RO+ T cell numbers. Preferential migration of CLA+, CD45RO+, CD4+ cells has previously been demonstrated in PPD induced DTH lesions (46,50). However, the increase of CD4+, CD45RO+ T cells at the later time points is probably due to proliferation rather than migration since 19% of this subset expressed Ki67 reactivity at 7 days after initiation of the reaction, coinciding with the peak in

T cell numbers. Furthermore, this expansion of the CD4+ T cell subset was selective since no Ki67 reactivity was found in CD8+ T cells in any of the samples tested.

After antigenic stimulation the induction of cell cycling is driven by cytokines such as IL-2 and IL-7 (79,84,244). In addition it has recently been shown that IL-15 which utilises both the γ- and the β chain of the IL-2 receptor complex also triggers proliferation in activated T cells (80-83). We therefore investigated if the substantial proliferative activity in T cells at 7 days after PPD challenge was associated with the presence of these cytokines. We found that IL-15 was upregulated in epidermal KC 12 hours after injection of PPD and that epidermal and dermal DC expressing IL-15 were also present at 12 and in greater numbers at 72 hours. This confirmed previous observations demonstrating the presence of IL-15 in epidermal KC and LC in normal skin, and in T cell rich areas in Rheumatoid synovium (80,88). Further, the strong cytoplasmic staining in large oval perivascular and interstitial cells was consistent with reports that activated monocytes/macrophages are a major source of this cytokine (81,82,246). There was a marked reduction in staining intensity for IL-15 in both KC and monocytes/macrophages on day 7 when T cell numbers and T cell proliferation were maximal. This suggests that this cytokine may only contribute to the induction of the T cell proliferation during the early phases of the DTH response and that other cytokines may then take over this role at later stages after PPD challenge. We found that in contrast to IL-15, IL-2 and IL-7 expression were low 12 hours after intradermal PPD and appreciable amounts were only observed at 72 hours. The pattern of staining obtained with IL-7 was different to that obtained with IL-2 and IL-15. Less T cell surface expression was observed, and the cells with cytoplasmic staining appeared neither dendritic nor like small lymphocytes. Rather, they were large, had oval or round nuclei and resembled lymphoblasts or macrophages. IL-7 is generally regarded as being derived from stromal or epithelial cells (87,247,248). However, T cell derived IL-7 has recently been

implicated in the generation of inflammation in IL-7 transgenic mice with colitis (248), suggesting that lymphoblasts may be a major source of IL-7 in perivascular areas in MR. At 7 days after challenge when maximal proliferation and T cell numbers were evident, maximal perivascular IL-2 and IL-7 expression were observed. These data, although circumstantial, are compatible with the possibility that during the Mantoux reaction, IL-15, a non T cell derived cytokine may promote the initial proliferative drive until T cells themselves synthesise IL-2 and IL-7 which maintains the proliferative activity.

The cytokine micro-environment may be important not only in induction, but also in amplification and subsequent termination of the DTH response. Thus, the high levels of IFN-γ observed in perivascular areas at 3 and 7 days may promote macrophage activation and differentiation, resulting not only in upregulation of macrophage MHC class II and ICAM-1 expression, and facilitation of antigen presentation, but also increasing their TNF-α synthesis, enhancing cytotoxic activity (244,249). In addition to its possible pro-apoptotic effects, the high levels of TNF-α observed on days 3 and 7 may promote endothelial cell activation, upregulation of cytokine and adhesion molecule expression, and T cell recruitment into the lesions (244,250). It is of interest that high levels of IL-6 and TNF-α were observed at the peak of the response on day 7, since in the presence of IL-2, IL-6 and TNF-α have been shown to synergize, upregulating IL-2R expression on activated T cells, thereby increasing their responsiveness to IL-2 mediated proliferation, and their production of IL-2 and IFN-γ (244). Furthermore, this combination of cytokines has been shown to cause activation, proliferation, and differentiation of non-antigen specific, bystander T cell populations (91) which may serve to amplify the response.

The clearance of T cells during the resolution of the DTH response may be due to both the efflux of cells or to the death of cells *in situ*. We found that the numbers of T cells were significantly reduced 14 days after the initiation

of the PPD challenge. At this time, significant numbers of TUNEL+, apoptotic T cells were observed. These apoptotic cells, identified morphologically on staining with propidium iodide, could also be detected inside macrophages. It is likely, therefore, that the numbers of apoptotic cells detected represent a substantial underestimate of the total extent of apoptosis taking place (245,251). It is well recognised that activated T cells require the continued presence of certain cytokines to prevent apoptosis (3-6). For mature activated human T cells, IL-2 and IL-15 are the most efficient, whereas IL-4 is the least efficient at preventing death (9,170,171,252). It is of interest therefore, that on day 14, when substantial apoptosis was detected, IL-2, IL-7 and IL-15 were all significantly decreased as compared to day 7 when maximal proliferation and T cell numbers were observed. The persistence of high levels of IL-4 at this time point is perhaps not surprising, a) because it is relatively inefficient at rescuing T cells from apoptosis in humans (9) and b) because it has been shown to be required during the late stages of a number of intracellular infections in animal models, in order for the pathogen to be cleared (253). Interestingly, IL-4 has additionally been shown to stimulate fibroblast collagen synthesis (but not proliferation) as efficiently as TGF-β (254), suggesting that it may be involved in the increase in collagen synthesis previously detected at later time points in Mantoux reactions (255,256).

The strikingly decreased Bcl-2 which was observed on day 14 is compatible with previous observations that γ chain cytokines regulate apoptosis via the induction of this molecule (3,6,9). This suggests that when maximal levels of IL-2, IL-15 and IL-7 are present, T cell proliferation may occur. Conversely, when levels of these cytokines are limiting, T cells undergo apoptosis due to cytokine withdrawal. We also investigated T cell expression of Bcl-2 and Bax in a single subject where the PPD response was investigated at multiple time points. The reduction of Bcl-2 after the peak of the response preceded the fall in T cell numbers, further suggesting that cytokine deprivation was involved in the resolution of the response. Furthermore, the maintenance of

higher Bcl-2 expression in CD8 cells on day 14, suggests that it is the expanded CD4 cell population which is destined to die as a result of cytokine withdrawal. However, apoptosis was also observed at the peak of the response on day 7, when high levels of proliferation, IL-2, IL-7, IL-15 and Bcl-2 were observed implying that other mechanisms were responsible for the induction of apoptosis at this time.

The apoptosis of mature, activated, cycling T cells may occur as a consequence of CD95 mediated AICD (6,8,121,168). We therefore investigated the expression of CD95-ligand during the Mantoux reaction. The kinetics of CD95-L expression indicated that maximal expression was found in the perivascular infiltrates at the peak of the response (day 7) but was significantly reduced at 14 days. This coincided with increased T cell HLA DR expression, and HLA DR+ T to T cell interactions on day 7 may favour CD95-mediated apoptosis (174,240). Furthermore, the expression of TNF-α, which can induce T cell apoptosis as a consequence of binding to its receptor was also high on day 7, but was reduced on day 14. Although the percentage of apoptotic T cells identified was lower on day 7, than on day 14, the much greater number of infiltrating T cells at this time point suggests that maximal apoptosis occurred at the height of the response, and that both CD95 and TNF-α mediated death are likely to be involved. However the relative contribution of each of these pathways is not clear. In contrast, at 14 days, when there has been clinical resolution of the response, presumably as a result of antigenic clearance, and when T cell proliferation, CD95-ligand and TNF-α expression are substantially reduced, it is unlikely that AICD plays a major role.

These results collectively suggest that, while apoptosis occurring during the peak of the PPD response may involve the interactions of either CD95 or TNFR with their ligands, apoptosis during the resolution phase may be controlled by the regulation of Bcl-2/Bax levels by cytokines. Interestingly,

maximal T cell HLA DR expression was observed on day 14, suggesting that T cell anergy may also contribute to downregulation of the response (239-241).

For effective immunological memory to be generated a proportion of antigen-specific T cells must be "rescued" from cytokine-deprivation induced apoptosis at the end of the response. Long term memory requires T cells to revert to rest, and a number of different mechanisms are likely to be involved in this process. The role of stromal cell factors, such as type I interferons, may be pivotal, since they have been shown both *in vitro* and *in vivo*, to rescue activated T cells from apoptosis independently of Bcl-2, by upregulating their Bcl-x_L and returning them to a resting (G_0/G_1) state (178,257 and 10, submitted). Our findings suggest that other stromal cells, namely epidermal keratinocytes and endothelial cells, actively participate in the generation of the response by secreting cytokines such as IL-15 (KC) and IFN-γ/TNF-α (EC). We were therefore interested to know whether fibroblast-mediated IFN-β production was involved in the resolution phase of the DTH response.

There is mounting evidence to suggest that fibroblasts function as key regulators of the inflammatory process in many tissues (230,258). Like lymphocytes they appear to consist of subpopulations with unique phenotypes and functions, and may be activated via CD40 to express a variety of cytokines and chemokines, including IL-6, IL-8, RANTES and eotaxin (230,258-260) enabling them to participate in leukocyte recruitment at sites of inflammation. Several previous studies have shown enhanced expression of IL-1-β, TNF-α, TGF-β, PDGF and increased synthesis of new collagen (pro-collagen I) during the course of the Mantoux reaction (255,256). This suggests that fibroblasts may not only actively participate in the inflammatory process, but that DTH and wound healing responses share similar features, differing principally in the degree of remodelling and

collagen removal occurring at later time points. Interestingly, T cell-fibroblast interactions appear to be "bi-directional" , since activated T cells can also regulate fibroblast behaviour, promoting fibroblast adhesion and proliferation (261). In this study we found increasing numbers of fibroblasts, particularly during the resolution phase of the response. This increase in fibroblast numbers was preceded by high intralesional IFN-γ and TNF-α expression, both of which are known to promote fibroblast activation (230,262). We found evidence to suggest that fibroblast accumulation was likely to be at least in part due to *in situ* proliferation. Such proliferation, as well as collagen synthesis, can be promoted by TGF-β and TNF-α (262), both of which showed high levels of expression on day 7. Interestingly, TNF-α has also been shown to enhance fibroblast IFN-β production *in vitro* (263) and may therefore contribute to the increasing IFN-β expression observed at later time points. Our findings also suggested that numbers of infiltrating DDC increased during the resolution phase. Although their role in resolution is uncertain, they may provide a source of both TNF-α and IFN-β. The exact nature of the interstitial cells producing IFN-β therefore remains uncertain, however, maximal IFN-β expression coincided with resolution on day 14, and high levels were also detected in endothelial cells and keratinocytes from day 3 to day 14, suggesting that this cytokine may indeed be responsible for rescuing a proportion of antigen specific T cells destined to maintain memory at the end of the immune response.

The reaction was not followed beyond 14 days and the rate of clearance and fate of infiltrating fibroblasts remain uncertain. Rates of fibroblast proliferation appeared to be reduced on day 14 and since MR do not usually leave long-lasting scarring, it is likely that these cells are cleared at later time points. As in wound healing responses these fibroblasts will probably be removed, at least in part, by apoptosis (261). Although preliminary investigations identified very occasional apoptotic fibroblasts on day 14, further studies would be required at later time points to establish whether their apoptotic

clearance occurs.

In summary, we have shown that in the Mantoux reaction, the generation of the response involves not only recruitment, but also T cell proliferation, while resolution occurs in part by induction of apoptosis in infiltrating T cells. We hypothesise that the proliferative phase and the resolution of the response appear to be controlled by different levels of the same group of (IL-2R γ chain) cytokines, the presence of which promotes proliferation, while the absence of these mediators leads to apoptosis. At the end of the response, increased IFN-β production may allow the survival of a small number of primed T cells enabling specific memory to be maintained. These findings suggest not only that dysregulation of T cell apoptosis may contribute to chronicity of inflammation in cutaneous diseases such as atopic eczema, but also that IFN-β, if present in excess, may contribute to this dysregulation. Studies into the regulation of T cell apoptosis in atopic eczema are clearly pertinent to its future management.

4. ATOPIC ECZEMA-AN EXAMPLE OF PERSISTENT CUTANEOUS INFLAMMATION

4.1 INTRODUCTION

The aetiopathogenesis of atopic eczema (AE) is still incompletely understood. The persistent T cell infiltrate in the dermis of AE patients may in part be due to ongoing percutaneous absorption of a variety of antigenic stimuli, including aeroallergens and staphylococcal antigens and/or superantigens (207,208). Such antigens may be internalised and efficiently presented to T cells by IgE receptor bearing epidermal and dermal DCs in patients with circulating antigen-specific IgE (reviewed in 189,210,228). The T cell activation and cytokine production thereby induced would favour further T cell recruitment and the generation of more inflammation. Whether the initial trigger for this "vicious circle" of T cell activation and recruitment is a genetic abnormality involving the regulation of IgE synthesis is still uncertain; however, it is likely that such an abnormality is present in some patients (191). Another possibility, is that the processes which regulate the normal clearance of T cells from the skin, in particular T cell apoptosis, are defective. This could occur either as a result of genetically determined abnormalities in the apoptotic pathways themselves, or because of an abnormal cutaneous cytokine microenvironment. Since other abnormalities of the lymphoreticular system are not usually a feature of the disease, it seems unlikely that a global dysregulation of apoptotic pathways occurs. Furthermore, a number of studies suggest that abnormal cytokine microenvironments occur in the skin of AE patients (189,222,228). The possibility that this microenvironment influences T cell clearance from the skin in AE has not previously been addressed.

Having established that resolution of a normal cutaneous immunological response, namely the MR, is achieved at least in part by T cell apoptosis, we sought to compare this resolving response with chronic lesional AE, in order to establish whether a dysregulation of T cell apoptosis might contribute to the persistence of the cutaneous T cell infiltrate in this chronic disease

(specific aim 4). We examined AE lesions present for at least two weeks so that they might, theoretically, be comparable to the resolution phase (day 14) of the MR.

Our studies in the MR suggested that fibroblasts might be actively involved in regulation of the inflammatory response. Since there is mounting evidence to suggest that fibroblast-mediated T cell survival contributes to chronic inflammation in the joints of patients with rheumatoid arthritis (11,13,178 and 10, submitted), we were interested to know whether such a mechanism might also be involved in the pathogenesis of AE. We therefore investigated the numbers, distribution and proliferation of infiltrating fibroblasts, and the expression of TGF-β and IFN-β in AE **(specific aim 5)**.

4.2 RESULTS

Patients were recruited and biopsies were taken as detailed in section 2.1 and Table 2.2. An example of the clinical features of AE is demonstrated in Plate 16A.

4.2.1 HISTOLOGY

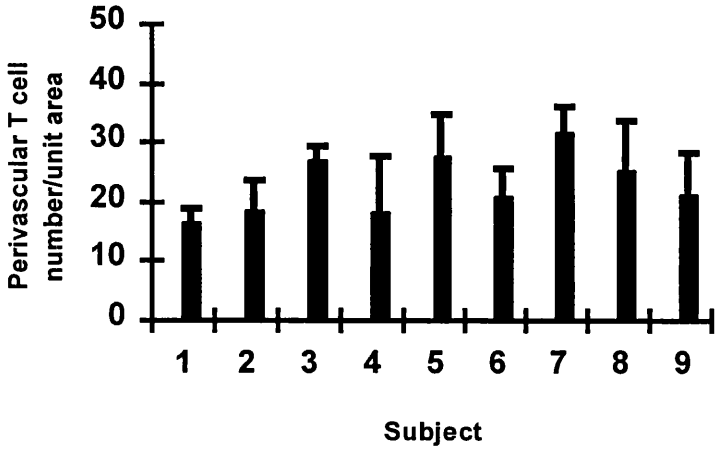
Characteristic histologic features of AE were present in all biopsies, namely epidermal hyperkeratosis and acanthosis, with variable degrees of spongiosis, fusion of rete ridges and in some sections vesiculation. A predominantly perivascular inflammatory cell infiltrate was present in the papillary and upper reticular dermis. The deep dermis was largely uninvolved. Infiltrating cells were seen extending up into the dermal papillae in a majority of sections, and the vascularity was increased, particularly in the papillary dermis when compared to normal skin and DTH responses. A diffuse interstitial infiltrate, containing numerous spindle cells was also observed in the papillary and upper reticular dermis.

4.2.2 CHARACTERISTICS OF INFILTRATING T CELLS

4.2.2.1 T cell numbers and distribution

Infiltrating T cells were concentrated in perivascular areas, predominantly in the papillary and upper reticular dermis (Plate 16B), although occasional infiltrates were present in the deeper reticular dermis. In general, the density of perivascular T cells was less than at the height of the Mantoux reaction (MR). Occasional T cells were present in the interstitium and within in the papillary dermis extending into dermal papillae, however, the majority of infiltrating cells in these areas were not T cells. There was significant variation between mean perivascular T cell numbers in the 9 different subjects (range 16.7-31.6 cells/UA, $p = 0.005$; Fig 4.1a).

a)



b)

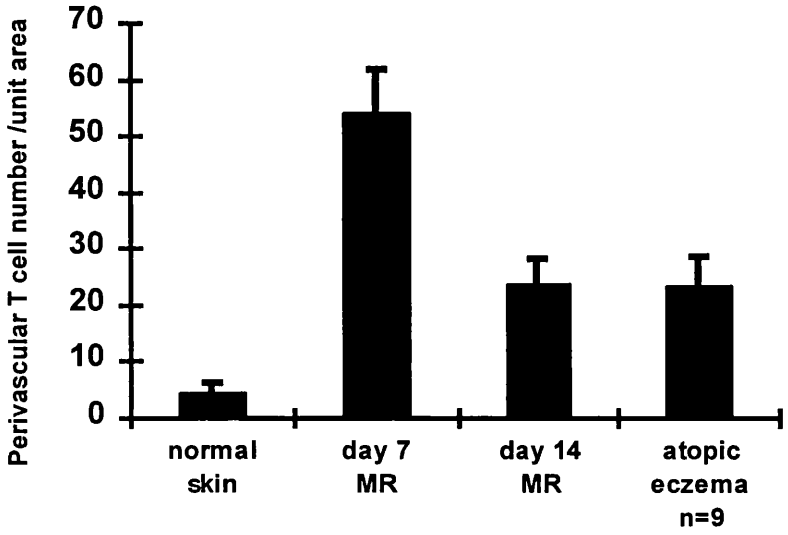


Figure 4.1 Mean numbers of infiltrating T cells in perivascular areas.

T cells were stained by an indirect immunoperoxidase method and quantified in each section using an image analysis system, per circular frame area centred on the 5 largest dermal perivascular inflammatory cell infiltrates.

a) T cell numbers in chronic atopic eczema (AE) subjects, **b)** comparison of T cell numbers in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

However, this variability was less than that observed when comparing the eczema patients as a group with the different time points of the DTH response. Thus the mean perivascular T cell number in AE was significantly greater than in normal skin (23.1 +/- 5.0 vs 4.5 +/-1.9 cells/ UA; p= 0.0001), but lower than at the height of the MR (23.1 +/- 5.0 vs 54.2 +/- 4.7 cells/ UA, p=0.0001; Fig 4.1b). It was similar to that on day 14 of the MR (23.8 +/- 6.3/UA, p= 0.68). Since the majority of T cells were present perivascularly, and in order to compare the phenotypic characteristics of AE and DTH responses, these areas were further examined (n=9 unless otherwise stated).

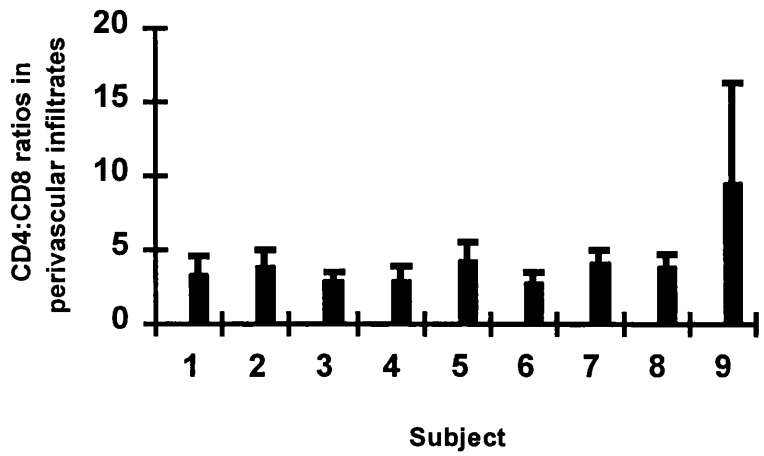
4.2.2.2 CD4:CD8 ratios

Numbers of CD4+ cells exceeded CD8+ cells in all subjects (Plate 16C). In perivascular infiltrates, there was significant inter-subject variability in CD4:CD8 ratios (range 2.9-9.6, p = 0.003; Fig 4.2a), however, CD8+ cells were more often situated at the periphery than at the centre of the infiltrates. This variability was taken into account when comparing the eczema patients as a group with the different time points of the DTH response. Thus the CD4:CD8 ratio in AE (mean = 4.24 +/- 2.1) was not significantly different from the 7 or 14 day MR (p = 0.12 & p = 0.62 respectively; Fig 4.2b).

4.2.2.3 Characteristics of CD45RO+ cells

In the upper dermis, a mean of 22.4 +/-6.1 cells /UA (range 16.5-37/UA; Fig 4.3a) perivascular cells were CD45RO+ and occasional positive interstitial cells were seen. Fewer infiltrating cells were positive in mid-reticular dermal infiltrates when compared with those in the papillary and upper reticular dermis. Perivascular CD45RO+ cell numbers were similar to day 7 MR (p= 0.1; Fig 4.3b), but significantly greater than those observed in normal skin (3.5 +/-2 cells/ UA, p = 0.0001) and lower than in 14 day MR (27 +/- 6.6 cells/ UA, p=0.02).

a)



b)

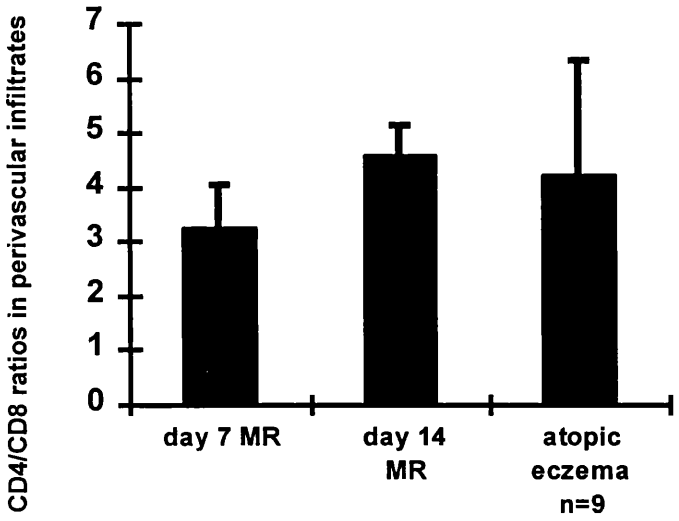
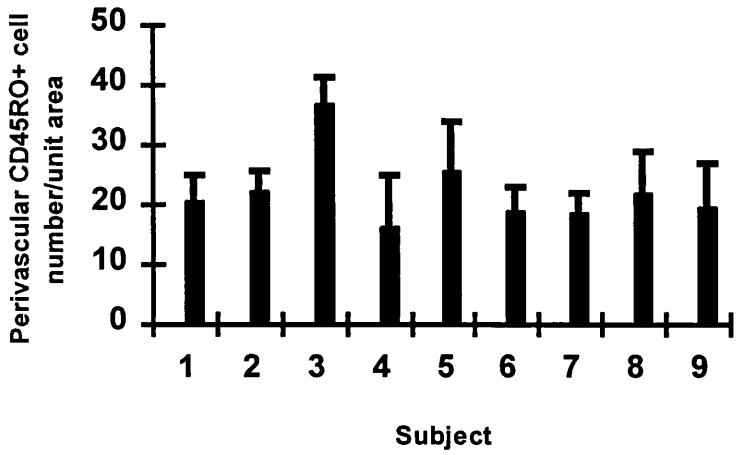


Figure 4.2 Mean ratios of CD4/ CD8 cells

These were quantified by dual immunofluorescence (IMF) and proportions of positive cells in the five largest dermal perivascular inflammatory cell infiltrates were counted in each section using a Zeiss fluorescence microscope

a) CD4/CD8 ratios in chronic atopic eczema (AE) subjects, **b)** comparison of CD4/CD8 ratios in 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. (CD4 cells predominated in normal skin but the numbers of cells were too small to give meaningful ratios). Error bars indicate standard deviations.

a)



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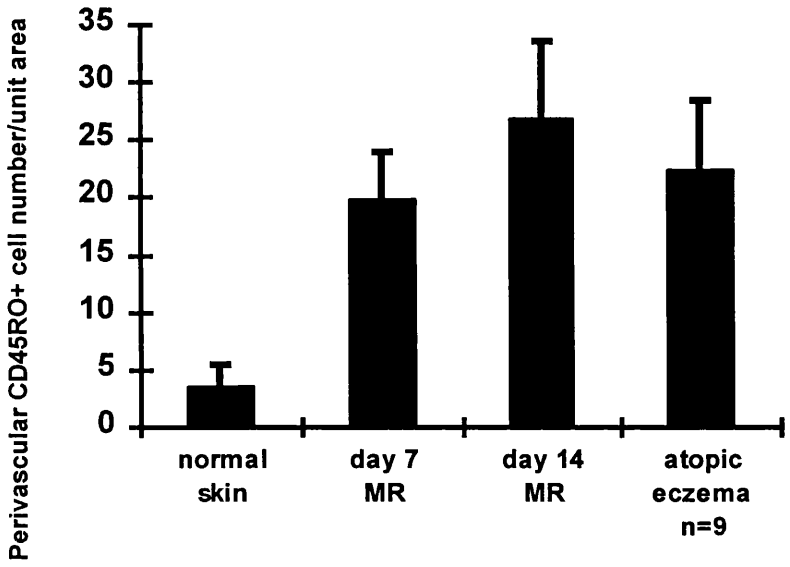


Figure 4.3 Mean numbers of CD45RO+ cells in perivascular areas.

CD45RO+ cells were stained by an indirect immunoperoxidase method and quantified in each section using an image analysis system, per circular frame area centred on the 5 largest dermal perivascular inflammatory cell infiltrates.

a) CD45RO+ cell numbers in chronic atopic eczema (AE) subjects, **b)** comparison of CD45RO+ cell numbers in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

Dual immunofluorescence studies revealed that a majority of perivascular CD4 cells were CD45RO+ (mean % CD4 cells expressing CD45RO = 86.0 +/-3.1%), and that there was remarkably little inter-subject variability (range 79.3-89.4% p = 0.30; Fig 4.4a). This high proportion of CD4+CD45RO+ cells resembled that seen 14 days after intradermal PPD (86.9 +/- 4.2%, p = 0.66), and was significantly greater than in normal skin (45.3 +/- 25.5%, p=0.0001) and 7 day MR (80.7 +/- 14.3 %, p = 0.003; Fig 4.4b) .

In summary, in chronic lesional AE a majority of infiltrating T cells were observed in close proximity to dermal blood vessels. Mean perivascular T cell numbers were lower than at the height of the MR, but similar to those on day 14. As previously reported, a majority of infiltrating cells were CD4+CD45RO+ cells (48,176,189,204). Thus, the phenotypic characteristics and numbers of infiltrating cells in two week old AE lesions were similar to those observed during the resolution phase (day 14) of PPD induced DTH responses.

4.2.3 T CELL PROLIFERATION IN ATOPIC ECZEMA

In order to investigate whether proliferation *in situ* plays a part in the accumulation of T cells in lesional AE we used dual immunofluorescence studies to ascertain the proportion of proliferating perivascular T cells. The mean percentage of T cells expressing Ki67 was 6.9 +/- 2.1% (range 3.6-9.6%, p=0.34, Fig 4.5a & Plate 16D). Again this figure was significantly lower than on day 7 after PPD (18.8 +/- 3.7%), but greater than in normal skin (0%, p = 0.0001 in both cases). Although slightly higher, it resembled that observed on day 14 of the MR (4.2 +/-3.4%, p = 0.04; Fig 4.5b).

Plate 16 Clinical features and characteristics of infiltrating T cells in atopic eczema

A Clinical features of atopic eczema, showing erythema, lichenification and some excoriation on the trunk in subject 6.

B Indirect immunoperoxidase staining with T mix in subject 6.

Dual immunofluorescence studies were used in **C-E**.

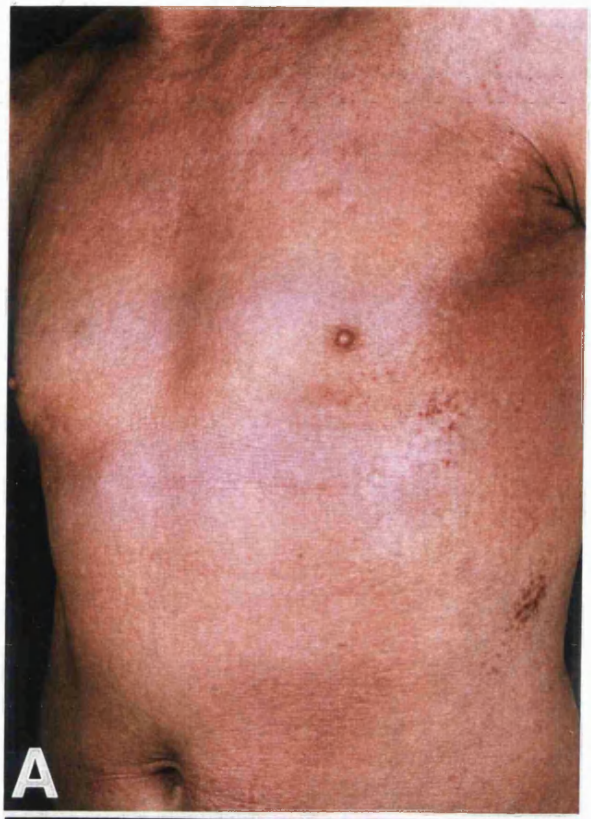
C Proportions of CD4+ cells (red) and CD8+ cells (green) in a dermal perivascular inflammatory cell infiltrate (subject 5).

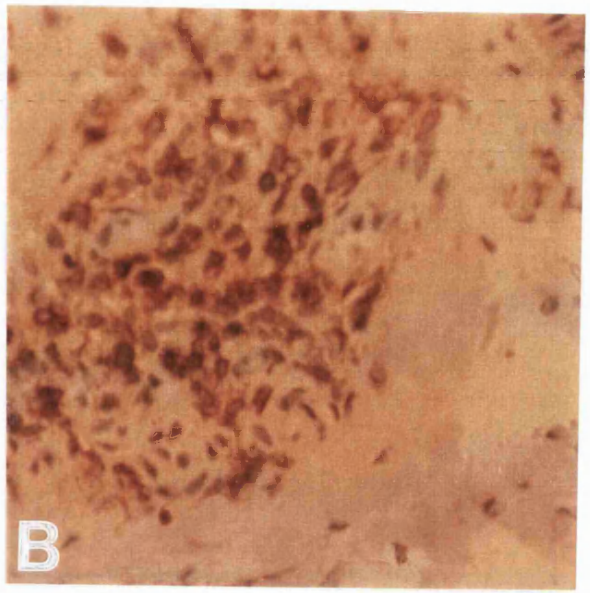
D Proportions of T cells (red) expressing Ki67 (green) in a dermal perivascular inflammatory cell infiltrate (subject 6).

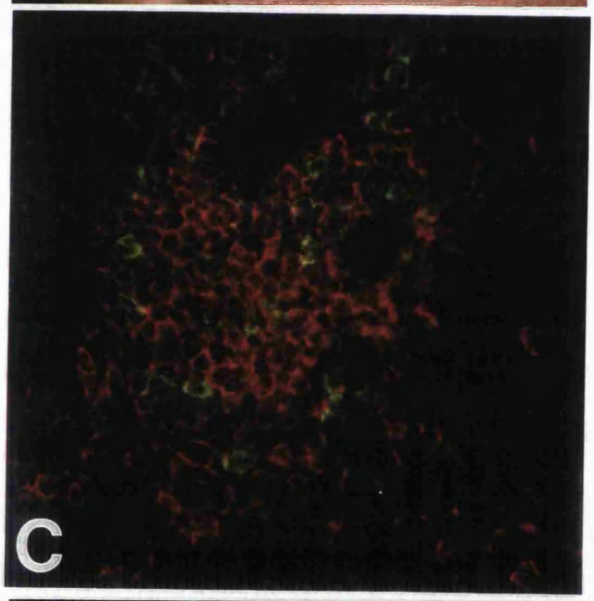
E Proportions of T cells (red) expressing HLA DR (green) in a dermal perivascular infiltrate (subject 6).

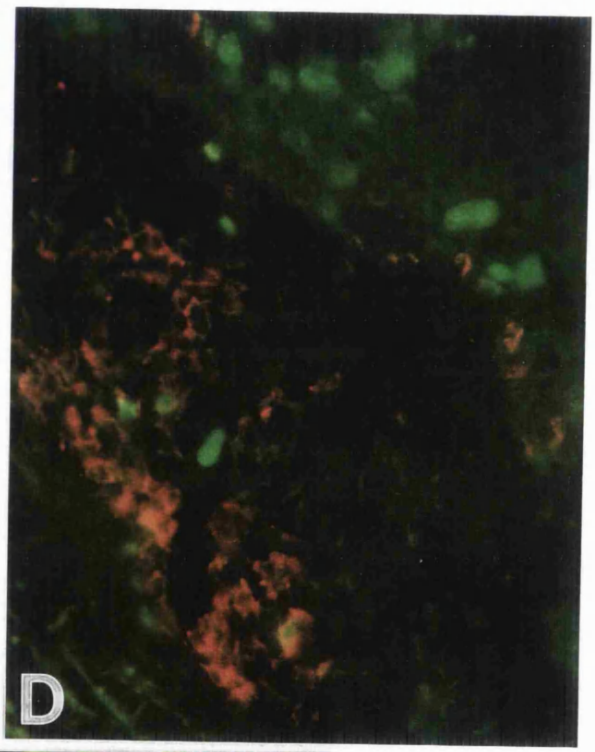
B-E x400 magnification.

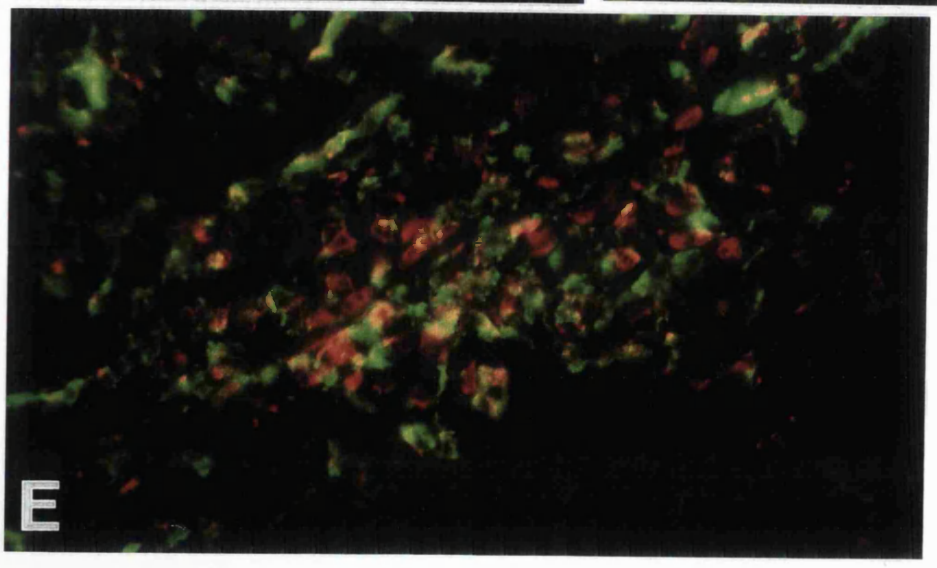
PLATE 16



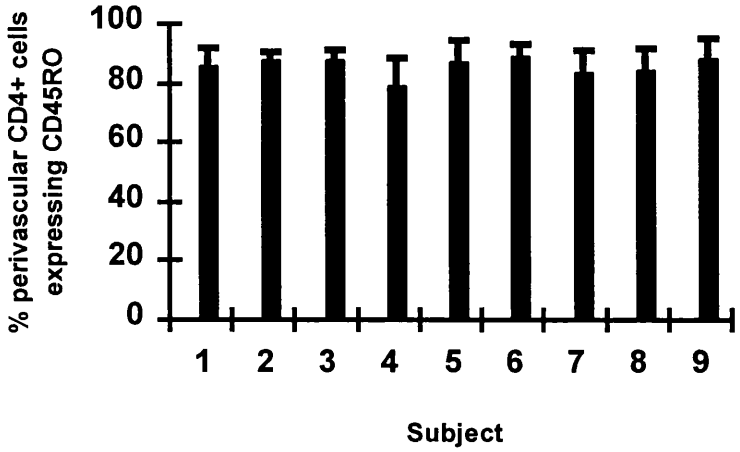








a)



b)

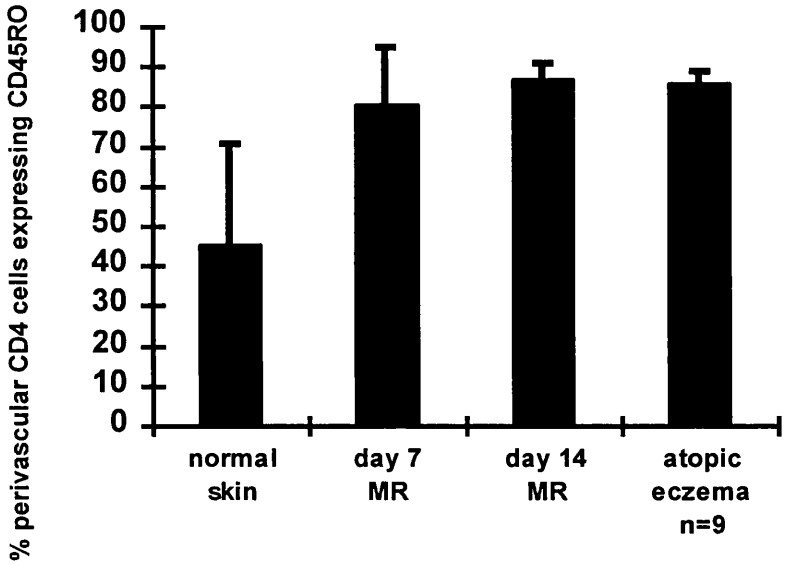
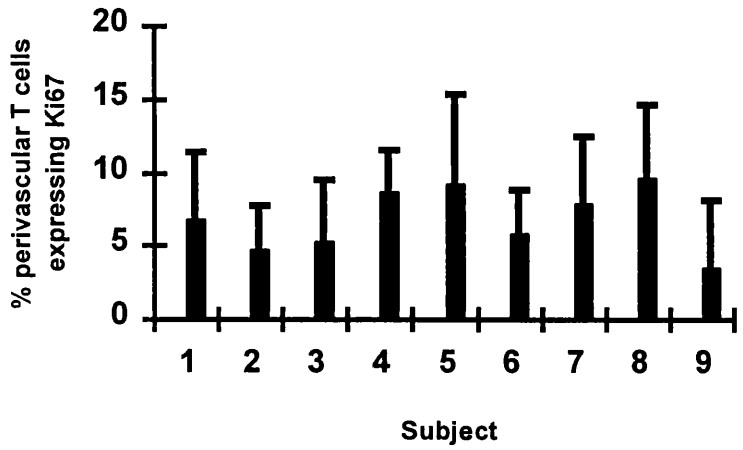


Figure 4.4 Mean percentages of perivascular CD4+ cells expressing CD45RO.

Dual immunofluorescence was used to count proportions of double positive cells in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

a) % CD4+ cells expressing CD45RO in chronic atopic eczema (AE) subjects, **b)** comparison of % CD4+ cell CD45RO expression in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

a)



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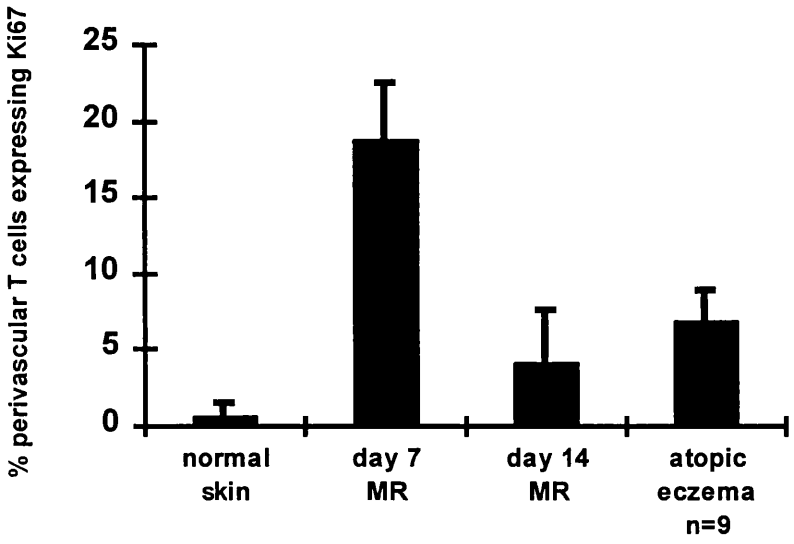


Figure 4.5 Mean percentages of proliferating T cells.

Dual immunofluorescence was used to count proportions of Ki67+ T cells in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

a) % T cells expressing Ki67 in chronic atopic eczema (AE) subjects, **b)** comparison of % T cells expressing Ki67 in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

4.2.4 DISTRIBUTION AND CHARACTERISTICS OF HLA DR POSITIVE CELLS

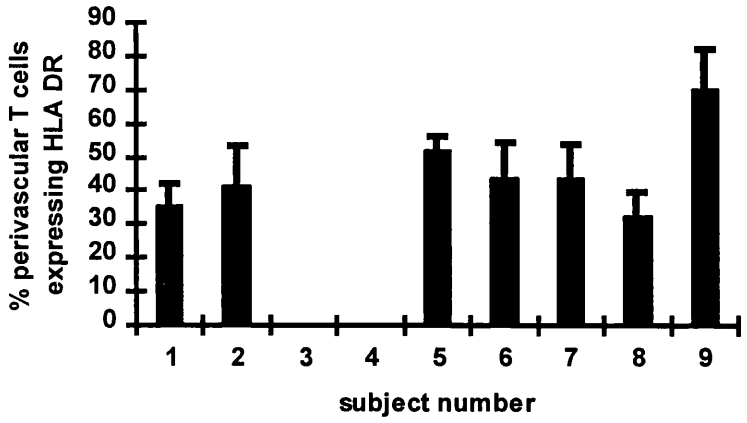
Numerous HLA DR positive epidermal LC were present in all sections and in one section positive epidermal KC were detected. In perivascular areas, the numbers of HLA DR+ cells greatly exceeded the number of T cells present. A mean of 45.7 +/- 12.6% of perivascular T cells were HLA DR+ (range 32.8-70.4%, n=7; Fig 4.6a & Plate 16E). Despite the inter-subject variability observed (p=0.0001), overall analysis revealed that T cell HLA DR expression was similar to that in normal skin (33.2+/-14.6% p=0.05), but significantly lower that on days 7 and 14 of the MR (60.3 +/-5.8% and 74.3 +/- 12.9% respectively, p=0.0001; Fig 4.6b). However, in AE numerous positive dendritic processes were present in close apposition to negative staining perivascular T cells. Positive perivascular cells included endothelial cells (EC) and dendritic cells. Such HLA DR+ cells were also identified in the interstitium and a sparser infiltrate of positive spindle-shaped fibroblasts was present in the deeper dermis.

4.2.5 FAILURE OF RESOLUTION- MARKERS OF APOPTOSIS

4.2.5.1 TUNEL+ T cells

To investigate whether a failure of T cell apoptosis may contribute to the persistence of the T cell inflammatory infiltrate in chronic AE we employed a combination of indirect immunofluorescence and TUNEL methodologies. Resolution of the MR was associated with an increase in the proportions of TUNEL+ perivascular T cells to1.83 +/-0.74% (range 0.81-2.48%) on day 7 and 2.5 +/- 0.93% (range 1.5-3.75%) on day 14. In contrast, the percentage of apoptotic T cells identified in AE subjects were low (mean 0.23 +/- 0.3 % range 0-0.73% p = 0.5; Fig 4.7a) and Plate 17A), and significantly different to those obtained on days 7 and 14 or the MR (p = 0.0001, Fig 4.7b).

a)



b)

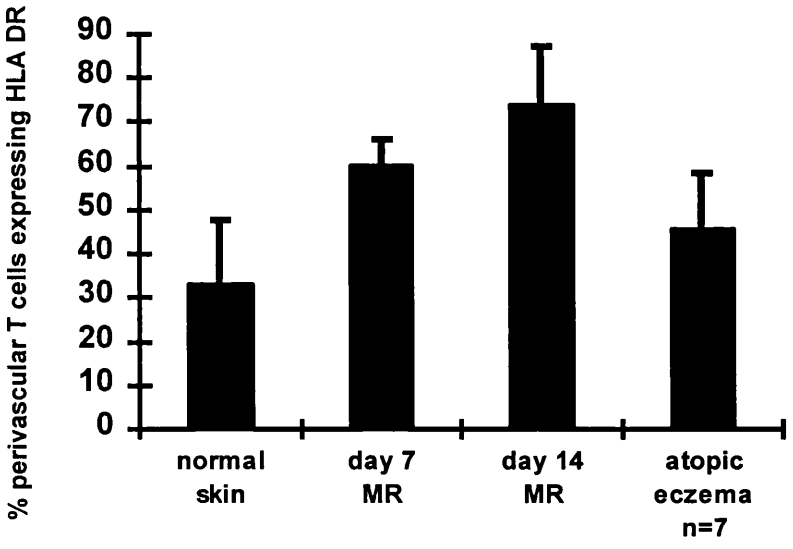


Figure 4.6 Mean percentages of HLA DR expressing T cells.

Dual immunofluorescence was used to count proportions of HLA DR+ T cells in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

a) % T cell HLA DR expression in chronic atopic eczema (AE) subjects (data was not available for subjects 3 and 4), **b)** comparison of % T cells expressing HLA DR in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

a)

Subject	Mean % TUNEL+ T cells in perivascular areas	Standard deviation
1	0	0
2	0	0
3	0.25	0.57
4	0.73	1.01
5	0	0
6	0.61	1.36
7	0.47	1.04
8	0	0
9	0	0

b)

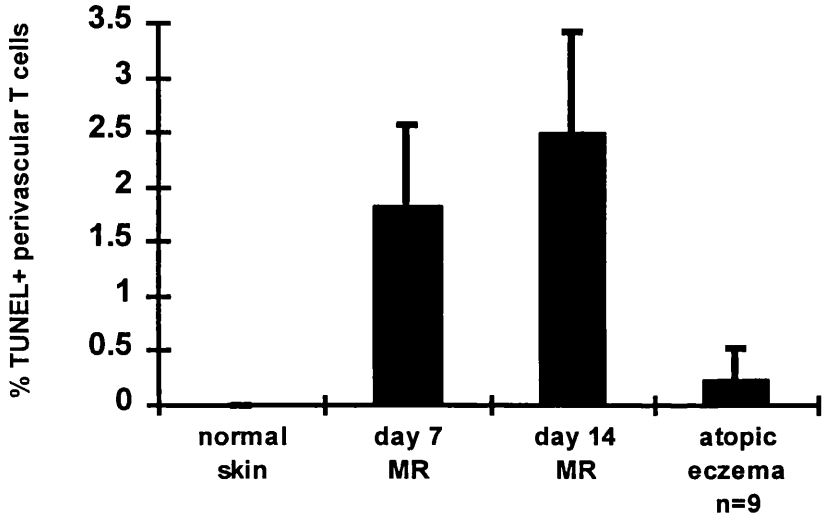


Figure 4.7 Mean percentages of TUNEL+ perivascular T cells.

A combination of indirect IMF and TUNEL methodologies were employed to quantify the proportions of double positive cells in the five largest dermal perivascular infiltrates in each section using a Zeiss fluorescence microscope

a) % TUNEL+ T cells in chronic atopic eczema (AE) subjects, **b)** comparison of % TUNEL+ T cells in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations

4.2.5.2 T cell Bcl-2 and Bax expression

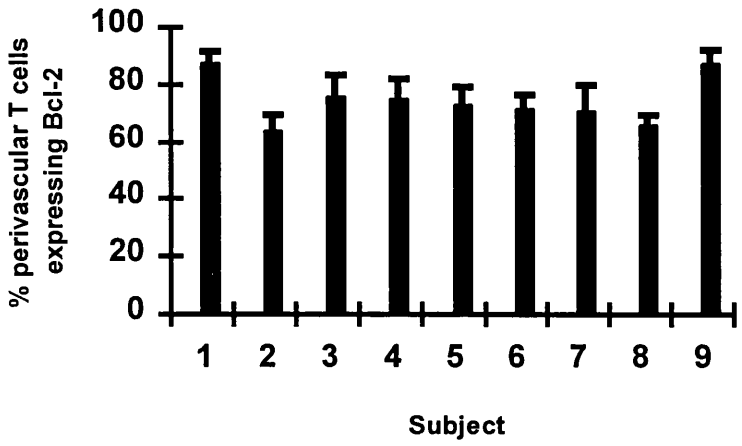
Since the γ chain cytokines promote T cell survival by upregulating the expression of Bcl-2 relative to Bax (9,167,171,175), we investigated the percentages of T cells expressing Bcl-2 and Bax in perivascular infiltrates to see if the low rates of T cell apoptosis observed might be due to upregulation of Bcl-2. Dual immunofluorescence studies revealed that a mean of 74.54 +/- 8.2% perivascular T cells expressed Bcl-2 whilst >98% expressed Bax. Levels of Bcl-2 expression showed significant inter-subject variability (range 63.8-87.5% p=0.0001; Fig 4.8a), whereas Bax expression was uniformly high (data not shown). Nevertheless, the variability in Bcl-2 expression was less than that observed when comparing the eczema patients as a group with the different time points of the DTH response. Thus the level of T cell Bcl-2 expression in AE was similar to that in 3 (76.3% +/- 7.5%), and 7 day (70.9% +/- 6.1% p = 0.25) MR. It was significantly greater than in normal skin and greater than that seen 14 days after intradermal PPD (38.7 +/-15.7% and 23.5% +/-3.3% respectively, p = 0.0001; Fig 4.8b).

4.2.5.3 CD95 ligand expression

An alternative pathway to apoptosis involves the interaction of CD95 and its ligand (CD95-L). We therefore used the biotin/streptavidin method to investigate whether reduced CD95-mediated cell death might also be occurring in AE (n=8).

Weak to moderate cytoplasmic CD95-L was expressed in endothelial cells in all sections. Epidermal KC expression was variable with strong cytoplasmic positivity occurring in 2/8, and weak-moderate staining in 6/8 subjects. In 3/8 subjects interstitial spindle shaped cells showed weak-moderate cytoplasmic staining.

a)



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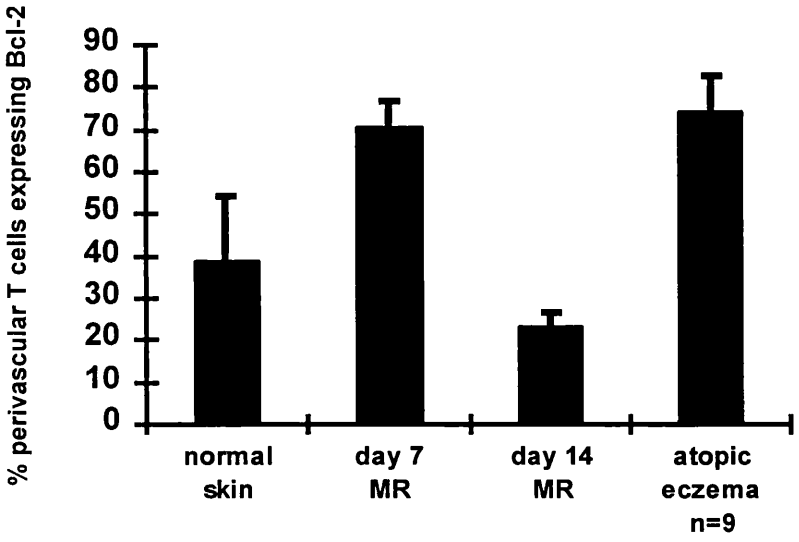
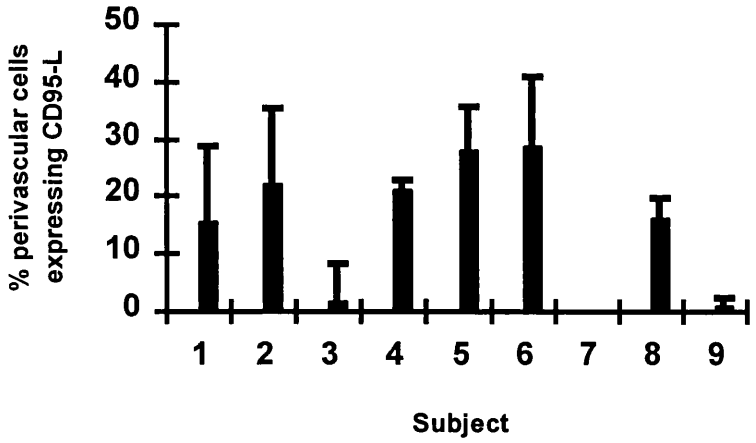


Figure 4.8 Mean percentages of Bcl-2+ perivascular T cells.

Dual IMF studies were performed using CD5+CD8 (T cells) and Bcl-2 and proportions of double positive cells were counted in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

a) % Bcl-2+ T cells in chronic atopic eczema (AE) subjects, **b)** comparison of % Bcl-2+ T cells in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations

a)



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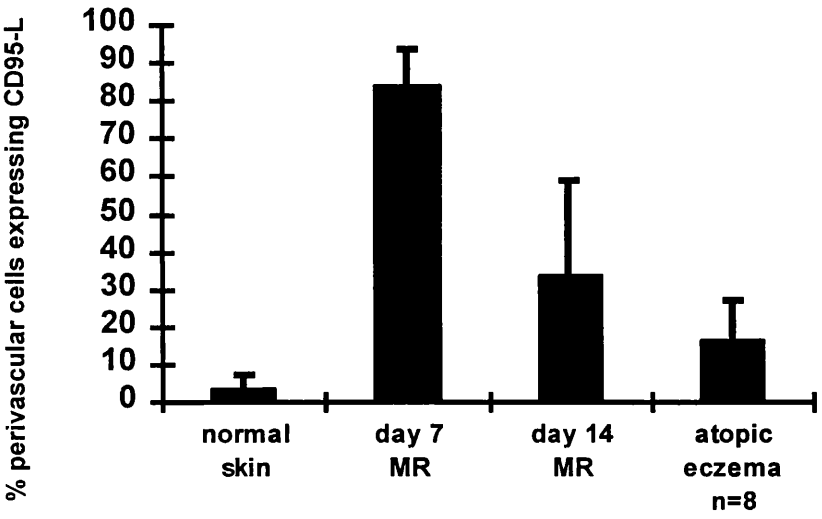


Figure 4.9 Mean percentages of CD95-L+ perivascular cells.

A biotin/streptavidin method and an image analysis system were used to determine CD95 ligand expression in the five largest dermal perivascular infiltrates. Cells with cytoplasmic or membrane positivity were counted. Error bars indicate standard deviations.

a) % CD95-L+ cells in chronic atopic eczema (AE) subjects (no data was available for subject 7), **b)** comparison of mean % CD95-L+ perivascular cells in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE.

Plate 17 TUNEL positivity, CD95-L and TNF-α expression in atopic eczema

A T cells were labelled with TRITC (red) and then apoptotic cells were stained using TUNEL reaction mixture(green), x400 magnification. Few TUNEL positive T cells were identified in perivascular areas in AE (subject 5).

B-F A biotin/streptavidin method was used in to determine the cutaneous distribution of CD95-L and TNF-α in AE (all x400 magnification).

B An example of maximal CD95-L expression observed in AE (subject 8). CD95-L was expressed in keratinocytes and a majority of perivascular cells showed weak surface staining. Occasional cells with strong cytoplasmic staining were present perivascularly and in the interstitium.

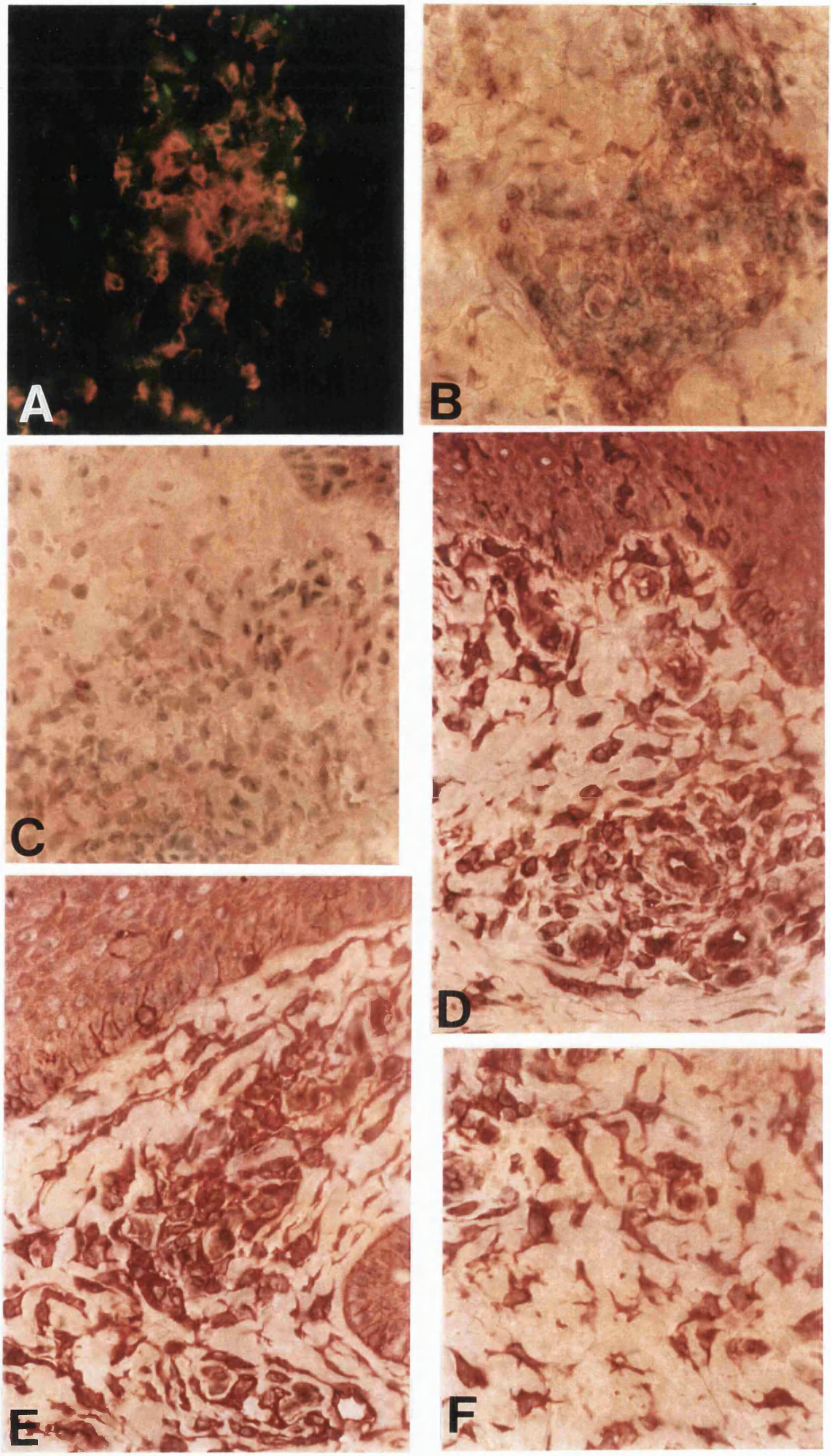
C In this subject (number 3) very low levels of CD95-L expression were observed.

D Moderate TNF-α expression in keratinocytes. Strong expression present in epidermal dendritic cells, endothelial cells, and numerous perivascular cells (subject 1).

F Large numbers of TNF-α expressing interstitial dendritic and stellate cells in the upper dermis (subject 1).

E Positive dendritic cells in the basal layer of the epidermis and dendritic and spindle cells just below the dermo-epidermal junction (TNF-α, subject 5).

PLATE 17



In perivascular areas, although a mean of 16.9 +/- 10.6% of cells expressed CD95-L, a majority showed weak surface staining, and this was more obvious in larger perivascular infiltrates (Plate 17B). Few cells expressed moderate-strong cytoplasmic CD95-L, those that did resembled macrophages in that they were large with round or oval nuclei. Intensity of expression showed considerable inter-subject variability (Plate 17C), both in terms of the numbers of cells with strong cytoplasmic staining (range 0.6-9.8 cells/UA p = 0.0001), and the percentage of positive cells (range 1.2-28.6% p = 0.0002; Fig 4.9a). However, when this variability was taken into account, the number of strongly positive cells and percentage of positive perivascular cells were nevertheless significantly greater than in normal skin, but lower than 7 and 14 days after intradermal PPD (16.9 +/- 10.6% vs. 1.3 +/-0.5%, 83.5 +/- 9.4% and 34.2 +/- 16.0 respectively, p=0.0001, Fig 4.9b)

Thus, although inter-subject variability occurred, CD95-L expression was lower in all AE subjects examined (n=8), both in terms of numbers of positive cells and intensity of staining, when compared to 7 and 14 day MR.

4.2.5.4 TNF-α expression

TNF-α has also been implicated in the induction of apoptosis. We therefore used the above method to ascertain its expression in AE and found a striking uniformity of high TNF-α expression, predominantly in upper dermal cells with dendritic/stellate morphology, in all the sections examined (n=7; Plate 17D-F). These cells were of similar morphology to those staining with the Dianova anti-fibroblast antibody both in 7 and 14 day MR (see section 3.2.10) and in AE (see section 4.2.10) and may represent activated DDCs or fibroblasts.

Epidermal KC expressed moderate, and endothelial cells moderate to strong cytoplasmic TNF-α (Plate 17D). In all sections large numbers of interstitial and perivascular cells with strong cytoplasmic expression were present in the

papillary and upper reticular dermis. A majority of these cells were of stellate or dendritic morphology (Plate 17D- F). In 5/7 sections strongly positive spindle and dendritic cells were also scattered throughout the deeper reticular dermis. In the epidermis, occasional strongly positive DC were present (Plate 17D &E), and in one subject positive lymphocytes were observed in an area of epidermal spongiosis. In all subjects a majority (85-95%) of perivascular cells comprising large stellate and DC and small lymphocytes strongly expressed cytoplasmic TNF-α, in addition some lymphocytes with surface staining were observed (Plate 17D and Table 4.1).

4.2.6 IL-2R γ CHAIN SIGNALLING CYTOKINES

Of the γ-chain signalling cytokines, IL-15 and IL-2 are the most effective at rescuing T cells from apoptosis in humans (9), and IL-15 is additionally known to promote T cell chemotaxis and proliferation (81-83,242). Our previous findings suggested that this cytokine in particular, is involved both in the induction and the resolution phase of Mantoux reactions. Since high levels of T cell Bcl-2 were detected in chronic lesional AE, we were interested to know the relative expression of the IL-2R γ chain signalling cytokines in these lesions.

4.2.6.1 Distribution of IL-15

Some variability in IL-15 expression occurred between subjects with AE (n=9).In 7/9 subjects however, the distribution and intensity of staining obtained resembled those in 3 and 7 day MR (Plate 18A-D and Table 4.1).

In 4/9 subjects epidermal KC showed cytoplasmic expression (Plate 18A) and numerous strongly positive epidermal DC were present (Plate 18B). In addition, large numbers of DC with strong cytoplasmic IL-15 expression were present within dermal papillae (Plate 18A). In the papillary and upper reticular dermis a majority of perivascular cells (75-90%) strongly expressed IL-15 (Plate 18C).

Table 4.1 CYTOKINE EXPRESSION IN PERIVASCULAR AREAS IN ATOPIC ECZEMA

	TNF-α	IL-15	IL-2	IL-7	IL-4	IFN-γ	IL-6	TGF-β
% perivascular expression†	89.4%	54.3%	14.8%	8%	52.7%	49.5%	40.7%	79.4%
Range	85-95%	20-90%	0-35%	5-15%	25-80%	20-80%	20-70%	65-90%
Intensity*	+++	++/+++	+/-	+	++/+++	+	+/-	++/+++

† Both cells with membranous and cytoplasmic staining were taken into account when calculating the mean and range percentages of positive perivascular cells.

* Intensity of staining was graded as follows: +/- = very weak + = weak, ++ = moderate, +++ = strong

These cells comprised large oval and dendritic cells with cytoplasmic staining and small lymphocytes with membrane staining. Intensity of staining and distribution of IL-15 in these subjects resembled maximal IL-15 expression on day 3 of the MR.

In 3/9 subjects epidermal KC showed moderate-strong cytoplasmic staining, but fewer interstitial and perivascular cells exhibited cytoplasmic IL-15. However, a majority of perivascular lymphocytes (50-65%) expressed membrane bound IL-15 similarly to results obtained in the 7 day MR specimens.

In the remaining 2 subjects epidermal KC and perivascular cells were largely negative (<5% positive cells). Occasional DC with strong cytoplasmic staining were present in the epidermis and in the interstitium of the papillary dermis.

4.2.6.2 Distribution of IL-2

KC showed little or no staining with IL-2. In the papillary dermis a minority of perivascular cells expressed weak membranous IL-2 and occasional large cells with moderate cytoplasmic expression were present (0-35% of cells, Plate 18D & Table 4.1). No strongly positive cells were identified. IL-2 expression was therefore markedly down-regulated in chronic lesional AE (n=9) when compared with the height of the MR (3 and 7 days).

4.2.6.3 Distribution of IL-7

In all AE subjects examined (n=5) strong IL-7 expression occurred in the basement membrane zone of the epidermis and hair follicle epithelium, as it did in normal skin and MR.

Very occasional positive interstitial cells with weak cytoplasmic staining were

present. Within perivascular areas 5-15% of cells expressed IL-7. Very occasional, strongly positive, large, round cells with cytoplasmic staining were present. A majority of the positive cells exhibited variable degrees of membrane, rather than cytoplasmic staining (Plate 18E and Table 4.1).

Much less IL-7 expression was present in the AE specimens when compared with 3,7 or 14 day Mantoux reactions

4.2.6.4 Distribution of IL-4

Considerable inter-subject variability in IL-4 expression occurred, however, as with IL-15, subjects in whom epidermal KC staining was more intense also exhibited greater dermal IL-4 expression. 25-80% of perivascular cells expressed IL-4, however, the intensity of expression in all subjects was less intense than in 3, 7 and 14 day MR (n=8, Table 4.1).

In 4/8 subjects strong cytoplasmic KC IL-4 expression was present and strongly positive DC were present within the dermal papillae. In perivascular infiltrates in the papillary and upper reticular dermis a minority of cells strongly expressed cytoplasmic and a majority expressed membranous IL-4 (55-80%, Plate 18F).

In 1/8 subjects moderate KC IL-4 expression was observed and occasional lymphocytes with strong cytoplasmic staining, but no membrane staining were present in perivascular infiltrates.

In the remaining 3 subjects, KC were only weakly positive, and a minority of cells with surface staining (10-25%) were present in perivascular infiltrates. In these subjects large round cells with indistinct margins and strong cytoplasmic staining, which may represent mast cells, were observed (Plate 18G).

Plate 18 IL-2R γ chain cytokine expression in atopic eczema

A biotin/streptavidin method was used in to determine the cutaneous distribution of IL-15, IL-2, IL-7 and IL-4 in AE (x400 magnification unless otherwise stated).

A-C IL-15 expression

A IL-15 expression in dendritic cells in the dermal papillae (subject 6).

B Moderate keratinocyte and strong Langerhans cell IL-15 expression (subject 5).

C IL-15 expression in a majority of perivascular cells (subject 4).

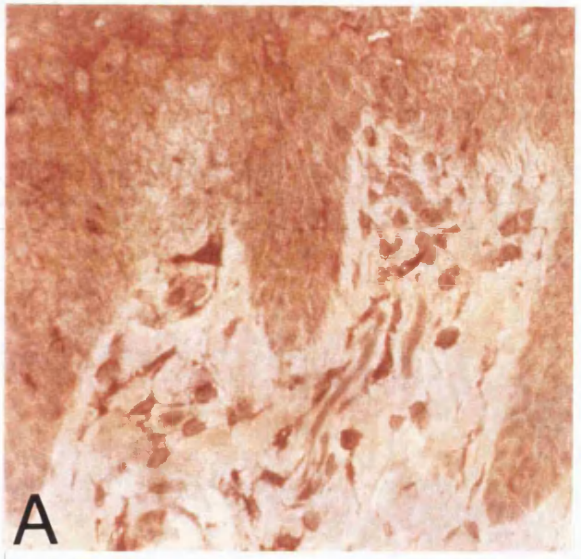
D Very low levels of IL-2 expression (subject 5, x250 magnification).

E Perivascular lymphocytes expressing weak surface staining with IL-7 (subject 7).

F Marked IL-4 expression in keratinocytes, perivascular cells and interstitial dendritic cells (subject 6).

G Strong cytoplasmic IL-4 expression in large perivascular cells (subject 2).

PLATE 18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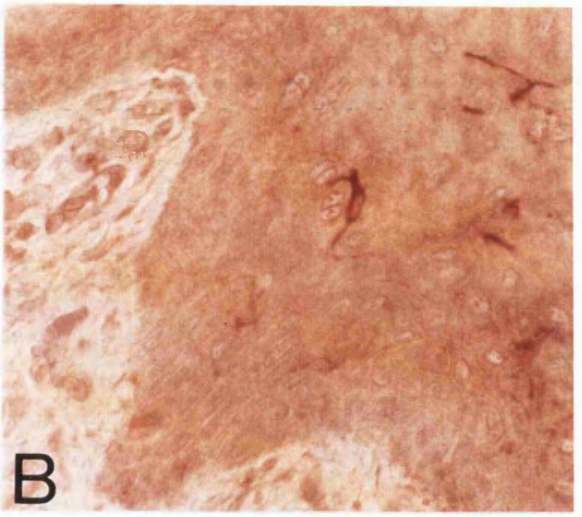
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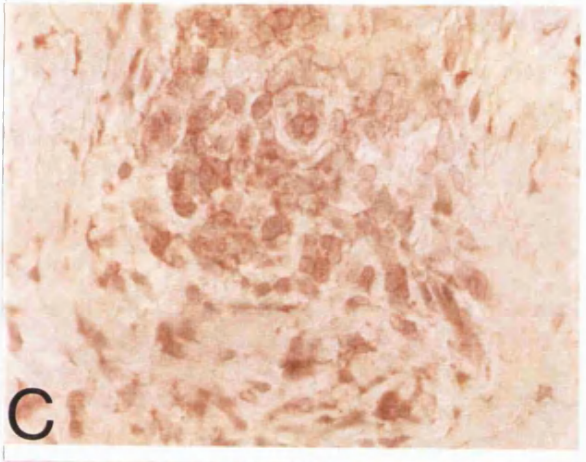
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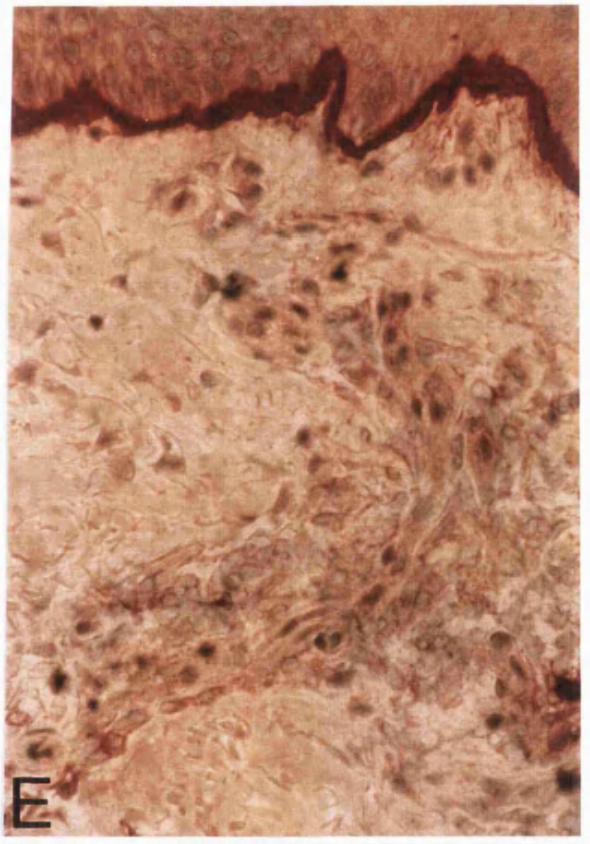
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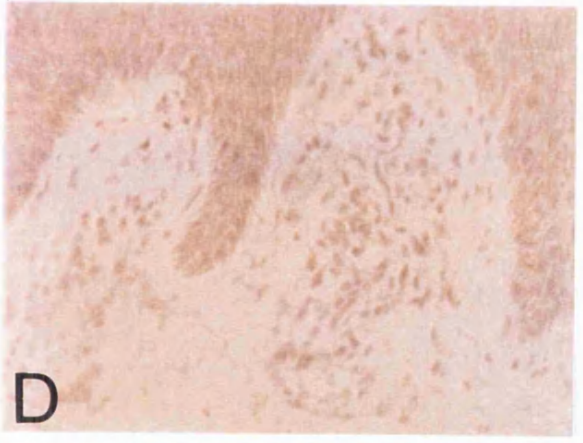
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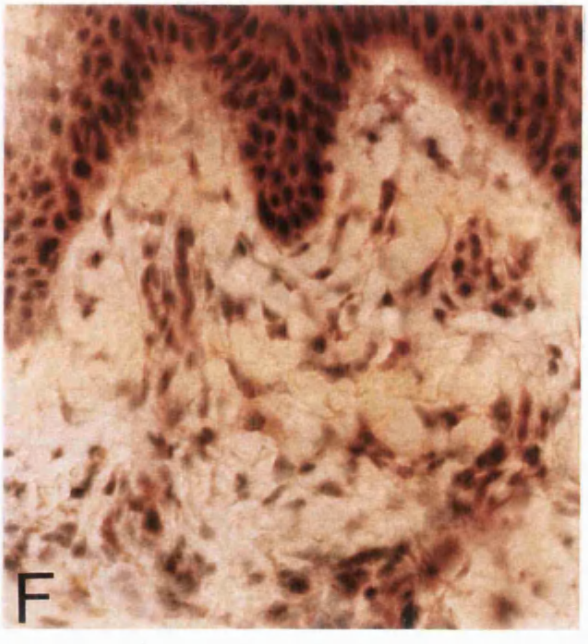
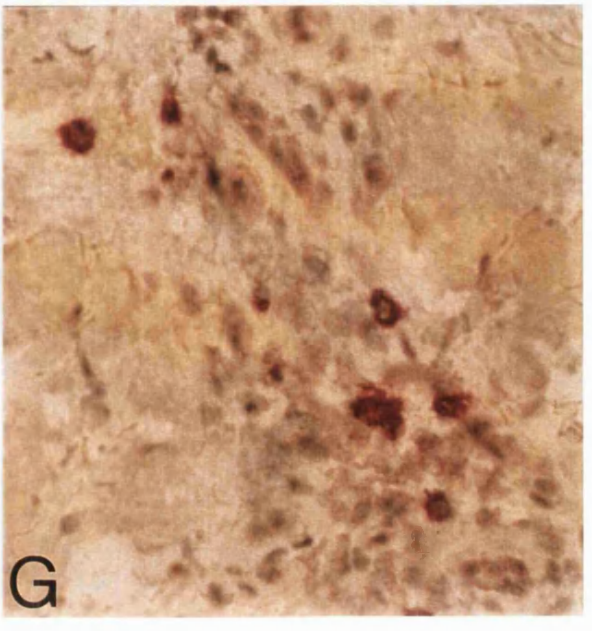

F


G

4.2.7 DISTRIBUTION OF IFN-γ

In view of the conflicting reports regarding IFN-γ expression in chronic AE we were interested to compare its levels and distribution in AE with those in DTH responses. In all AE subjects examined (n=7) intensity of IFN-γ expression was reduced in perivascular lymphocytes compared to 7 day Mantoux reactions. IFN-γ was variably down-regulated on day 14 of the MR, and levels of expression in AE subjects were greater than in some MR subjects at this time point.

IFN-γ was expressed by epidermal KC and strongly by endothelial cells in all subjects examined (Plate 19A & B). In the papillary dermis occasional strongly positive cells with dendritic morphology were present (Plate 19A), often just below the dermo-epidermal junction. Marked inter-subject variability occurred with 20-80% of perivascular cells showing some positivity (Table 4.1). However, only very occasional cells with cytoplasmic staining were seen and for the most part these positive cells were lymphocytes (morphologically) exhibiting weak-moderate membrane staining (Plate 19B).

4.2.8 DISTRIBUTION OF IL-6

In order to further support the notion that high IL-15 expression may be contributing to chronicity, and for comparison with the DTH responses, the expression of IL-6 (n=6), which does not signal via the IL-2R was investigated.

Epidermal KC showed weak or no cytoplasmic IL-6 expression. In 3/6 subjects weakly positive DC were present in the papillary dermis, and weak cytoplasmic and membrane staining were present in 40-70% of cells in perivascular areas (Plate 19C and Table 4.1). In 3/6 subjects variable numbers of large round cells with strong cytoplasmic IL-6 expression were

present scattered within the papillary dermis and in perivascular areas, but fewer other cells (20-40%) were positive (Plate 19D). This pattern of staining was also obtained in the DTH responses and may be occurring in mast cells or neutrophils.

Thus, much lower levels of IL-6 expression were present in the AE specimens examined, than in 7 and 14 day MR. The distribution and intensity of staining varied between subjects but was similar to those observed in 12 and 72 hour MR.

4.2.9 DISTRIBUTION OF TGF-β

As with TNF-α there was marked uniformity in distribution and intensity of staining with TGF-β in AE subjects (n=9), and levels were similar to those in 7 day MR.

Epidermal KC showed moderate-strong cytoplasmic TGF-β expression in all subjects. Strong cytoplasmic staining was present in numerous interstitial cells, a majority of which were dendritic or spindle-shaped, in the papillary and upper reticular dermis (Plate 19E). In perivascular areas a large majority of cells expressed cytoplasmic or membranous TGF-β (65-90%), although relative proportions of the latter varied between subjects (Plate 19F and Table 4.1).

Plate 19 IFN-γ , IL-6 and TGF-β expression in atopic eczema

A biotin/streptavidin method was used in to determine the cutaneous distribution of IFN-γ , IL-6 and TGF-β in AE (x400 magnification unless otherwise stated).

A IFN-γ expression in keratinocytes, endothelial and occasional dendritic cells (subject 9).

B Weak-moderate surface staining with IFN-γ in perivascular cells (subject 4).

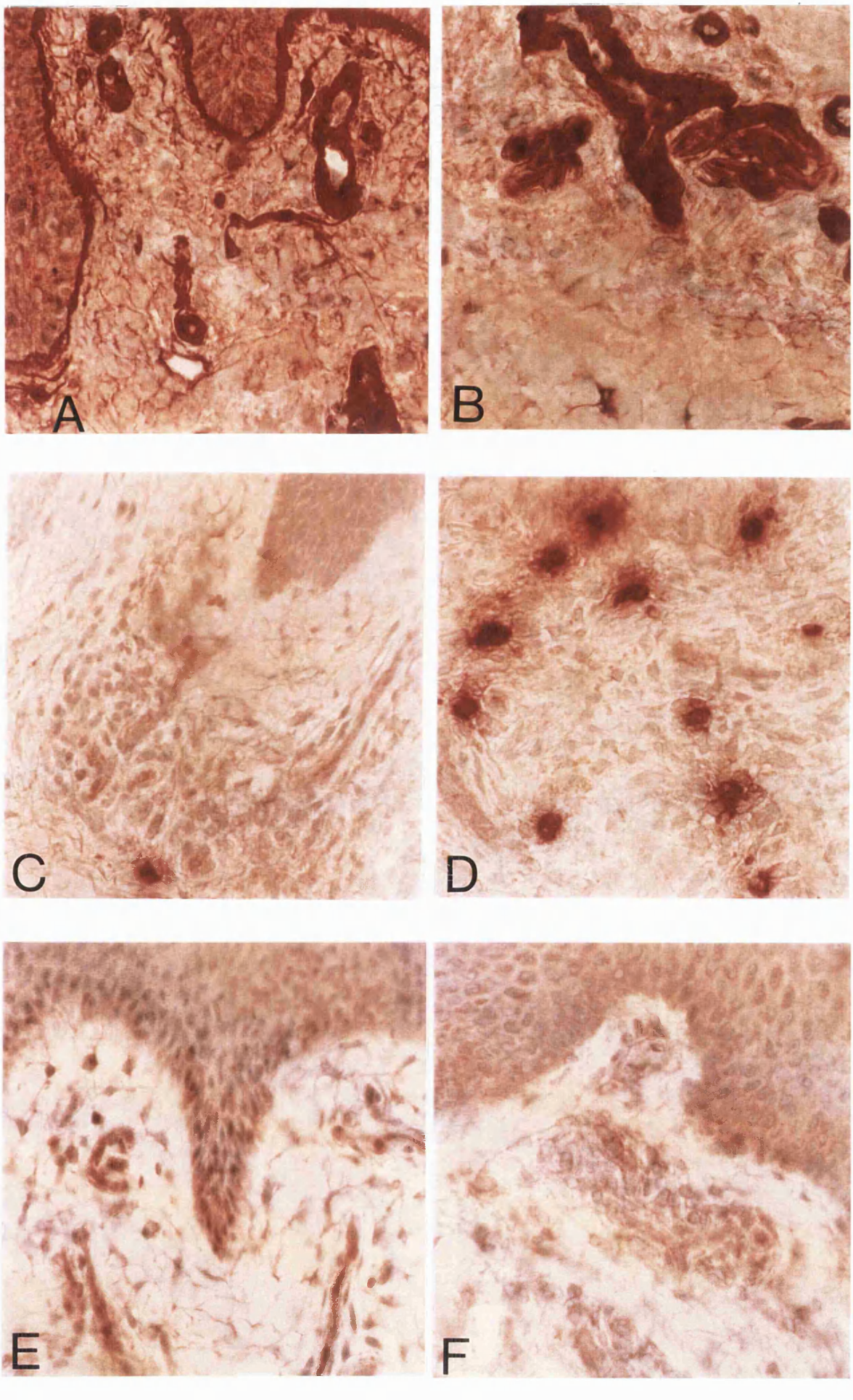
C Weak IL-6 expression in keratinocytes, interstitial dermal dendritic cells and perivascular areas (subject 6 x250 magnification).

D Strong cytoplasmic IL-6 expression in large round perivascular cells (subject 4).

E TGF-β expression in keratinocytes, endothelial cells and interstitial dendritic/spindle cells (subject 6).

F Moderate cytoplasmic and membranous perivascular TGF-β expression (subject 7).

PLATE 19



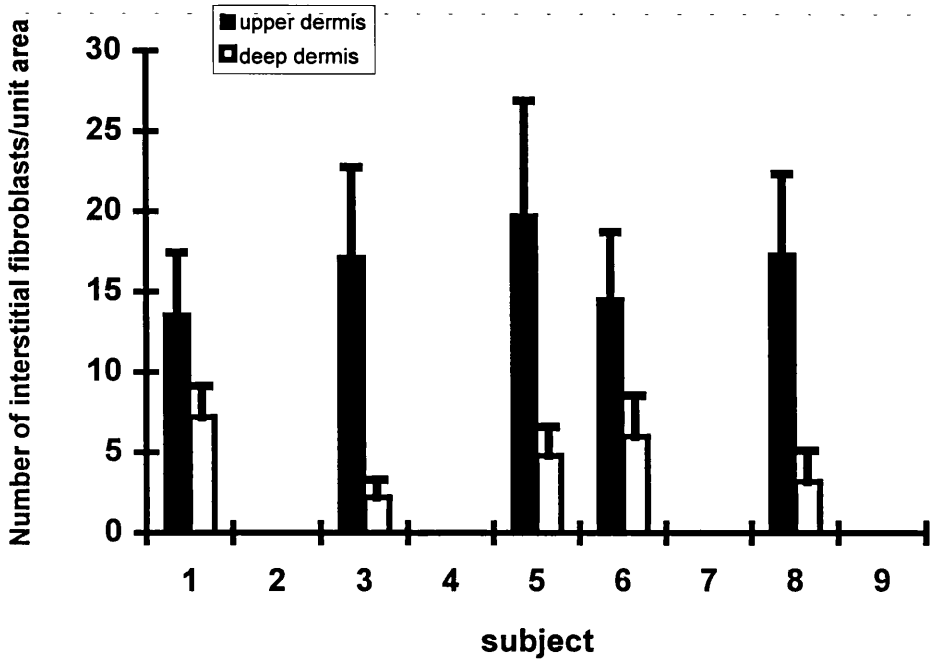
4.2.10 CHARACTERISTICS OF INFILTRATING FIBROBLASTS

4.2.10.1 Fibroblast numbers

- Dianova antibody

Indirect immunoperoxidase staining with the Dianova anti-fibroblast antibody (Dia 110) revealed an extremely dense infiltrate of positive spindle-shaped and dendritic cells present throughout the papillary and upper reticular dermis in all AE subjects (n=9, Plate 20A). Large numbers were present perivascularly and scattered throughout the interstitium (Plate 20B). In the deeper reticular dermis, in contrast to the small numbers of T cells observed, large numbers of positive cells were present both perivascularly and in the interstitium (Plate 20C). Again because of the difficulty in clearly distinguishing labelled cells in perivascular areas with the immunoperoxidase technique, positive cells with identifiable nuclei were counted in the interstitial areas both in the upper and lower dermis. In the upper dermis numerous dendritic processes, not obviously "attached" to a cell body, were also identifiable, suggesting that numbers of Dia 110+ cells present were greater than those which were counted. Labelled interstitial cells were quantified in 5 subjects, although samples from the remaining 4 subjects showed similar numbers and distribution. In the upper dermis an overall mean of 16.5 +/- 2.5 fibroblasts/UA was detected (range 13.6-19.8/UA; Fig 4.10a). This was significantly greater than the numbers observed in this area in normal skin (4.1 +/- 2.7/UA), and on day 7 (3.8 +/-1.9/UA, p=0.0001 in both cases) and day 14 (11.7 +/- 1.9/UA p=0.005) of the MR (Fig 4.10b). In the deep dermis numbers were smaller (4.7 +/- 2/UA, range 2.2-7.2/UA; Fig 4.10a). Overall analysis revealed that Dia 110+ cell numbers in this area were greater than in normal skin or 7 day MR (3.1 +/- 0.4/UA, and 3.0 +/- 2.7/UA respectively, p=0.01 in both cases), and resembled those present on day 14 of the MR (5.85 +/- 2.2/UA p=0.08).

a)



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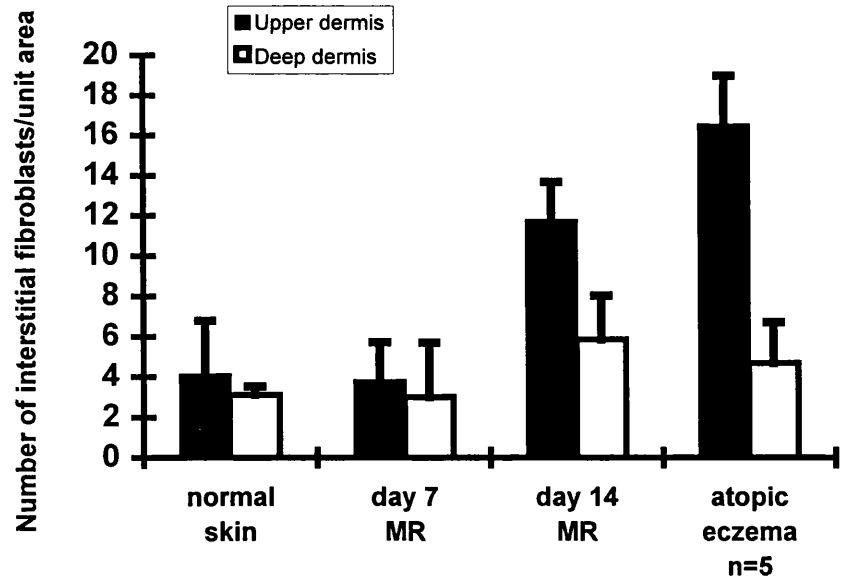


Figure 4.10 Mean interstitial Dia 110+ fibroblast numbers.

Fibroblasts were stained with Dia 110 by an indirect immunoperoxidase method and quantified in each section, per rectangular frame area centred on areas in between perivascular infiltrates, both in the upper dermis and in the deep dermis using an image analysis system, 5 times per section. Only cells with clearly identifiable nuclei were counted.

a)Interstitial fibroblast number in chronic atopic eczema (AE) subjects (no data was available for subjects 2, 4, 7 & 9), **b)** comparison of interstitial fibroblast number in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

- Cross-reactivity of Dia 110 with Factor XIIIa and RFD1

Dual immunofluorescence studies were performed in three AE subjects with Dia 110 and FXIIIa. Numerous Dia 110 and FXIIIa positive interstitial cells were present, particularly in the papillary and upper reticular dermis. As in DTH responses, the close relationship between cells and dendritic processes made it difficult to quantify dual staining cells accurately. Up to 50% of upper dermal interstitial Dia110+ cells appeared to also be positive for FXIIIa, and greater proportions of FXIIIa+ cells (50-75%) appeared to be dual stained (with Dia 110) than on day 14 of the DTH response (Plate20D). In the deep dermis a smaller proportion of Dia110+ cells (up to 30%) were dual stained with FXIIIa (Plate 20E). In the upper dermis, immunoperoxidase staining with anti-CD34 again showed positivity in endothelial cells, but only very occasional positive perivascular or interstitial cells were detected (data not shown), suggesting that, as in DTH responses, the FXIIIa+ cells were unlikely to be fibrocytes.

In view of the cross reactivity observed in DTH responses RFD1 expression was also investigated in 3 AE subjects. On immunoperoxidase staining RFD1+ cells were observed in perivascular and interstitial areas in the upper reticular and papillary dermis (Plate21A). In contrast to the findings in DTH responses, occasional positive cells were also present in the deep reticular dermis. Dual immunofluorescence studies showed that up to 20% of Dia110+ cells also stained with RFD1 (Plate 21B), a higher proportion than in resolving DTH responses.

In summary, significant cross-reactivity was again demonstrated with the Dia 110 antibody. The higher proportions of dual stained cells, particularly in the upper dermis (50% FXIIIa+, 20% RFD1+) compared to day 14 of the DTH response may reflect the presence of greater numbers of DDCs in AE lesions.

Plate 20 Fibroblast staining with Dia 110 and specificity of the Dia 110 antibody in atopic eczema

A-C An indirect immunoperoxidase method was used to stain fibroblasts with Dia110.

A Low power view showing large numbers of positive perivascular and interstitial cells (subject 5 x160 magnification).

Large numbers of positive interstitial spindle and dendritic cells in the upper **B** and deep dermis **C** (subject 5)

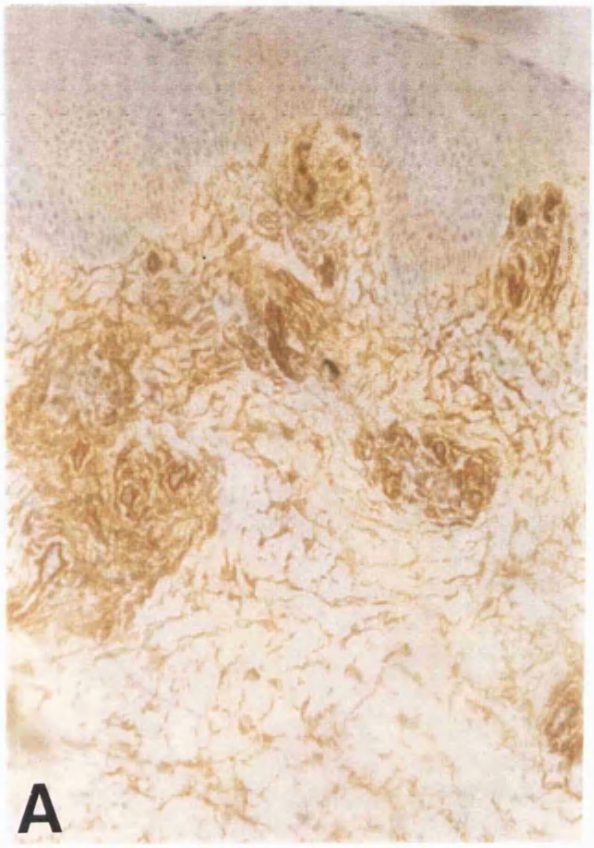
D & E Dual immunofluorescence studies were employed to label Dia110+ cells with TRITC and FXIIIa+ cells with FITC.

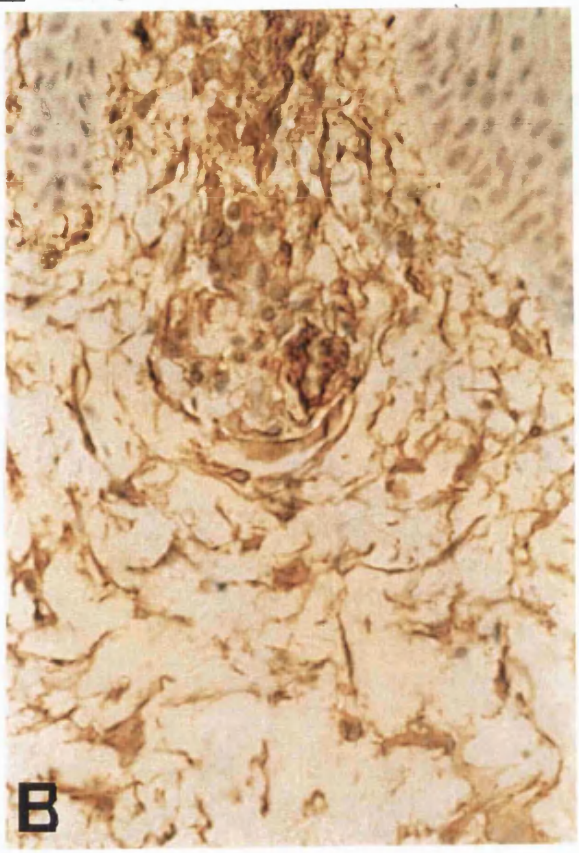
D Up to 50% of Dia110+ upper dermal interstitial cells (red) also stained with FXIIIa (green) [subject 1].

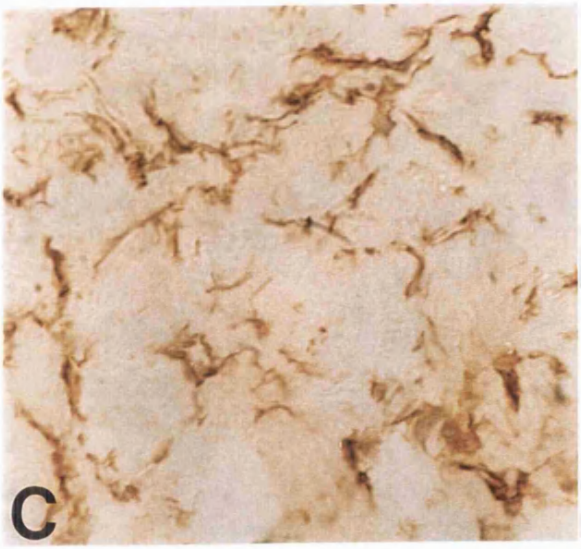
E Smaller proportions of dual stained cells in the deep dermis (subject 6).

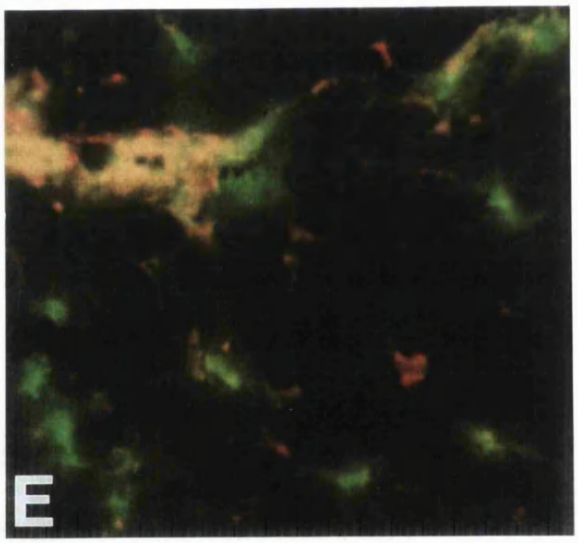
(x 400 magnification unless otherwise stated)

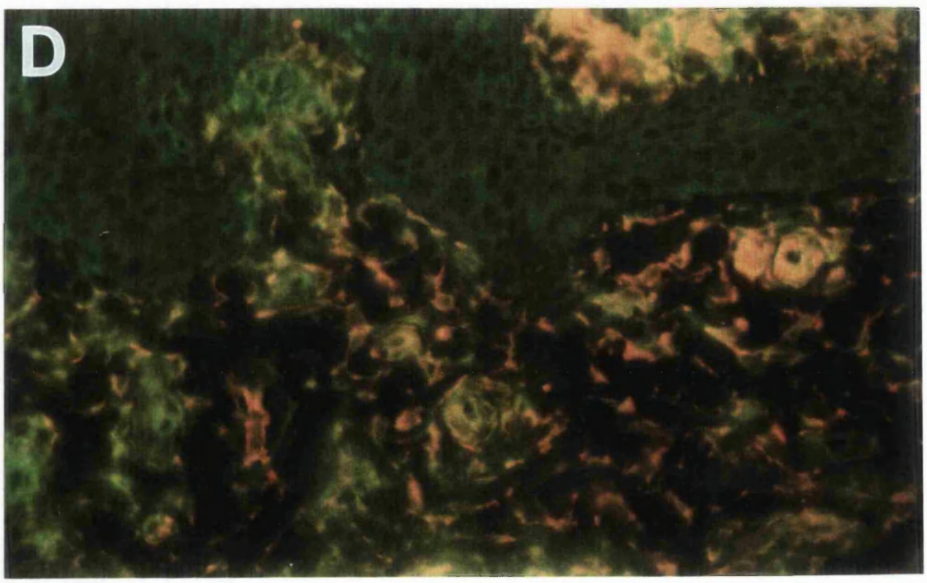
PLATE 20











- Staining with other anti-fibroblast antibodies

Sufficient material was available to stain for fibroblasts with the LHF5 and LHF4 antibodies in 3 AE subjects. As in DTH responses, these antibodies appeared to stain slightly different cell populations. LHF5 positivity was observed in both spindle shaped cells and larger cells with more granular cytoplasmic staining. A majority were interstitial and within the papillary dermis, small numbers were also observed in perivascular areas. With the LHF4 antibody greater numbers of predominantly spindle shaped cells were detected in interstitial areas of the upper dermis. Again, neither of these antibodies stained the large numbers of stellate/dendritic cells observed in the interstitium with the Dianova antibody. This may reflect the fact that all three antibodies stain different subpopulations of fibroblasts, or that the Dianova antibody lacks specificity.

Numbers of positive interstitial cells were quantified in the upper and deep dermis as before. With LHF5, one subject showed similar numbers of positive upper dermal interstitial cells as with LHF4 (8.8 +/-2,2/UA, Plate 21C). In the other 2 subjects few positive cells were identified (3.2 +/- 1.8/UA and 3.8 +/- 1.9/UA, Fig 4.11). Since LHF5 predominantly stains post-mitotic, quiescent cells, this may reflect the low levels of fibroblast proliferation observed (see below). There was less variability with LHF4. With this antibody, the mean number of positive cells in the upper dermis in AE (n=3) was similar to those on day 14 of the DTH response (8.3+/- 0.3/UA Vs 8.2 +/- 2.9/UA respectively) and greater than in normal skin (1.4+/- 0.5/UA), 12 hour (0.6 +/- 0.9/UA) and 72 hour (2.2 +/-0.8/UA) DTH responses (Fig 4.12 and Plate 21D). Lower numbers of positive cells were detected in the deep dermis with both antibodies (Fig 4.11 & 4.12 and Plates 21D & E). In view of the small number of subjects examined, no statistical analyses were performed on this data. However, collectively these findings suggest that greater numbers of fibroblasts were present in chronic AE lesions than in normal skin or the induction phase of the MR.

Plate 21 RFD1/ Dia110 cross reactivity and staining with LHF5 and LHF4 anti-fibroblast antibodies in atopic eczema

A RFD1+ cells in perivascular and interstitial areas in the upper dermis (indirect immunoperoxidase method, subject 6 x250 magnification).

B RFD1+ cells were labeled with TRITC and Dia110+ cells with FITC. Up to 20% of Dia110+ cells (green) were also positive for RFD1 (red), subject 6, x400 magnification.

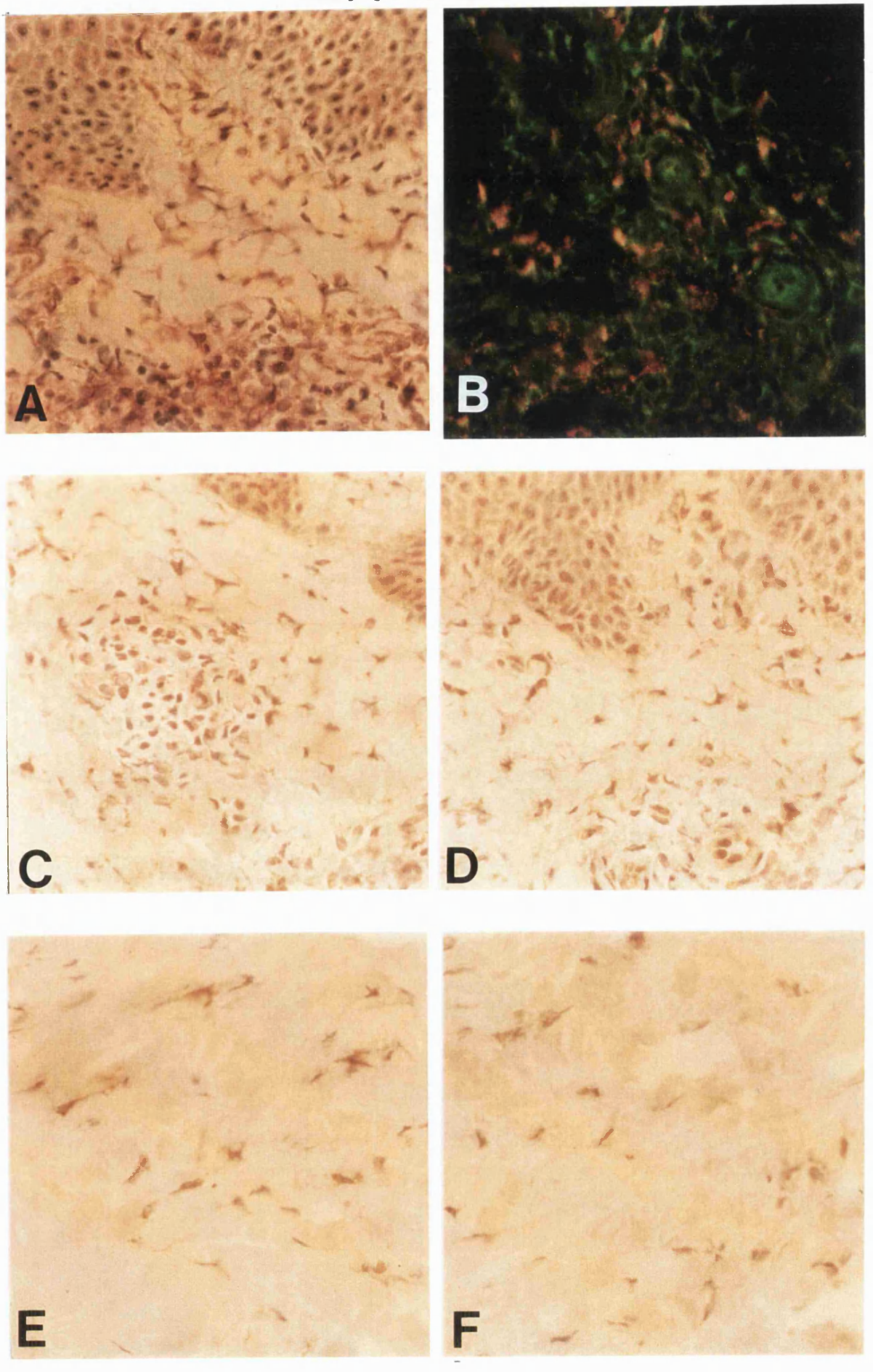
C-F An indirect immunoperoxidase method was used to stain fibroblasts with LHF5 and LHF4 (x400 magnification).

C greater numbers of upper dermal positive cells with LHF5 in subject 1.

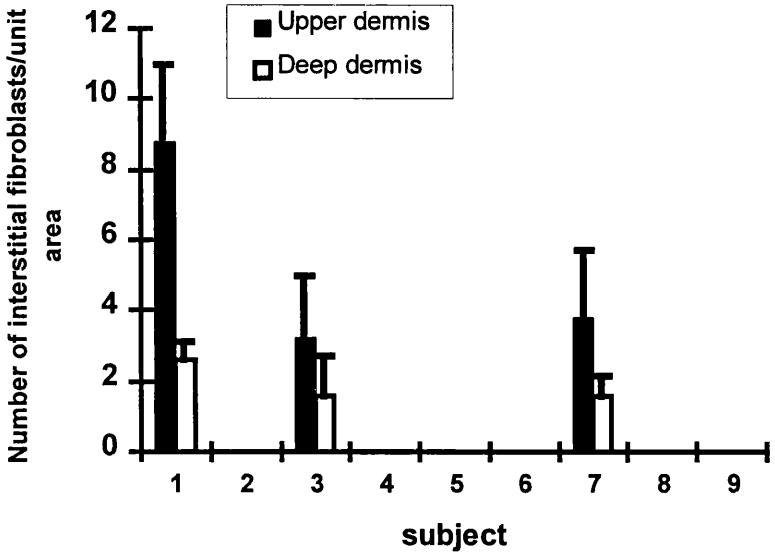
D Upper dermal positivity with LHF4 in the same subject.

Positive cells in the deep dermis, **E** with LHF5 and **F** with LHF4 (subject 1).

PLATE 21



a)



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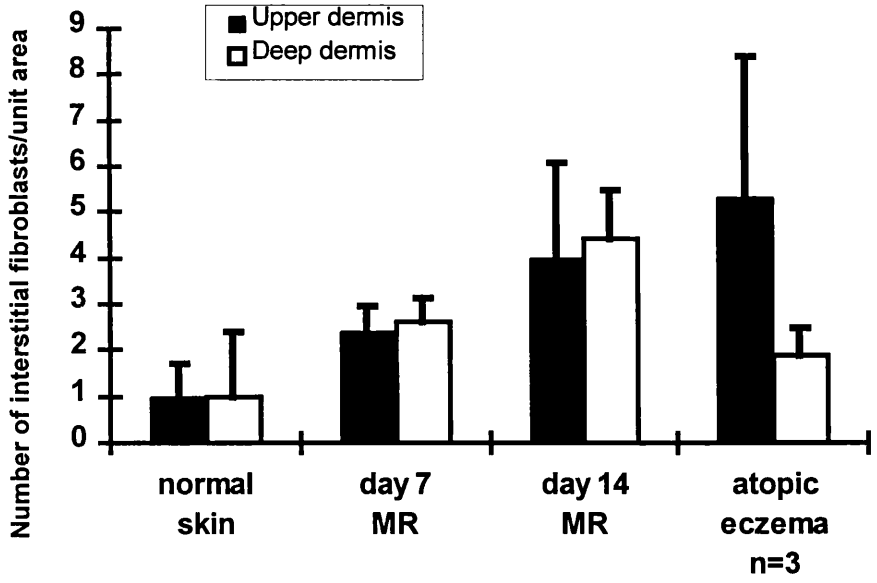
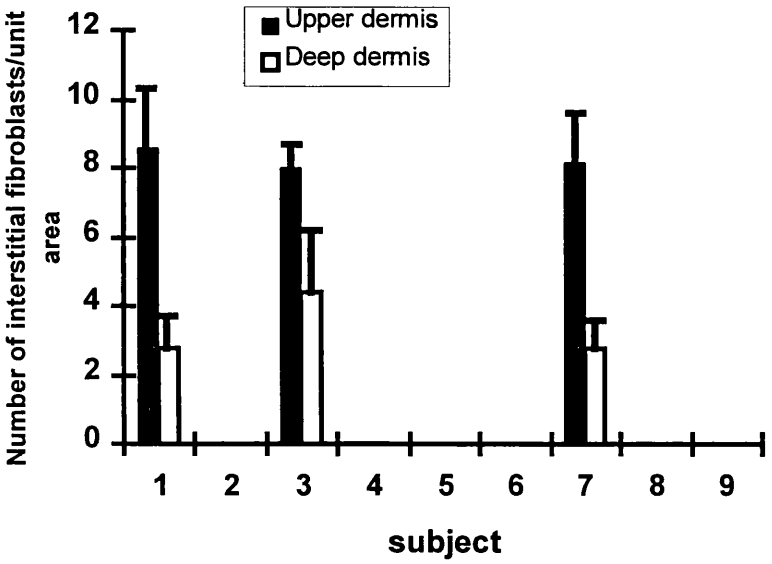


Figure 4.11 Mean interstitial LHF5+ fibroblast numbers.

Fibroblasts were stained with LHF5 by an indirect immunoperoxidase method and quantified in each section, per rectangular frame area centred on areas in between perivascular infiltrates, both in the upper dermis and in the deep dermis using an image analysis system, 5 times per section. Only cells with clearly identifiable nuclei were counted.

a)Interstitial fibroblast number in chronic atopic eczema (AE) subjects (data was only available for subjects 1, 3 & 7), **b)** comparison of interstitial fibroblast number in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

a)



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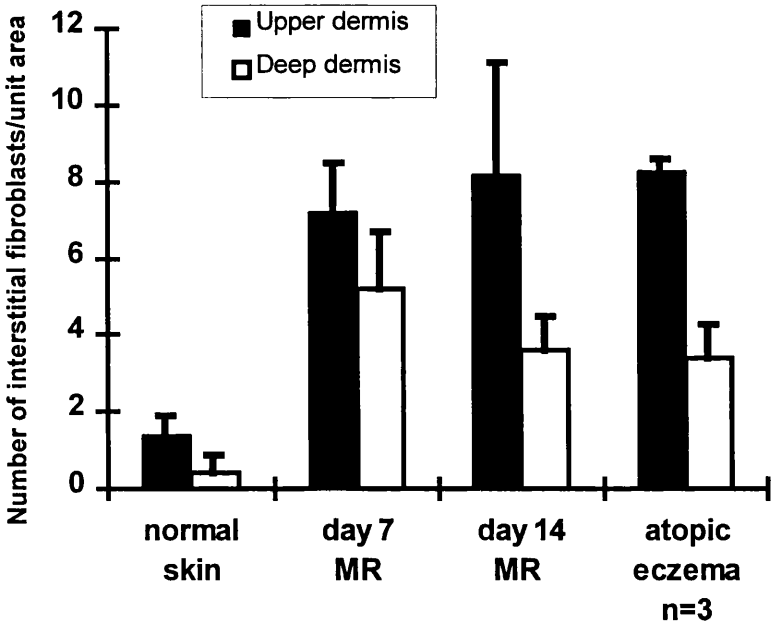


Figure 4.12 Mean interstitial LHF4+ fibroblast numbers.

Fibroblasts were stained with LHF4 by an indirect immunoperoxidase method and quantified in each section, per rectangular frame area centred on areas in between perivascular infiltrates, both in the upper dermis and in the deep dermis using an image analysis system, 5 times per section. Only cells with clearly identifiable nuclei were counted.

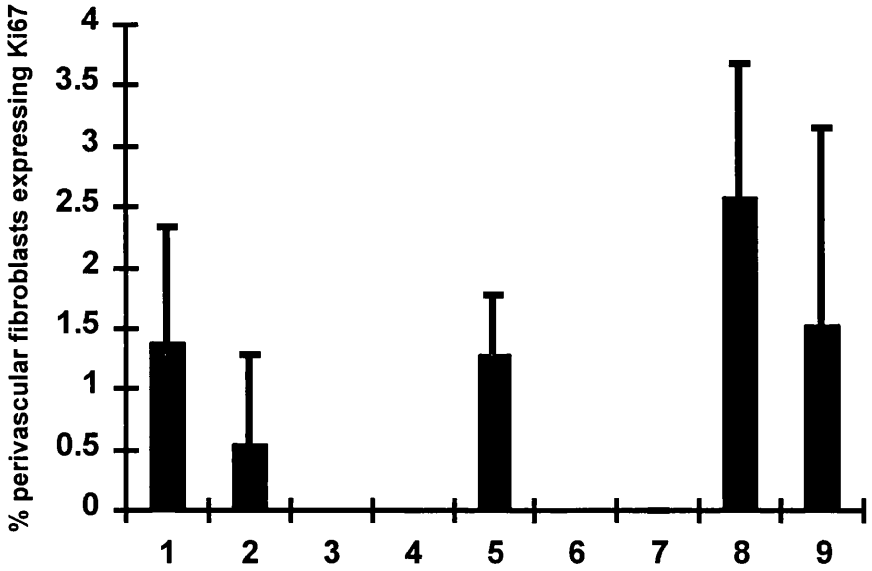
a) Interstitial fibroblast number in chronic atopic eczema (AE) subjects (data was only available for subjects 1,3 & 7), **b)** comparison of interstitial fibroblast number in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

4.2.10.2 Fibroblast proliferation

As in the DTH responses the Dia 110 antibody was employed in dual immunofluorescence studies with Ki67. These revealed an overall mean of 1.2 +/-0.9% (range 0-2.6% n=6, Fig 4.13a) of Dia 110+ perivascular cells were proliferating. Occasional Ki67+ interstitial Dia110+ cells were also identified (mean 0.6 +/-0.6 Ki67+ Dia110+ cells/high power field, range 0-3). Proliferation was confined to the papillary and upper reticular dermis and not seen in the deeper reticular dermis. This rate of proliferation was not significantly different from that observed in normal skin (0.9 +/-1.5% p=0.59), and was significantly lower than that detected on days 7 and 14 of the MR (5.86 +/-2.7 p=0.0001 and 2.2 +/-1.9 p=0.01 respectively; Fig 4.13b).

In view of the relative lack of specificity of the Dia110 antibody, dual staining was also performed with Ki67 and both the LHF5 and LHF4 antibodies. Sufficient material was available to perform these investigations in 3 AE subjects. Although Ki67 positivity was observed in basal KC and perivascular areas in all sections, no fibroblast proliferation was detected with the LHF5 antibody. With LHF4 occasional proliferating interstitial fibroblasts (<1%) were present in the upper dermis in one subject (subject2), and 4.0+/-3.5% of perivascular fibroblasts were Ki67+ in another (subject 6). In the third subject no fibroblast proliferation was detected (data not shown). These results may reflect, either the small number of subjects examined, or the fact that the three anti-fibroblast antibodies employed label different subpopulations of fibroblasts. It is also possible, in view of the relative lack of specificity of Dia 110, that some proliferating cells detected with this antibody were in fact dermal dendritic cells.

a)



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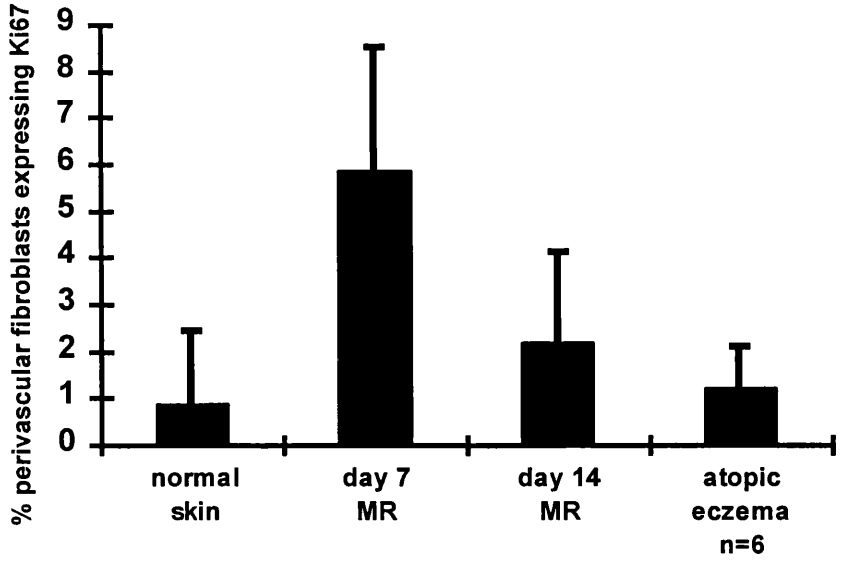


Figure 4.13 Mean percentages of proliferating Dia 110+ fibroblasts in perivascular infiltrates.

Dual IMF studies were performed using Ki67 and the Dia 110 anti-fibroblast antibody. Proportions of double positive cells were quantified in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

a) mean % Ki67+ perivascular fibroblasts in chronic atopic eczema (AE) subjects (n=6,no proliferation was observed in subject 7, no data was available for subjects 3, 4 & 6), **b)** comparison of mean % Ki67+ perivascular fibroblasts in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

4.2.11 DISTRIBUTION OF IFN-β

IFN-β was strongly expressed in KC throughout the epidermis in all sections (Plate 22A-C). Endothelial cells showed moderate-strong cytoplasmic expression and a majority of perivascular lymphocytes had surface staining (Plate 22A). Spindle and dendritic cells, and occasional cells with intense cytoplasmic staining were also identifiable in perivascular areas (Plate 22C). However, in view both of the difficulty in distinguishing these morphologically from other perivascular cells, and of the large number of clearly identifiable IFN-β expressing cells in interstitial areas, we counted the latter in the papillary and upper reticular dermis (the same areas of the "upper dermis" counted in the PPD responses). These IFN-β+ cells were predominantly dendritic and spindle-shaped and of similar morphology to the interstitial cells staining with the Dianova anti-fibroblast antibody (Plate 22A & C). Intensity of staining was greater than in normal skin and appeared similar to, or greater than that observed in cells of similar morphology on day 14 of the MR. IFN-β was also present in the deep dermis and was confined in this area to spindle shaped cells (Plate 22D) . An overall mean of 15.8 +/- 2.3 IFN-β+ cells/UA (range 12.4-18.4 IFN-β+ cells/UA, p=0.17) were present in the upper dermal interstitium paralleling the mean number of cells staining with the Dianova anti-fibroblast antibody in this area (Fig 4.14a). Furthermore, IFN-β expression was significantly greater than in normal skin, and than on day 7 (p=0.0001 in both cases) and day 14 (p=0.002) of the MR (Fig 4.14b). As in DTH responses, it is likely that both activated fibroblasts and DDCs provide a source of IFN-β in chronic AE.

Plate 22 IFN-β expression in atopic eczema

A biotin/streptavidin method was used in to determine the cutaneous distribution of IFN-β in AE. (x400 magnification).

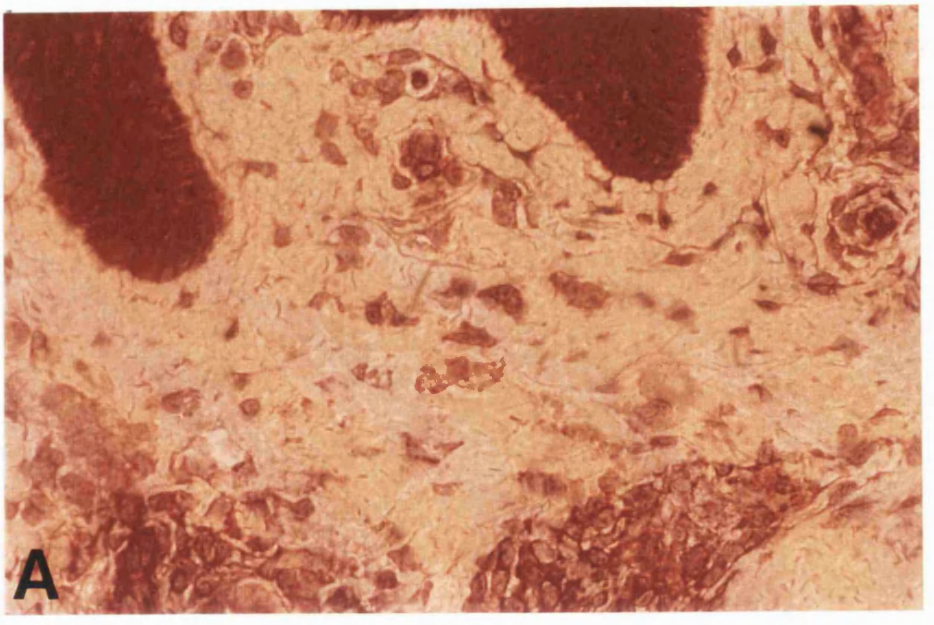
A IFN-β expression in keratinocytes, endothelial cells, and surface staining on perivascular lymphocytes.

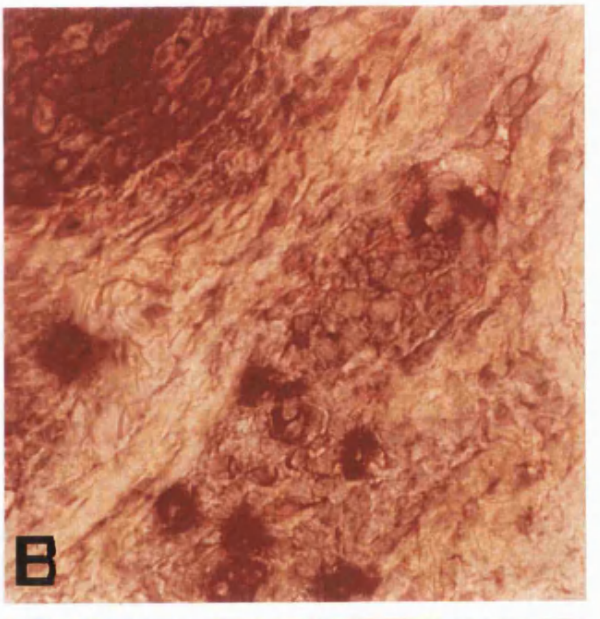
B Positive spindle and dendritic cells, and cells with intense cytoplasmic staining in perivascular areas.

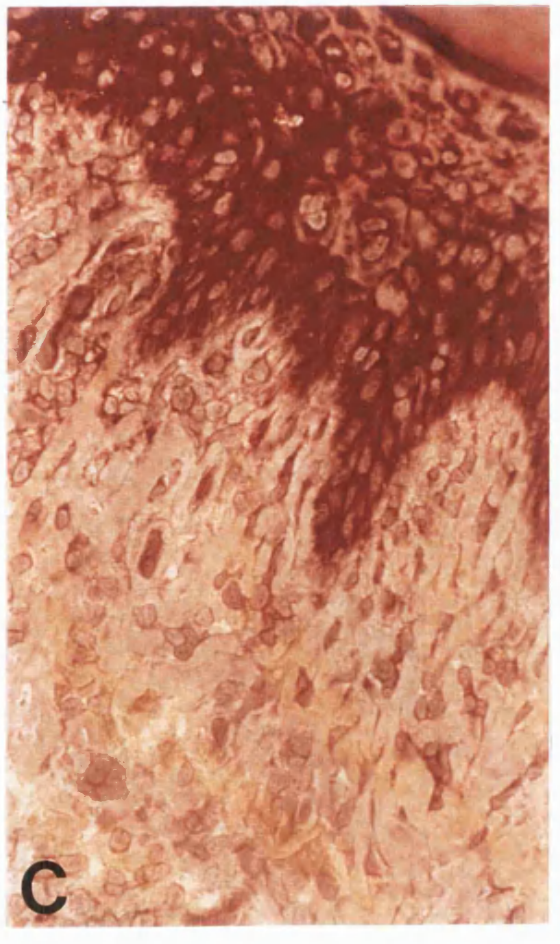
C Positive interstitial dendritic and spindle-shaped cells in the upper dermis.

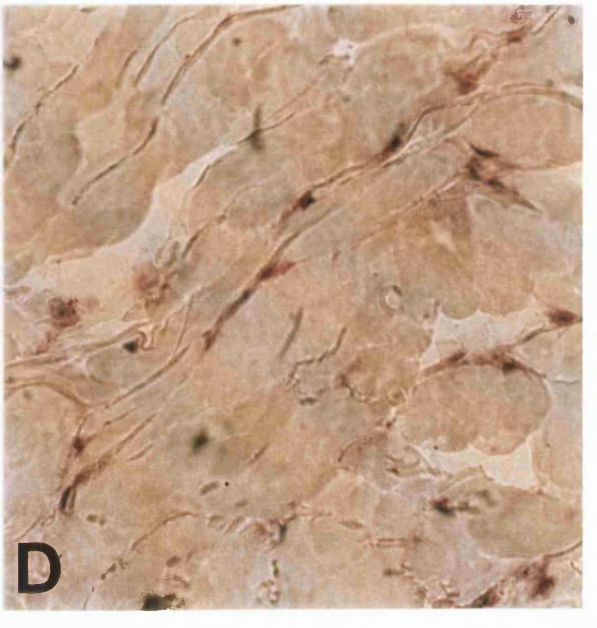
D IFN-β expression in spindle cells in the deep dermis.

PLATE 22









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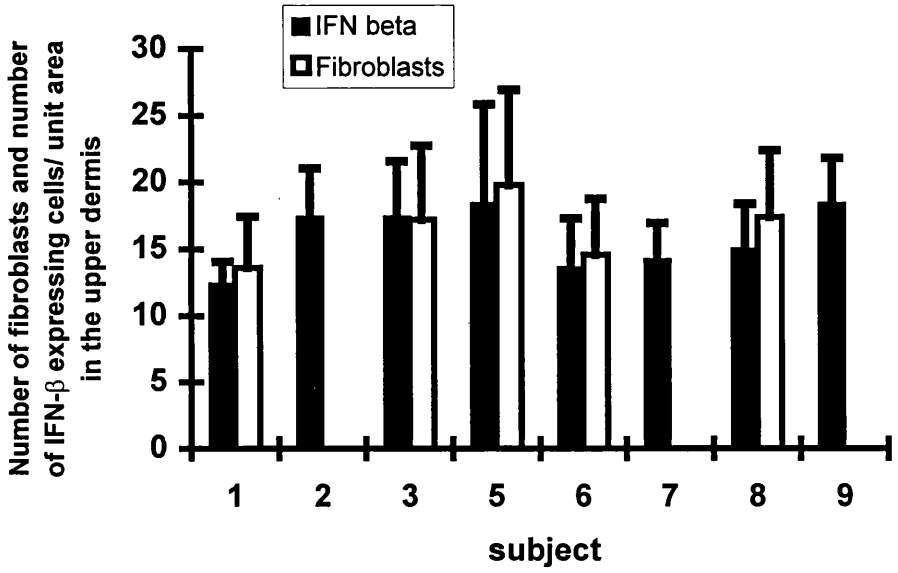

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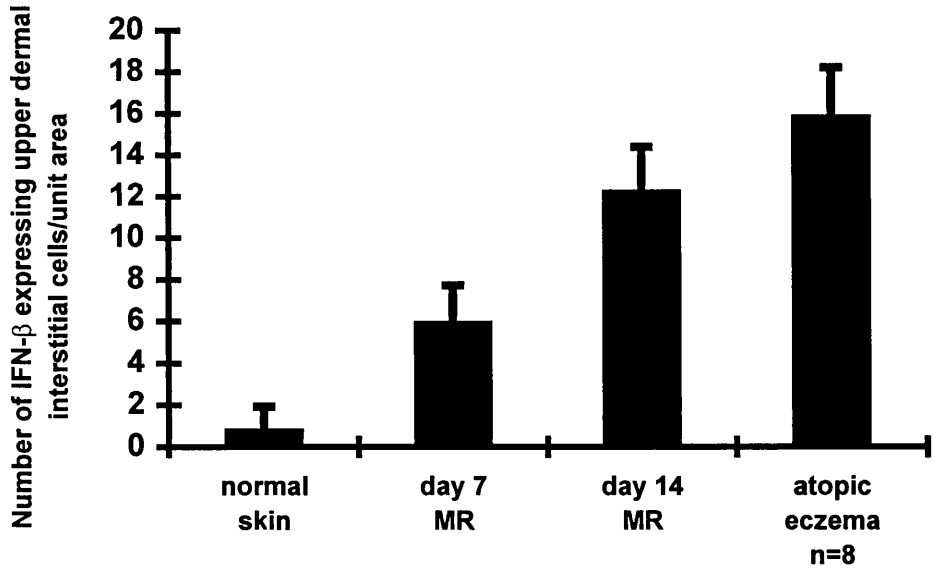

Figure 4.14 Mean numbers of interstitial cells expressing IFN-β in the upper dermis.

A biotin/streptavidin method and an image analysis system were used to determine IFN-β expression in interstitial cells, per rectangular frame area centred on areas in between perivascular infiltrates in the upper dermis, 5 times per section.

a) Comparison of IFN-β expressing interstitial cells and Dia 110+ fibroblast numbers (see Fig 4.10) in the upper dermis in chronic atopic eczema (AE) subjects, **b)** comparison of IFN-β expressing interstitial cells in normal skin, 7 day (peak) and 14 day (resolving) Mantoux reactions (MR), and chronic AE. Error bars indicate standard deviations.

4.3 DISCUSSION

The persistence of infiltrating activated T cells in chronic AE could occur both as a result of ongoing presentation of percutaneously absorbed antigens, and/or as a result of reduced cell clearance. Similarly, inability to clear T cells might be due both to reduced T cell efflux from the lesions and/or to a failure of *in situ* cell death.

The chronic AE lesions examined showed characteristic histologic features (206). The distribution and phenotypic characteristics of infiltrating T cells resembled those observed in MR, supporting the notion that AE represents a type of DTH response (189,210,228). Perivascular inflammatory cell infiltrates were present largely in the papillary and upper reticular dermis. The proportion and overall numbers of T cells in these infiltrates were lower than at the height of the MR and similar to those observed on day 14, during the resolution phase. As in previous studies a majority of T cells expressed CD4 and CD45RO (48,176,189,204), confirming that they were memory rather than naïve cells (28). This is pertinent since these cells are known to be more susceptible to apoptosis than their naïve counterparts (51,169,172,174). Such cells are also known to migrate preferentially into sites of cutaneous inflammation (176), but their accumulation in AE is additionally likely to be due to low-grade (relative to the MR) *in situ* proliferation since a mean of only 6.9% were in cycle.

Of the cytokines known to drive T cells into cycle (79,81,82,84,244), only IL-15 was present in appreciable quantities in our AE subjects, suggesting that as in the MR, this cytokine plays an important role, not only in T cell chemotaxis (242) but also in T cell proliferation within the lesions (81,82). The high levels of IL-15 expression in KC and epidermal and dermal DC in 7/9 subjects are of particular interest, since in humans, this cytokine is the most efficient of the IL-2R γ chain signalling cytokines at rescuing T cells

from apoptosis (9). We found significantly higher levels of T cell Bcl-2 expression (mean 74.5 +/- 8.2%) and lower proportions of apoptotic T cells (mean 0.23 +/- 0.3 %) in our AE subjects than during the resolution phase of the MR. These data are compatible with the possibility that in chronic AE, T cell apoptosis is inhibited as a result of the upregulation of Bcl-2 relative to Bax expression. Since IL-15 was the only γ chain cytokine to be up-regulated (by comparison with peak expression during the Mantoux reaction), this cytokine is implicated in the relative failure of T cell apoptosis in chronic AE. The reason for IL-15 upregulation remains unclear. It could represent a primary genetic abnormality. Alternatively, the high levels of IL-15 expression observed in epidermal and dermal DC might occur as a result of their ongoing activation by percutaneously absorbed antigens via IgE/IgE-receptor mediated mechanisms. It is not known whether such interactions cause IL-15 upregulation, however, recent *in vitro* work in monocytes and monocyte derived DC from AE patients has shown that they are capable of upregulating synthesis of TNF-α and IL-8 after cross-linking of FcεRI on their surface (264).

The possibility that γ chain cytokine withdrawal-mediated apoptosis is dysregulated in AE, is further supported by a recent Japanese study which showed reduced B cell apoptosis, associated with high B cell Bcl-2 expression in AE patients with high serum IgE levels and severe disease (265). It would be of interest, therefore, to know whether a down-regulation of cutaneous IL-15 and T cell Bcl-2 expression occurs in AE patients after successful therapy

Excess IL-15 may not only inhibit cytokine withdrawal-mediated cell death in this context, it may also influence the local production of other cytokines. IL-15 has been shown to induce IL-5 mRNA and protein synthesis in der f II (house dust mite) specific CD4+ T cell clones from atopic patients (266), and may therefore contribute to the high levels of IL-5 previously reported in AE

lesions (223). Furthermore, the presence of IL-15 may influence cutaneous TNF-α expression since IL-15 can induce TNF-α production in synovial T cells and macrophages from rheumatoid joints *in vitro* (80,267).

We were also interested to know whether a dysregulation occurred in the "active" apoptotic pathways which involve the interaction of CD95 and TNF-α with their ligands (7,8,92,93). Despite inter-subject variability, CD95-L expression was markedly reduced in all AE subjects examined, both in terms of the numbers of positive cells and intensity of staining, when compared to 7 and 14 day MR. Although the exact relationship between apoptotic susceptibility and proliferation remains unclear, CD95-mediated death is known to occur predominantly in cycling cells (7,8,116). Our study suggests that this type of cell death occurred predominantly at the height of the Mantoux reaction, when very high levels (19%) of T cell proliferation were observed. It is therefore possible that the lower rates of proliferation observed in AE are insufficient to activate this apoptotic pathway. In addition, since we biopsied AE lesions which had been present for at least two weeks, the possibility that CD95-mediated apoptosis may occur in more acute lesions cannot be excluded. Another possible explanation may be that infiltrating T cells in AE are less able to express CD95-L, as has previously been suggested in T cells which predominantly secrete IL-4 rather than IFN-γ (131). Collectively these data suggest that the reduced lesional CD95-ligand expression observed may contribute to the persistence of the T cell infiltrate in chronic AE lesions.

In comparison to the Mantoux reaction perivascular distribution and intensity of staining with TNF-α was similar to that 7 days after intradermal PPD. However, since numbers of apoptotic cells were low, it seems unlikely that TNF-α provides a major contribution to T cell death in AE. On the contrary, since TNF-α has been implicated in T cell recruitment and the generation of cutaneous inflammation (250), its presence may reflect a pro-inflammatory

role. Intriguingly, the numbers of positive cells and intensity of staining in interstitial dendritic and stellate cells throughout the dermis, and in particular in the papillary dermis, were greater in the eczema subjects than at any time point examined in MR. This excess of intralesional TNF-α may exert a number of effects. It may promote the migration of antigen bearing LC and DDC to regional lymph nodes, resulting in the generation of increased numbers of antigen specific skin-homing T cells (250,268). It may also facilitate their subsequent entry into the skin, since TNF-α and IFN-γ, both of which were expressed in intralesional endothelial cells (EC), can increase E-selectin expression in human dermal microvascular EC, both *in vitro* via a mechanism involving up-regulation of CD40 (269), and *in vivo* (250). In addition, intralesional TNF-α expression have an impact on T cell survival, since it has been shown *in vitro* to up-regulate fibroblast IFN-β production (263) (see below).

Increased fibroblast numbers and proliferation have been implicated in the generation and maintenance of chronic inflammation in the joints of patients with rheumatoid arthritis . Our study suggests that the numbers of infiltrating fibroblasts in the upper dermis in chronic AE were similar to, or greater than those in a resolving cutaneous DTH response. Although rates of proliferation were low, the absolute numbers of infiltrating fibroblasts suggest that proliferation may contribute to their intralesional accumulation. It is of interest, therefore, that both fibroblast proliferation and activation may be promoted by the increased intralesional expression of TNF-α and TGF-β observed (230,262).

Activated fibroblasts express a number of cytokines and chemokines enabling them to participate in leukocyte recruitment at sites of inflammation (230,258). Furthermore, the expression of RANTES and eotaxin by dermal fibroblasts can be up-regulated *in vitro* by TNF-α and IL-4 (259,260), suggesting that in AE, fibroblasts may be involved in the recruitment of

lymphocytes and eosinophils into the lesions, as well as in promoting T cell survival.

The mechanism whereby fibroblasts rescue T cells from apoptosis involves up-regulation of their IFN-β production (12,13,169,178 and 10, submitted). We found greater numbers of IFN-β producing cells in AE than at any time point during the MR. Furthermore, the intensity of IFN-β expression was similar to, or greater than the maximal levels observed in 14 day MR. Fibroblast-mediated lymphocyte survival is thought, under normal circumstances, to generate a population of T cells that are quiescent, less likely to die by apoptosis, and destined to provide long-term memory (12,13,169,261). Such cells can subsequently be re-activated by CD3 antibody in the presence of appropriate co-stimulatory molecules such as CD28 (12). Although the relative contributions of fibroblasts and DDCs to IFN-β production remains uncertain, the presence of excess IFN-β producing cells in chronic AE may contribute to the persistence of inflammation by promoting the survival of increased numbers of infiltrating lymphocytes in a state in which they may subsequently be reactivated without dying. Furthermore, levels of T cell HLA DR expression were significantly reduced compared to those in resolving MR, suggesting that fewer anergic, suppressive T cells may be generated in these lesions (239,241).

In summary, we have shown that the distribution and phenotypic characteristics of infiltrating T cells in chronic AE are similar to those in resolving MR, but that a reduction in normal apoptotic T cell clearance occurs. Low levels of CD95-L expression in chronic AE and in resolving MR suggest that the CD95-mediated apoptotic pathway is not active in these lesions. Two different groups of anti-apoptotic cytokines are now recognised, those of the IL-2R γ chain family and type I interferons. In DTH responses different waves of expression occurred in these two groups of cytokines, at the height and during the resolution phase respectively. In chronic AE,

however, members of both groups, namely IL-15 and IFN-β, were expressed concomitantly. Over-expression of IL-15 may not only contribute to the failure of cytokine-withdrawal mediated death by up-regulating Bcl-2, but also to T cell recruitment and proliferation. In addition, increased fibroblast- and DDC-mediated IFN-β expression may cause excess numbers of T cells to be rescued from apoptosis and maintained in a state in which they can be reactivated and contribute further to the cutaneous inflammatory response.

5. CONCLUSIONS AND FUTURE CONSIDERATIONS

5.1 CONCLUSIONS

- This study suggest that both the induction and resolution phases of the DTH response are controlled by different levels of expression of the same group of anti-apoptotic, IL-2R γ chain signalling cytokines. During the induction phase high levels of expression, initially of IL-15 and later of IL-2 and IL-7, favour T cell recruitment, proliferation, upregulation of Bcl-2 and by inference, survival. During the resolution phase, whilst antigen is cleared and T cells become increasingly differentiated, a reduction in γ chain cytokine and T cell Bcl-2 expression, promote an increased susceptibility of T cells to cytokine withdrawal mediated death.

- Active antigen-driven cell death, mediated by the interactions of CD95 and TNF-R with their ligands is thought to occur in situations of antigenic excess and active T cell proliferation (7,8). The presence of maximal CD95-L and high TNF-α expression on day 7, suggest that AICD is induced at the height of the response. and is the mechanism whereby excessive immune stimulation is prevented.

- Survival of a proportion of T cells destined to establish and maintain memory at the end of the response is favoured by increased expression of IFN-β, a member of the second group of anti-apoptotic cytokines which do not induce proliferation, but maintain T cells in a primed but quiescent state.

- In the DTH response different waves of expression occurred in these two groups of anti-apoptotic cytokines suggesting an interaction between T cells, fibroblasts and dermal dendritic cells during the course of the reaction. This interaction may at least in part be mediated by cytokines such as IL-15, TNF-α, and IFN-β secreted sequentially by cells of different lineages.

- In chronic atopic eczema low levels of apoptosis and CD95-L, and high Bcl-2 expression were observed in a population of CD4+CD45RO+ T cells which one would normally expect to be apoptosis prone (51,169,172).

- Concomitant expression of members of the two different groups of anti-apoptotic cytokines, IL-15 and IFN-β, in this disease may lead to the survival of abnormally large populations of primed T cells and contribute to the persistence of the inflammatory process. These cytokines appear to be derived both from stromal cells, in particular keratinocytes and fibroblasts, and from dermal dendritic cells.

A model for the generation of cutaneous inflammation in AE may therefore be proposed (Fig 5.1) in which persistent percutaneous antigenic challenge results in increased synthesis of IL-15 and TNF-α by keratinocytes and activated epidermal (LC) and dermal dendritic cells (DDC). This would not only promote T cell recruitment, activation and proliferation, but also result in IL-15-mediated up-regulation of T cell Bcl-2. Increased TNF-α expression may additionally induce fibroblast activation, and their synthesis of cytokines such as TGF-β, TNF-α and IFN-β. The factors responsible for increased IFN-β expression in DDC, keratinocytes and endothelial cells remain uncertain. However, the microenvironment thus generated would not only favour the continued recruitment of T cells and promote their survival, but also result in increasing fibroblast numbers, activation and cytokine production, supporting the proposal that T cell-fibroblast interactions are important in the development of persistent inflammation. If such a mechanism operates in situations of chronic inflammation, how is it "switched off" at the end of a "normal", self-limiting immunological response? We have demonstrated that resolution of the Mantoux reaction occurs at least in part as a result of T cell apoptosis. Thus, a combination of antigenic clearance and T cell death may remove the stimuli for dendritic cell and fibroblast activation and subsequently result in downregulation of the response (Fig 5.2).

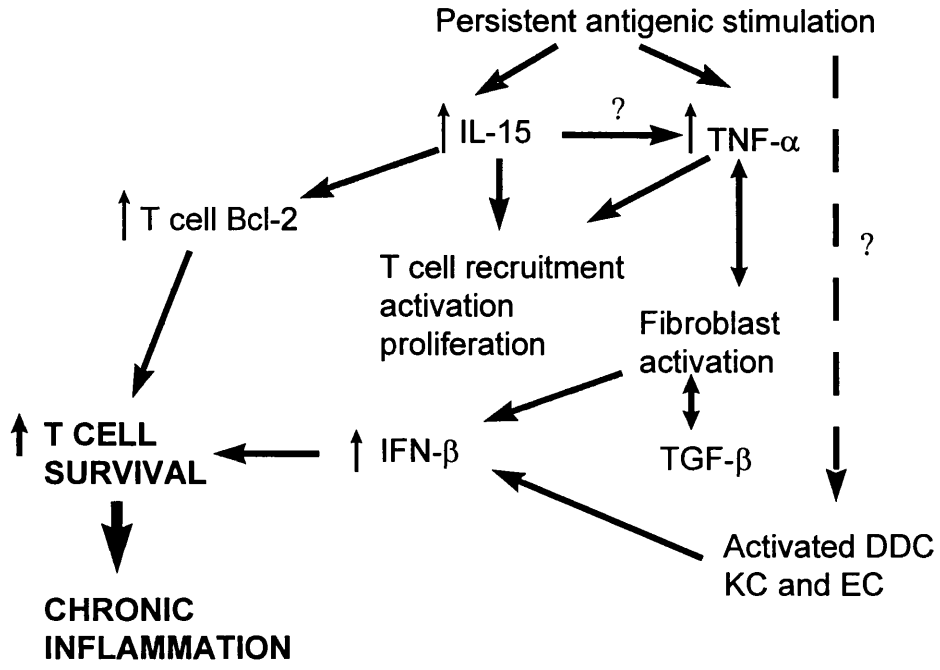

Figure 5.1 Proposed mechanisms involved in the persistence of the T cell infiltrate in situation of chronic inflammation

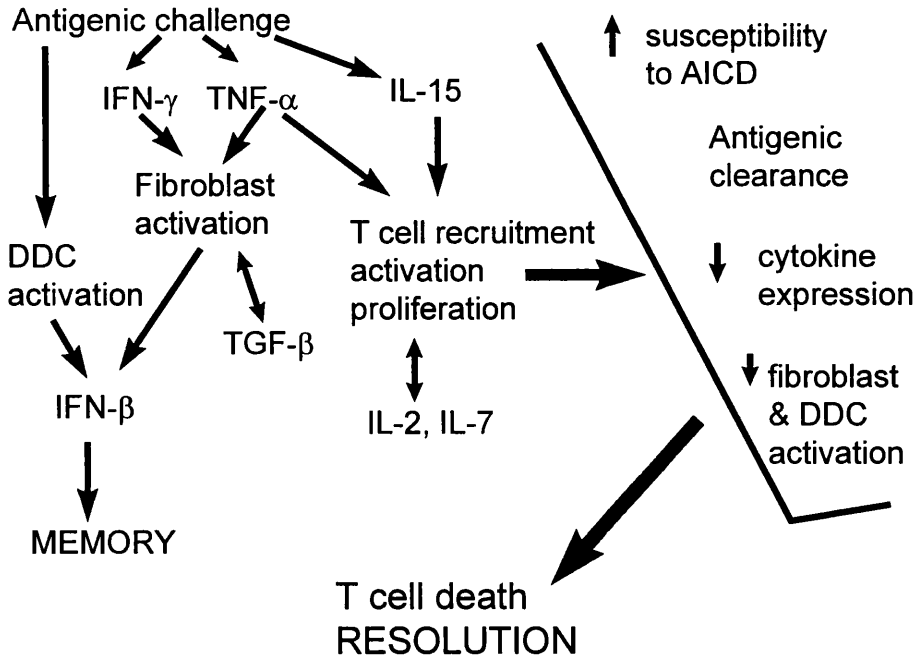

Figure 5.2 Proposed mechanisms involved in generation and resolution of a self-limiting T cell mediated response

5.2 FUTURE CLINICAL CONSIDERATIONS

Our results suggest that the generation and resolution of acute T cell mediated inflammatory responses is controlled at least in part by the co-ordinated expression of different groups of anti-apoptotic cytokines. In contrast, concomitant expression of such cytokines, namely IL-15 and IFN-β, may favour the development of chronic inflammation. Two key questions may thus be generated. The first is whether prolonged T cell survival, mediated either by IL-2R γ chain cytokines, or type I interferons, or both, is a ubiquitous mechanism involved in the generation and maintenance of chronic inflammation. The second, is whether dysregulated or abnormal T cell-fibroblast interactions at sites of inflammation provide the trigger for the generation of chronicity.

A number of chronic inflammatory conditions are characterised by an infiltrate composed of CD4+CD45RO+ T cells and large numbers of fibroblasts. Rheumatoid arthritis is one such example, in which the cytokine microenvironment in joints is strikingly similar to that which we have observed in AE, namely, high levels of TNF-α, TGF-β and IFN-β (267,270 & 10 submitted) and in some studies, high IL-15 expression (80,270). There is now mounting evidence to suggest that this microenvironment favours a reduction in T cell apoptosis, resulting in a failure to clear T cells from involved joints and a perpetuation of the inflammatory response (10, submitted and 11-13).

The relevance of these findings to other cutaneous inflammatory conditions remains to be evaluated. Psoriasis is characterised by keratinocyte hyperproliferation and a dermal infiltrate, again predominantly composed of CD4+CD45RO+ T cells (271,272). Although reduced Bcl-2 expression and increased apoptosis have been reported in psoriatic keratinocytes (273), there is little information regarding either the apoptotic susceptibility of

infiltrating lymphocytes, or intralesional IFN-β expression. However, both type I interferons (α and β) have been reported to cause exacerbations of the disease in some patients (274), suggesting that IFN-β might promote T cell survival and inflammation in psoriatic skin. Fibroblasts may also be important since it has been proposed that an IL-8 paracrine loop between keratinocytes and fibroblasts may be involved in generating the keratinocyte hyperplasia observed (275).

In systemic sclerosis (SSc), early endothelial cell damage and perivascular inflammation, are followed later by the development of fibrosis (276,277). Although the fibroblastic response observed in late stage disease is far in excess of that observed in AE, the progression from an infiltrate of macrophages and T cells, to one composed primarily of fibroblasts, may reflect different degrees of a similar pathological process. As in AE, a majority of T cells are CD4+, and T cell clones generated from cutaneous lesions predominantly secrete IL-4 rather than IFN-γ (276). Furthermore, over-expression of TNF-α and TGF-β, appear to play a central role in disease progression (277,278). A literature search has failed to reveal any reports of IL-15 or IFN-β in association with SSc, and their relevance to its pathogenesis therefore remains uncertain. However, the similarities in T cell phenotype and cytokine microenvironments detected in SSc (276-278), at sites of cutaneous inflammation in our study, and in rheumatoid joints by others (267,270), raise the possibility of a similar underlying pathogenetic mechanism, involving T cell-fibroblast interactions, in the generation of chronic inflammation. Further studies will be needed to accurately determine the relative contributions of T cells, fibroblasts and dermal dendritic cells to this process, and to establish whether the susceptibility to develop chronic inflammation is determined primarily by the intralesional cytokine microenvironment, or by an abnormal susceptibility of infiltrating cells to that environment.

5.3 FUTURE EXPERIMENTAL CONSIDERATIONS

5.3.1 FURTHER CHARACTERISATION OF INTERFERON-β PRODUCING CELLS

Further work is needed to fully characterise the cell types secreting IFN-β. In AE specimens in particular, this cytokine was expressed by numerous dendritic and spindle-shaped cells. Although their morphology is identical to that of the cells staining with the Dianova anti-fibroblast antibody employed, we have shown that the latter cross-reacts both with Factor XIIIa+ and RFD1+ dermal dendritic cells (DDC), as well as endothelial cells. Thus, although activated fibroblasts are a well recognised source of IFN-β (10, submitted), it is likely that activated DDC (as well as keratinocytes and endothelial cells) contribute to its synthesis in situations of cutaneous inflammation.

In order to properly clarify this issue, firstly the specificity of the Dia 110 antibody should be further tested by dual/triple staining for factor XIIIa, CD1a and CD14 to establish which population of DDCs (27) are cross-reactive. The cell types producing IFN-β could then be investigated by performing dual staining with IFN-β plus a range of anti-fibroblast antibodies, and IFN-β plus the DDC markers mentioned above. Although there was insufficient time to take this further, towards the end of the study we devised an immunofluorescence method for dual staining cytokines and other cell markers which should allow the above studies to be performed. This type of study would also allow confirmation of whether the increased production of TGF-β, TNF-α and IL-6 observed in dermal spindle-shaped cells during the resolution phase of the DTH response was occurring in fibroblasts or DDC.

5.3.2 THE NATURE OF FIBROBLAST MEDIATED T CELL SURVIVAL: ARE THE FIBROBLASTS OF ATOPIC ECZEMA PATIENTS ABNORMAL?

The current study raises the question of whether a primary abnormality of fibroblasts exists in patients with chronic inflammatory conditions such as

AE, or whether intralesional fibroblasts are simply responding to their microenvironment. In other words, are the fibroblasts from patients with AE inherently better at promoting T cell survival than those from non-atopic patients? One may take this further, and ask whether they promote the survival of a particular subset of T cells with a specific cytokine profile. In order to begin to address these questions *in vitro* studies would be required to investigate the relative capacity of skin fibroblasts from normal subjects and patients with AE to modulate apoptosis and cytokine synthesis of activated T cells. This could be tested by co-culturing apoptosis prone T cells on fibroblast monolayers derived from explants of normal and AE skin, and examining not only the proportions of apoptotic T cells but also their phenotypic characteristics and cytokine repertoires.

Armed with answers to the above questions it should then be possible to investigate whether overexpression of anti-apoptotic cytokines promotes T cell survival in other chronic inflammatory diseases of the skin, and to investigate the relative contribution of fibroblasts to this process.

5.3.3 ARE THE REDUCED LEVELS OF T CELL APOPTOSIS OBSERVED IN ATOPIC ECZEMA LINKED TO A FAILURE OF NORMAL CELLULAR SENESCENCE?

Finally, it would be of interest to investigate the relationship between apoptosis and senescence in situations of resolving and chronic inflammation. There is now evidence to suggest that human cells undergo a finite number of cell divisions, before entering a non-dividing senescent state (279,280). This process of regulation of cellular life-span is dependent on the progressive shortening of telomeres at the ends of chromosomes which occurs with each cellular division. It can be reversed by telomerase, an enzyme whose activity is not detectable in most somatic tissues, but which is present in a number of human tumours and immortal cell lines (279). More recently, inappropriate activation of telomerase in normal human cells has

been shown to greatly enhance their proliferative potential and life-span (280). It would therefore be of interest to know whether telomerase is activated in T cells during the induction phase of an acute inflammatory response, and whether it is subsequently switched off during the resolution phase. More highly differentiated T cells are known to be more susceptible to apoptosis by virtue of their low Bcl-2 and high CD95 expression (51,172). One may, therefore, envisage a relationship between senescence and apoptosis, such that senescent cells, with shorter telomeres, are also more susceptible to die by apoptosis at the end of the immune response. Appropriate telomerase activation might ensure that a proportion of T cells surviving as memory cells after resolution, would be able to proliferate upon future antigenic encounter. In contrast, inappropriate activation of telomerase might favour the survival of too many T cells, thereby promoting the generation of chronic inflammation. The technology to investigate telomerase activity *in situ* is now becoming available, which should allow this relationship to be investigated in the near future. If the above proves to be true, then inhibition of telomerase activity may provide a useful adjunct to therapy in the management of inflammatory disease.

PUBLICATIONS ARISING FROM THIS WORK

ABSTRACTS

1. **C Orteu**, M Rustin, A Akbar, LW Poulter. Loss of Bcl-2 precedes the decay of T cell populations in resolving Mantoux reactions. J Invest Dermatol 1996;107(3):470
2. **CH Orteu**, A Akbar, MHA Rustin, LW Poulter. Does failure to promote T cell apoptosis contribute to the chronicity of eczema. J Invest Dermatol 1997;108(4):663
3. **CH Orteu**, A Akbar, MHA Rustin, LW Poulter T cell apoptosis in delayed type hypersensitivity reactions and lesional atopic eczema. Immunology 1997;92 (Suppl 1):86
4. **CH Orteu**, AN Akbar, MHA Rustin, LW Poulter. T cell apoptosis in delayed type hypersensitivity reactions. Br J Dermatol 1998, 138 (4): 749
5. **CH Orteu**, AN Akbar, MHA Rustin, LW Poulter. T cell apoptosis in Mantoux reactions and atopic eczema J Invest Dermatol 1998;110 (4):640
6. **CH Orteu**, LW Poulter, AN Akbar, M Salmon and MHA Rustin. Increasing fibroblast numbers and IFN-b production in a resolving delayed-type hypersensitivity response. Immunology 1998;95 (Suppl1):27

PAPERS

CH Orteu, LW Poulter, MHA Rustin, CA Sabin, M Salmon, AN Akbar. The role of apoptosis in the resolution of T cell-mediated cutaneous inflammation. J Immunol 1998;161:1619-1629 (enclosed).

PAPERS IN PREPARATION

CH Orteu, AN Akbar, CA Sabin, M Salmon, LW Poulter, MHA Rustin. Over-expression of IL-15 and IFN-β promote T cell survival in chronic atopic eczema

CH Orteu, MHA Rustin, AN Akbar, CA Sabin, M Salmon, LW Poulter. T cell-fibroblast interactions participate in the generation and resolution of T cell mediated cutaneous inflammation.

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The Role of Apoptosis in the Resolution of T Cell-Mediated Cutaneous Inflammation[1]

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We have investigated cutaneous purified protein derivative-induced delayed-type hypersensitivity (DTH) responses in healthy volunteers to determine features associated with both the generation and resolution of the reaction. The clinical peak of the response occurred at day 3; however, T cell numbers were maximal on day 7. There was a preferential increase of $CD4^+CD45RO^+$ T cells on day 7, which was largely due to proliferation, since a mean of 19% was in cycle. The proliferation of this subset was associated with the presence of IL-15, which was expressed as early as 12 h, and IL-2, which showed peak expression at 7 days. By day 14, there was a significant decrease in both the mean T cell number/unit area and IL-2 and IL-15 expression in perivascular infiltrates. Maximal CD95 (Fas/Apo-1) ligand and TNF-α expression were observed at 7 days and were associated with the presence of 1.83% (range 0.81–2.48%) apoptotic T cells. At 14 days, CD95 ligand and TNF-α expression were reduced significantly, and the presence of 2.5% (range 1.5–3.75%) of apoptotic T cells at this time was probably due to cytokine deprivation, associated with decreased Bcl-2 relative to Bax expression. The induction and resolution of the Mantoux reaction may depend on the expression of cytokines, such as IL-2 and IL-15, which regulate both proliferation and apoptosis in T cells. Failure to control either of these phases of the Mantoux reaction may contribute to the chronicity of inflammatory responses in certain cutaneous diseases. *The Journal of Immunology,* **1998, 161: 1619–1629.**

T cell-mediated inflammatory reactions exhibit an infiltration and expansion of activated CD4 and CD8 T cell populations (1). Resolution is associated with a return to normal in terms of absolute cell numbers and relative proportions of these subsets within the tissue involved (2). It is now recognized that cell numbers are controlled in a wide array of biologic systems by the process of cell suicide or apoptosis (3–6); however, the contribution of this process to the resolution of cutaneous inflammatory responses has not been studied. Such investigations are of particular importance for understanding persistent inflammatory conditions such as atopic eczema, in which disease chronicity may be perpetuated by the inability to terminate the ongoing response.

The Mantoux reaction is a well-recognized delayed-type hypersensitivity (DTH)[3] reaction that peaks clinically at 48 to 72 h and resolves within 14 days (7, 8). This human model of cutaneous inflammation enables the study of the kinetics of the local immune response from onset to resolution. Previous studies have shown that within 12 h of the intradermal injection of PPD, interdigitating dendritic cells appear around dermal blood vessels, and that by 24 to 48 h, large numbers of infiltrating activated macrophages are present (9). T cells begin to accumulate perivascularly within 12 h of challenge and by 48 h, >60% of the mononuclear cell infiltrate is comprised of primed ($CD45RO^+$) T cells (9–11). The majority of studies of cutaneous DTH reactions in humans have not followed the reaction beyond 96 h, and the process by which activated T cells are removed and inflammation is resolved has not been characterized.

A persistent cutaneous inflammatory response may result from both continued recruitment of leukocytes into the involved tissue, and/or a lack of clearance of the infiltrating cells. Lymphocyte chemotactic factors such as IL-1 and IL-8 have been identified in epidermis overlying cutaneous PPD-induced DTH reactions and may be responsible in part for the infiltration of cells (12–14). In addition, recent studies have shown that the clearance of activated T lymphocytes by apoptosis can also be prevented by cytokines, in particular those such as IL-2, IL-4, IL-7, and IL-15, which share the IL-2R γ-chain as part of their receptor complexes (15–19). It is of interest that IL-15 also serves as a chemotactic factor for activated T cells (20–22). These cytokines were shown to prevent the down-regulation of intracellular regulatory molecules such as Bcl-2 and Bcl-x_L, which inhibit apoptosis, but do not alter the expression of Bax and Bcl-x_S, which induce death in activated T cells (16, 23–27). Apoptosis can also be induced by the ligation of CD95 on the surface of activated T cells by its ligand and also by the binding of TNF-α to its receptor (6). The regulation of expression of antiapoptotic cytokines or apoptosis-inducing CD95 ligand and TNF-α during the initiation and resolution of a cutaneous DTH response has not been characterized.

In this study, we have investigated the kinetics of T lymphocyte infiltration, proliferation, and apoptosis during a Mantoux reaction in relation to cytokine and apoptosis regulatory protein

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[3] Abbreviations used in this paper: DTH, delayed-type hypersensitivity; AICD, activation-induced cell death; DC, dendritic cells; EI, erythema index; KC, keratinocytes; PPD, purified protein derivative; TBS, Tris-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UA, unit area.

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Table I. *Abs used in the study*

Ab	Subclass	Code	Source	Specificity
Tmix (CD2, CD7, CD8, CD4, CD3)	IgG1 and IgG2a	RFTmix	RFH	T cells
CD45RO	IgG2a	UCHL-1	UCH[b]	Primed T cells
CD5	IgM	RFT1	RFH	T cells and some B cells
CD8	IgM	RFT8	RFH	Class I MHC restricted T cells
CD4	IgG1κ	MO716	Dako (High Wycombe, U.K.)	Class II MHC restricted T cells
CD4	IgG2a	MHCD 0400	Caltag (San Francisco, CA)	Class II MHC restricted T cells
CD3	IgG	UCHT1	UCH	T cells
Bcl-2	IgG1	MO887	Dako	Anti-apoptotic protein
Bax	Rabbit anti-human polyclonal IgG	N-20 sc-493	Santa Cruz Biotechnology (Santa Cruz, CA)	Pro-apoptotic protein
Ki67	IgG1κ	MO722	Dako	Proliferating cells (outside G_0)
Fas-ligand	IgG1κ	NOK-1	PharMingen (San Diego, CA)[c]	Membrane bound and soluble Fas-L; proapoptotic TNF family protein that binds to Fas
Anti-IL-15	IgG1	M112	Genzyme Diagnostics (Cambridge, MA)	T cell chemotaxis and proliferation
Anti-IL-2	IgG1	MCA 745	Serotec, Oxford, U.K.	T cell activation and proliferation
Anti-TNF-α	IgG1	80-3399-01	Genzyme Diagnostics	Endothelial cell and phagocyte activation, pro-apoptotic cytokine
Anti-IL-6	IgG1	1618-01	Genzyme Diagnostics	Ubiquitous pro-inflammatory cytokine, T cell activation

[a] All Abs were mouse anti-human monoclonals unless otherwise stated.
[b] Kindly provided by Prof. P. C. L. Beverley (University College and Middlesex School of Medicine, London, U.K.) and characterized in Reference 29.
[c] Kindly provided by Prof. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan).

expression to characterize features associated with resolution of inflammation.

Materials and Methods

Patients and control samples

Mantoux tests were performed on the volar surface of the nondominant forearm of 20 healthy volunteers previously immunized with Bacille Calmette-Guerin (15 males, age range 23–59, median 30 yr). Testing was first with 0.1 ml of a 1/10,000 solution of tuberculin PPD (Evans Medical, Leatherhead, U.K.), and then, if negative at 48 to 72 h, with 1/1000 strengths. Erythema and induration were measured at 72 h and on the day of the biopsy, as follows. Erythema was scored using a DermaSpectrometer (Cortex Technology, Hadsund, Denmark), a handheld system designed for measuring the erythema index (EI) of the skin by measuring light absorption coefficients (28). Erythema indices obtained were grouped and scored as 1 = EI < 5; 2 = EI 5–10; 3 = EI 10–15; and 4 = EI > 15. Induration was scored as: 1 = none detected; 2 = just palpable; 3 = easily palpable; 4 = marked; and 5 = very marked. The maximum diameter was measured at 72 h in millimeters and scored as 1, 4–9 mm, or 2, >10 mm. The sum of the erythema, induration, and diameter scores was then used to give each subject an overall score, both at the time of biopsy and at 72 h. We examined initiation and resolution of the reaction by biopsying early and late time points after PPD injection. Each volunteer had one 4-mm punch biopsy taken from the intradermal injection site at either 12 h, 72 h, 7 days, or 14 days after the procedure (five subjects per time point). Ethics committee approval and subjects' informed consent were obtained before performing the biopsies. Normal skin was obtained from surgical specimens in five patients.

In addition, one of the senior authors (L.W.P.) consented to intradermal injection with 1/10,000 PPD at six different sites on the volar aspect of the forearm, and had 4-mm punch biopsies performed at 6, 12, 24, 72 h, 7 days, and 14 days to ensure that the kinetics of the reaction could be observed in a single individual.

All biopsies were mounted in Cryo-M-Bed (Bright's instrument Company, Huntingdon, Cambs, U.K.) and snap frozen in isopentane cooled in a bath of liquid nitrogen. Samples were stored in liquid nitrogen until sectioned. Cryostat sections (6 mm) were cut onto poly(L-lysine)-coated slides, air dried for 2 h, and either used immediately or stored wrapped in cling film at −20°C before immunohistologic staining.

Immunohistology

The characteristics of the mAbs/polyclonal antiserum used in this study are documented elsewhere (29) and in Table I. All Abs were mouse anti-human monoclonals, unless otherwise stated.

The study used indirect immunoperoxidase, immunofluorescence, biotin/streptavidin alkaline phosphatase, and TUNEL methods.

Immunoperoxidase staining

An indirect immunoperoxidase technique was used to detect T cells, $CD45RO^+$ and $Ki67^+$ cell numbers, and distribution. Following a 10-min incubation with normal rabbit serum, skin sections were incubated with a pan anti-T cell IgG mAb mix (T mix), CD45RO or Ki67 diluted in PBS at pretitrated optimal concentrations for 45 min at room temperature. The slides were then washed in PBS, and a secondary peroxidase-conjugated goat anti-mouse IgG Ab (P161; IgG, Dako, High Wycombe, Bucks, U.K.) diluted 1/100 in PBS and containing 4% normal human serum was then applied. After an additional 45 min, the slides were again washed in PBS and the reaction was developed using diaminobenzidine. Sections were counterstained in hematoxylin and mounted in dibutyl polystyrene xylene (BDH Laboratory Supplies, Poole, U.K.) Three control preparations were used. Sections of normal human tonsil, in which the distribution and pattern of staining could be tested against tissue architecture, were used as positive controls in each experiment. In addition, control incubations to detect background staining were performed on sections of each skin sample, omitting the primary Ab. Third, isotype specificity was confirmed by comparison with staining with irrelevant mAbs of the same isotype as the mAbs used on tonsil sections.

Immunofluorescence

To determine CD4:CD8 ratios and proportions of T cell subsets expressing CD45RO and Ki67, sections were incubated for 45 min in a moist chamber with appropriate combinations of mAbs diluted in PBS. After rinsing in PBS, Ig isotype-specific FITC- or TRITC-conjugated affinity-purified goat anti-mouse (Southern Biotechnology, Birmingham, AL) second-layer Abs were applied at pretitrated optimal concentrations, and slides were incubated for 40 min. Slides were then rinsed in PBS fixed in 4% paraformaldehyde and mounted in Citifluor (AF1; Citifluor Products, Canterbury, U.K.). Using the above indirect dual immunofluorescence technique, sections were incubated with $CD5^+CD8$ (an IgM Ab mix used to stain T cells) and either Bcl-2 or Bax, and the percentage of T cells expressing Bcl-2 and Bax was determined. Controls were performed as described, but using the fluorochrome-conjugated second layers alone. Sections were fixed as described above.

Quantification of immunohistology

For immunoperoxidase studies, the number and distribution of positive cells were quantified in each section using an image analysis system (Seescan Imaging, Cambridge, U.K.; magnification ×320) per circular frame

area centered on the largest dermal perivascular inflammatory cell infiltrates, five times per section. For the purposes of statistical analysis and visual display of the data, results were scaled to a frame area of 1 unit area (UA).

For immunofluorescence studies, the distribution and percentages of T cells were estimated in each section using a Zeiss fluorescence microscope (×400 magnification) in the five largest dermal perivascular inflammatory cell infiltrates present in the sections.

Biotin/Streptavidin

To identify the distribution of IL-2 and IL-15, IL-6, TNF-α, and CD95 ligand, freshly cut cryostat sections were air dried for 2 h, ringed with polysiloxane, and fixed in precooled methanol:acetone 1:1 at −20°C for 10 min. After rinsing in PBS at room temperature, sections were incubated overnight with 100 ml of the appropriately diluted primary Ab in PBS +0.1% BSA. Sections were washed in Tris-buffered saline (TBS) at pH 7.6 and then incubated in a moist covered chamber with 50 ml of affinity-purified horse anti-mouse biotinylated second layer (IgG; Vector Laboratories, Peterborough, U.K.) diluted 1/100 in PBS-BSA for 1 h at room temperature. After rinsing in fresh TBS, sections were then incubated for 1 h with 50 ml of streptavidin-alkaline phosphatase-conjugated third layer (Vector Laboratories) diluted 1/100 in PBS-BSA at room temperature in a moist covered chamber. Sections were again rinsed in fresh TBS, and the reaction was developed by 15-min application of filtered substrate solution (0.005 g naphthol ASBI phosphate, 10 ml Tris-HCl (pH 8.2), 200 μl dimethylformamide, 0.01 g Fast Red (TR), and 10 drops Levamisole added last). Sections were then washed in tap water and counterstained with Mayer's hematoxylin before mounting in PBS glycerol (9:1). Controls were performed on skin sections as above using the streptavidin/biotin second and third layers alone. Isotype specificity was confirmed by comparison with staining with an irrelevant IgG1 mAb on skin sections. The proportion of perivascular cells with cytoplasmic or membrane staining was estimated using an image analysis system (Seescan Imaging; magnification ×320) in the five largest dermal perivascular inflammatory cell infiltrates in each section.

Identification of apoptotic T cells

The presence of apoptotic T cells within perivascular infiltrates in PPD reactions was confirmed using a combination of indirect immunofluorescence and the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) technique (30). Sections were stained as above, then fixed with 4% paraformaldehyde solution for 20 min at room temperature, and washed in PBS for 30 min. Permeabilization was performed by incubating with 0.1% Triton X-100 (Rohm & Haas, Philadelphia, PA), 0.1% sodium citrate for 2 min on ice. After rinsing in PBS, sections were incubated with 50 μl of TUNEL reaction mixture (in situ cell death detection kit, fluorescein; catalogue number 1684795, Boehringer Mannheim, Indianapolis, IN) for 60 min at 37°C in the dark. Sections were rinsed in PBS and mounted in Citifluor. The proportion of TUNEL-positive T cells was estimated in each section using a Zeiss fluorescence microscope in the five largest dermal perivascular inflammatory cell infiltrates. In each experiment, sections of normal human tonsil were used as positive controls, and negative controls were performed using Label solution (without terminal transferase) instead of TUNEL reaction mixture.

Statistical analysis

Measurements were taken from five perivascular infiltrates in each subject, and mean values and SDs were calculated. A minimum of three subjects was investigated at each time point. Using the ANOVA method, differences between the values at the five time points were tested for significance, including time and subject as factors in the analysis. For the purpose of visually displaying the data, the mean values for the different subjects were used to calculate the SD for each time point.

Results

Response to PPD of study subjects

All subjects responded to PPD with maximal reactions (as defined by erythema and induration scores) consistently occurring between 48 and 72 h. Of the lesions biopsied at 12 h, two showed a marked response and three a minimal response at that time. When reviewed at 48 to 72 h, all five subjects showed a positive test, even when inflammation induced by the biopsy itself was taken into account. Erythema and induration were scored at 72 h and at the time of biopsy. The maximum diameter of the reaction was scored

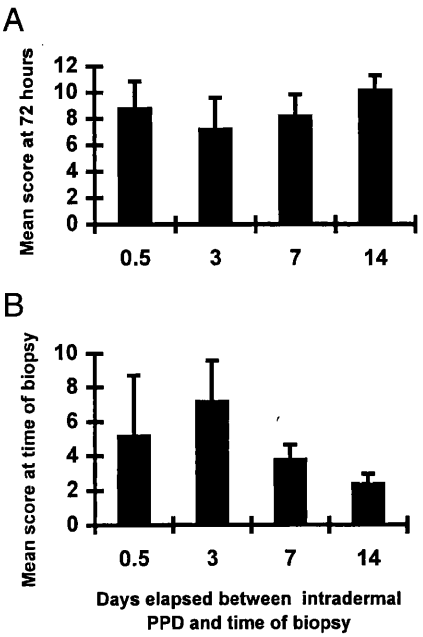

FIGURE 1. Response of study subjects to intradermal PPD. All subjects responded to PPD with maximal reactions consistently occurring between 48 and 72 h. Erythema and induration were measured at 72 h and on the day of the biopsy, as follows. Erythema was scored using a DermaSpectrometer as 1, <5; 2, 5–10; 3, 10–15; and 4, >15. Induration was scored as: 1 = none detected, 2 = just palpable, 3 = easily palpable, 4 = marked, and 5 = very marked. The maximum diameter was measured in millimeters (1, 4–9 mm, or 2, >10 mm) at 72 h. The sum of these scores for each individual was then used to calculate a mean score and SD for each time point (five subjects per time point): *A*, at 72 h after intradermal PPD, and *B*, at the time of biopsy. Error bars indicate SDs.

at 72 h. The sum of these scores for each individual was then used to calculate a mean score and SD for each time point (five subjects per time point) and is presented in Figure 1. There was no significant difference in the mean scores obtained at 72 h in each of the four groups of subjects who received intradermal PPD (ANOVA, $p = 0.12$), confirming that similar responses were obtained regardless of time of biopsy (Fig. 1*A*). The mean scores at the time of biopsy paralleled erythema measurements obtained with the DermaSpectrometer, but also took into account induration, which may better reflect the degree of inflammation occurring in the lesions. These scores were significantly lower at 14 days than at 3 days (ANOVA, $p = 0.02$), confirming that lesions biopsied at that time point were resolving (Fig. 1*B*).

Characteristics of infiltrating T cells

Normal skin contained small numbers of T cells (4.5 ± 1.9/UA). After intradermal PPD, T cells accumulated perivascularly within the dermis and numbers rose significantly by 12 h (15 ± 6.9/UA), and further by 72 h (39.6 ± 7.8/UA); there was also a further increase from this figure up to day 7 (54.2 ± 4.7/UA). T cell numbers then fell significantly by day 14, although they remained in excess of numbers in normal skin (23.7 ± 6.3/UA) (Fig. 2*A*). Overall analysis revealed that there were significant differences between T cell numbers at the five time points studied ($p < 0.0001$). Furthermore, T cell numbers at each time point were significantly different from those at the preceding and subsequent time points. Although a majority of infiltrating cells were present within perivascular areas, smaller numbers of T cells were seen infiltrating the interstitium and epidermis. In these areas, T cell numbers were maximal at 3 days, and subsequently declined up to

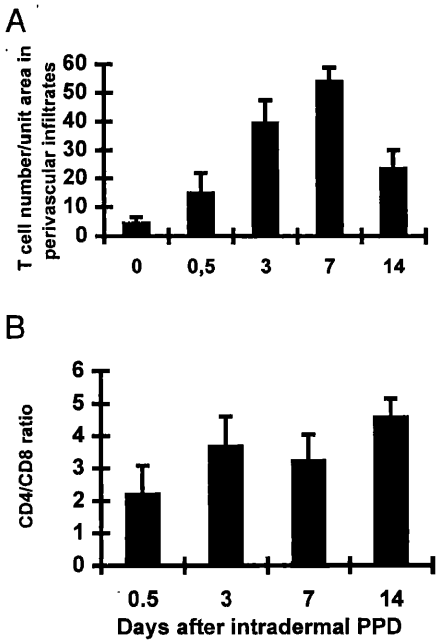

FIGURE 2. Mean T cell numbers per UA in dermal perivascular infiltrates were determined in Mantoux reactions (*A*). T cells were stained by an indirect immunoperoxidase method and quantified in each section, per circular frame area centered on the largest dermal perivascular inflammatory cell infiltrates, using an image analysis system, five times per section, in five subjects per time point. CD4 and CD8 cells were enumerated by dual immunofluorescence, and proportions of positive cells in the five largest dermal perivascular inflammatory cell infiltrates were estimated in each section using a Zeiss fluorescence microscope (*B*) five times per section ($n = 4$ at 72 h, $n = 3$ at other time points). CD4 cells predominated in normal skin, but the numbers of cells were too small to give meaningful ratios. Error bars indicate SDs.

day 14 after intradermal PPD (data not shown). Thus, although the clinical and overall histologic responses to intradermal PPD showed different kinetics, peaking at 3 and 7 days, respectively, by day 14 both were resolving.

In normal skin, $CD4^+$ cells predominated, but numbers were too small to calculate meaningful ratios. Throughout the course of the Mantoux reaction, the number of $CD4^+$ T cells exceeded the $CD8^+$ cells within the perivascular infiltrates. The ratio of CD4 to CD8 cells was lowest at 12 h (2.23 ± 0.85) and reflected proportions of CD4 and CD8 cells found within the peripheral circulation. Thereafter, the proportion of CD4 cells increased (Fig. 2*B* and Fig. 3*A*). This suggests that either active recruitment or proliferation of CD4 cells occurred. Dual immunofluorescence was performed on one representative section per time point with CD3 and CD8. No $CD3^-CD8^+$ cells were identified in any of the sections examined, indicating that the $CD8^+$ cells identified were T rather than NK cells. In addition, no $CD4^+CD8^+$ double-positive T cells were observed.

There were small numbers of $CD45RO^+$ cells in normal skin and at 12 h after intradermal PPD. The number of CD45RO-positive cells within the perivascular infiltrates increased between 12 and 72 h (6 ± 1.8 to 17.1 ± 6.3/UA), and again between day 7 and day 14 (19.8 ± 4 to 27 ± 6.6/UA). Overall analysis revealed that there were significant differences between values at 12 and 72 h, and between those at 7 and 14 days (ANOVA, $p < 0.0001$).

Double immunofluorescence studies showed that at 12 h, 68.7 ± 4.6% of CD8 cells within perivascular infiltrates were $CD45RO^+$. This figure rose to 83.4 ± 0.6% at 7 days after intradermal PPD (Fig. 4). This suggests that even from early time points during the reaction, CD8 cells recruited into the lesions were already primed.

In contrast, the proportion of primed ($CD45RO^+$) CD4 cells was lowest at 12 h (44.9 ± 22.2%), increased significantly by 72 h (74.4 ± 7.9%), and thereafter continued to rise gradually up to 14 days (86.9 ± 4.2%) (Fig. 4). Overall ANOVA, $p < 0.0001$.

T cell proliferation in Mantoux reactions

To determine whether in situ proliferation could account for increasing cell numbers within Mantoux reactions, we measured numbers of $Ki67^+$ cells within perivascular infiltrates. Numbers of $Ki67^+$ cells rose significantly from 0.2 ± 0.2/UA at 12 h to 7.3 ± 0.9/UA at 7 days, and then fell to 1.5 ± 1.4 at 14 days (overall ANOVA, $p < 0.0001$) (Fig. 5*A*). To investigate which cells were induced to proliferate, dual immunofluorescence studies were performed (Fig. 5*B*). At 12 h, very few T cells expressed Ki67. Seventy-two hours after intradermal PPD, 5.8 ± 3.3% of T cells were $Ki67^+$, and by 7 days, the percentage of proliferating T cells increased significantly to 18.8 ± 3.7%. However, at 14 days, this percentage fell to 4.2 ± 2.3% (overall ANOVA, $p < 0.0001$). The proliferating cells were $CD4^+CD45RO^+$ (Fig. 5, *B* and *C*, and Fig. 3*B*). At 72 h, 3.9 ± 1.5% of $CD4^+$ cells were actively proliferating; by day 7, this percentage had increased significantly to 19.3 ± 6.6%; and by day 14, it had fallen to 2.63 ± 2.65% (overall ANOVA, $p < 0.0001$). A similar trend was observed in the $CD45RO^+$ subset. No $Ki67^+CD8$ cells were identified in any of the sections examined. These results suggest that the increase in $CD4^+CD45RO^+$ T cells during the course of the Mantoux reaction was most likely to be due to the induction of proliferation within this subset.

Resolution of the Mantoux reaction markers of apoptosis

To investigate whether the reduction in T cell numbers and resolution of the DTH reaction occurred as a result of T cell apoptosis, we used a combination of indirect immunofluorescence and TUNEL methodologies. No TUNEL-positive T cells were seen within perivascular areas in normal skin. At 12 h after intradermal PPD, 0.05 ± 0.12% (range 0–0.27%) of perivascular T cells were TUNEL positive. Seventy-two hours after challenge, the percentage of TUNEL-positive perivascular T cells had increased (0.47 ± 0.44%), but not significantly. However, there was a significant increase at day 7 after intradermal PPD (compared with normal skin and the 12- and 72-h time points) to 1.83 ± 0.74% (range 0.81–2.48%), and a further rise to 2.5 ± 0.93% (range 1.5–3.75%) at day 14. This percentage increase at 7 and 14 days remained significant when the variation in T cell numbers between time points was taken into account (overall ANOVA, $p < 0.0001$). TUNEL-positive T cells were present in all of the 7- and 14-day specimens examined and were located predominantly at the periphery of perivascular infiltrates (Fig. 3*F*). Additional investigations revealed that a majority of $TUNEL^+$ cells at these time points were located within macrophages (data not shown), suggesting that the numbers of apoptotic T cells detected represented an underestimate of the total amount of apoptosis occurring.

Previous studies have shown that the propensity for T cells to die by apoptosis due to cytokine deprivation correlates with a reduction in their Bcl-2 expression relative to Bax (16, 23, 25–27, 31). We thus investigated the percentages of T cells expressing Bcl-2 and Bax within perivascular infiltrates to assess the extent to which the apoptosis observed could be due to lack of cytokines. Dual immunofluorescence studies revealed that the proportions of T cells expressing Bcl-2 perivascularly rose significantly between 12 h after intradermal PPD (43.4 ± 5.8%) and 3 days (76.3 ± 7.5%). This percentage remained relatively stable (70.9 ± 6.1%)

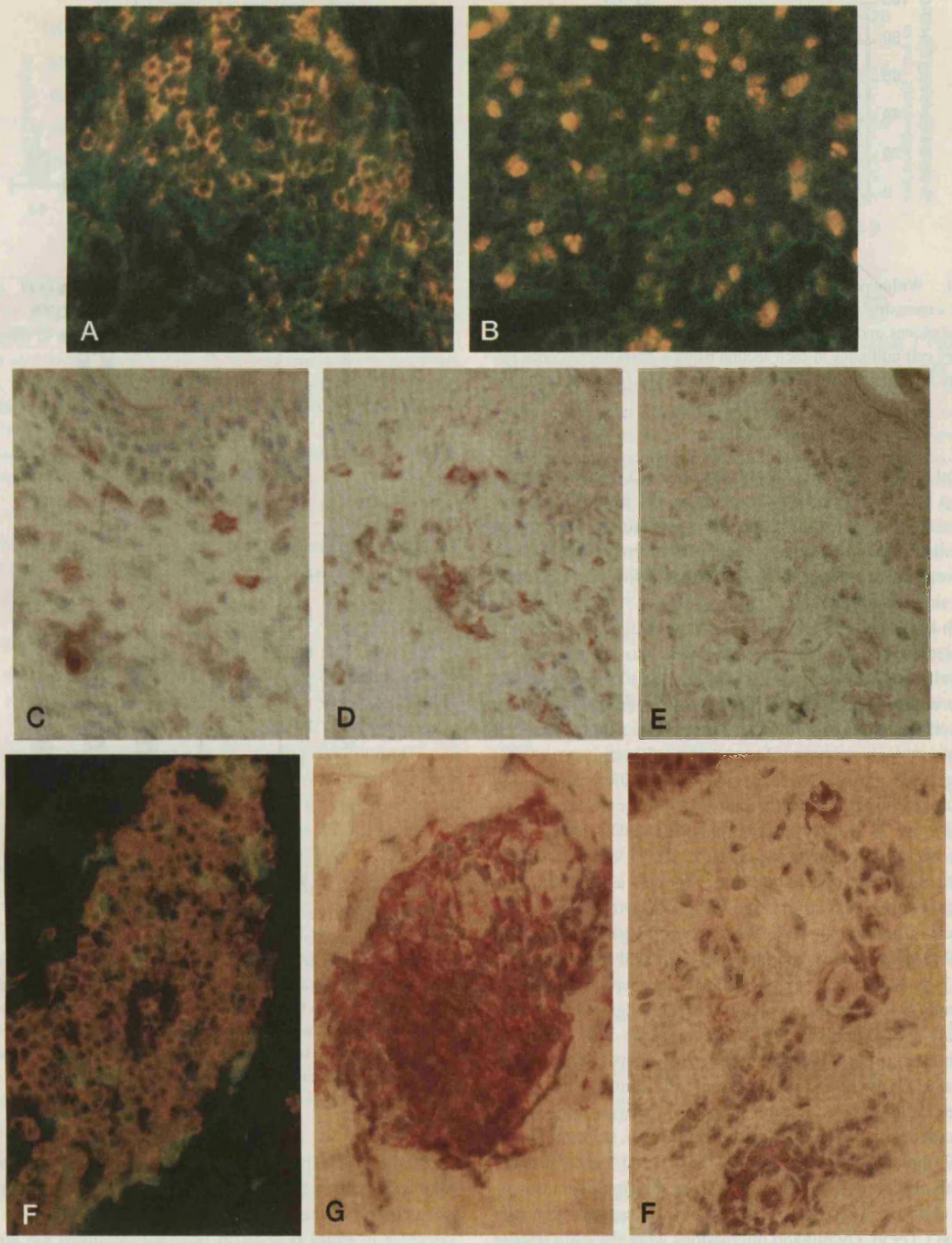

FIGURE 3. *A*, Proportions of $CD4^+$ and $CD8^+$ cells in a dermal perivascular inflammatory cell infiltrate 7 days after intradermal PPD. Using indirect immunofluorescence, CD4 cells were stained with FITC (green) and CD8 cells with TRITC (red). (×400 magnification.) *B*, Ki67 expression within $CD45RO^+$ cells in a dermal perivascular inflammatory cell infiltrate 7 days after intradermal PPD. Using indirect immunofluorescence, $CD45RO^+$ cells were stained with FITC (green) and $Ki67^+$ cells with TRITC (red). (×400 magnification.) *C–E*, Cutaneous distribution of IL-2 after intradermal PPD was determined within skin sections using a biotin/streptavidin method (×400 magnification). *C*, Seventy-two hours after challenge, a majority (>50%) of interstitial lymphocytes expressed cytoplasmic IL-2. *D*, At 7 days, cytoplasmic and/or membrane staining with IL-2 was detected in up to 75% of interstitial and perivascular lymphocytes. *E*, At day 14, IL-2 expression was markedly reduced. *F*, TUNEL-positive T cells in a dermal perivascular inflammatory cell infiltrate 7 days after intradermal PPD. Using indirect immunofluorescence, T cells were labeled with TRITC (red), and then apoptotic cells (green) were stained using TUNEL reaction mixture. (×400 magnification.) *G–H*, Cutaneous distribution of Fas ligand after intradermal PPD was determined within skin sections using a biotin/streptavidin method (×400 magnification). *G*, Intense cytoplasmic and membranous Fas ligand/CD95 ligand expression in a perivascular infiltrate at day 7. *H*, At 14 days, marked down-regulation of Fas ligand/CD95 ligand expression occurred.

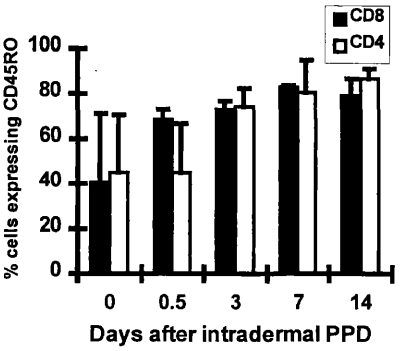

FIGURE 4. Within perivascular infiltrates, the proportions of $CD4^+$ and $CD8^+$ cells expressing CD45RO were measured using dual immunofluorescence techniques and estimated in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope five times per section. Error bars indicate SDs ($n = 3$ at each time point).

to day 7 and then fell significantly by day 14 (23.5 ± 3.3%; overall ANOVA, $p < 0.0001$) (Fig. 6). In contrast, the proportions of T cells expressing Bax within these areas remained constant at >98% at all of the time points studied, and although T leukocyte numbers were much smaller, high Bax expression was also found in normal skin (data not shown).

The high level of Bcl-2 expression on day 7 suggested that lack of this molecule, and by inference cytokines, was not responsible for the T cell apoptosis observed at this time. We therefore investigated the expression of CD95 ligand in perivascular infiltrates during the Mantoux reaction to determine whether this alternative pathway to apoptosis may contribute to death. Keratinocytes (KC) in normal skin expressed weak to moderate CD95 ligand, while interstitial and perivascular cells showed little or no expression (not shown). At 72 h after intradermal PPD, KC CD95 ligand expression was up-regulated, and 19.9 ± 12.7% of perivascular cells with moderate/strong cytoplasmic as well as membranous CD95 ligand expression was observed (Fig. 6). Cytoplasmic staining was present predominantly in large macrophage-like cells. On day 7, CD95 expression was markedly up-regulated in perivascular areas (84.3 ± 9.4%; Fig. 6). A majority of perivascular cells showed strong cytoplasmic and/or surface CD95 ligand expression (Figs. 6 and 3*G*). After 14 days, however, there was a marked reduction in both proportions of cells and the intensity with which they expressed CD95 ligand in perivascular infiltrates (Figs. 6 and 3*H*). These results suggest that the apoptosis that was observed on day 7 coincided with peak CD95 ligand expression, suggesting that CD95/CD95 ligand interaction may account for at least a proportion of death observed at this time. However, the significant down-regulation of CD95 ligand expression at day 14 suggests that it is unlikely that CD95/CD95 ligand interactions were the main trigger for apoptosis at this time.

We also investigated expression of TNF-α since this cytokine has also been implicated in the induction of apoptosis (6). We found that peak TNF-α expression occurred between days 3 and 7, and that although there was intersubject variability, at 14 days, proportions of cells in perivascular infiltrates with strong cytoplasmic staining were reduced (summarized in Table II). Thus, although it is possible that this cytokine may contribute to the death observed at the peak of the response on day 7, this cytokine may not have a major role on the apoptosis observed on day 14, when its expression is reduced (Table II).

The results obtained suggested that apoptosis at day 7 was not due to changes in Bcl-2 expression; however, the significant decreased Bcl-2 expression on day 14 was likely to contribute to apoptosis at this time. To further investigate whether apoptosis occurring after the peak of the Mantoux reaction was due to the decrease in Bcl-2 relative to Bax, we examined the kinetics of T cell accumulation together with expression of these molecules in six samples taken at different times from a single individual. This individual had a strongly positive response to PPD (>11 mm induration and >15 EI at 72 h), and showed slightly accelerated kinetics of the reaction. Nevertheless, the trends in T cell numbers and Bcl-2 and Bax expression were similar to the pooled data from different individuals at each time point. In this individual, T cell numbers were maximal 3 days after intradermal PPD, and then progressively declined to day 14. In contrast, Bcl-2 expression appeared maximal at day 1, and had declined significantly by 3 days, before the numbers of T cells were seen to be reduced (Fig. 7). Bax expression remained relatively constant throughout the reaction (data not shown). Thus, the fall in Bcl-2 preceded the fall in

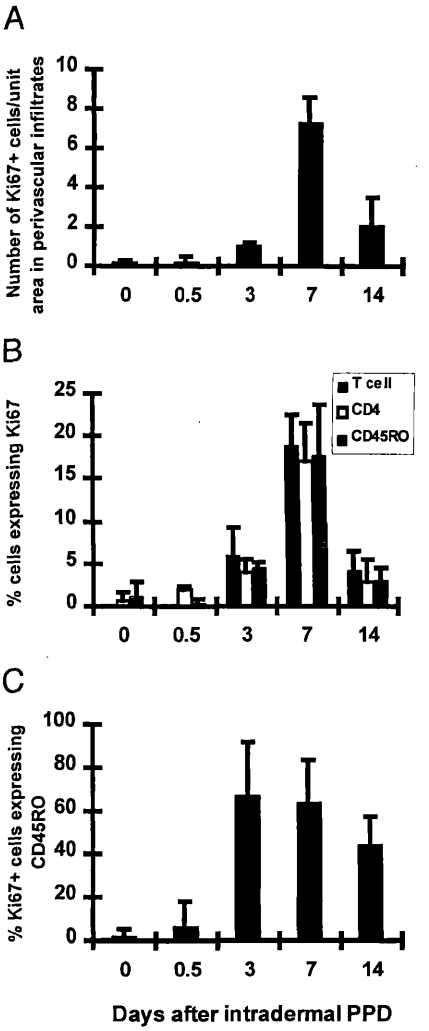

FIGURE 5. The mean number of proliferating cells (*A*); percentage of proliferating T, $CD4^+$, and $CD45RO^+$ cells (*B*); and mean percentage of $Ki67^+$ cells expressing CD45RO (*C*) were measured within perivascular infiltrates. An indirect immunoperoxidase technique and an image analysis system were used, and cell numbers per circular frame area centered on the largest dermal perivascular inflammatory cell infiltrates (five times per section) were found to be significantly increased at 7 days (overall ANOVA, $p < 0.0001$). In immunofluorescence studies, proportions of double-positive cells in the five largest dermal perivascular inflammatory cell infiltrates were estimated in each section using a Zeiss fluorescence microscope five times per section. Error bars indicate SDs ($n = 3$ at each time point).

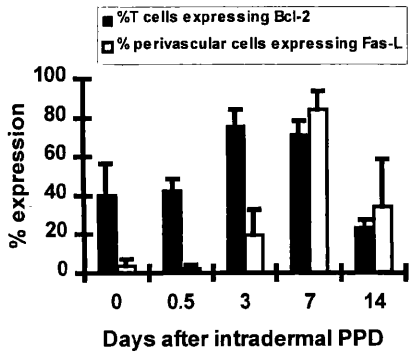

FIGURE 6. Dual immunofluorescence studies were performed using $CD5^+CD8$ (T cells) and Bcl-2, and proportions of double-positive cells were estimated in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope five times per section, in five subjects per time point. A biotin/streptavidin method and an image analysis system were used to determine Fas ligand expression in the five largest dermal perivascular infiltrates. The percentage of cells with cytoplasmic or membrane positivity were counted (n = 5 at day 14, n = 4 at other time points). Error bars indicate SDs.

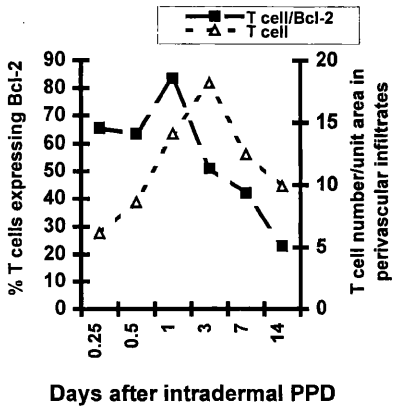

FIGURE 7. Mean T cell numbers and percentage of T cells expressing Bcl-2 in Mantoux reactions at different time points in a single subject. The kinetics of the reaction in this individual was accelerated compared with those of the group as a whole, and reflected a strongly positive response to PPD. However, the trends in cellular and Bcl-2 changes were similar. An indirect immunoperoxidase technique and an image analysis system were used, and cell numbers were counted per circular frame area centered on the largest dermal perivascular inflammatory cell infiltrates (five times per section). In dual immunofluorescence studies, the proportions of double-positive cells were estimated in the five largest dermal perivascular inflammatory cell infiltrates in each section using a Zeiss fluorescence microscope.

T cell numbers, suggesting an association between the down-regulation of this molecule and apoptosis at the later stages of the Mantoux reaction.

Cytokine studies

Withdrawal of cytokines such as IL-2 and IL-15 can induce T cell apoptosis by down-regulating their Bcl-2 expression relative to Bax (16). These cytokines, especially IL-15, are also involved in the induction of the immune response through promotion of T cell chemotaxis and proliferation (20–22, 32–34). We therefore investigated whether changes in IL-2 and IL-15 expression occurred throughout the course of the Mantoux reaction in three different subjects per time point. Although some variability occurred between individuals, the overall trends in IL-2 and IL-15 expression at different time points were the same. Normal skin showed no staining with IL-2 (Table II). At 12 h after intradermal PPD, occasional (<5%) dermal interstitial T cells expressed cytoplasmic IL-2, but no positive cells were seen in perivascular infiltrates or the epidermis. By 72 h, there was marked cytoplasmic expression in a majority (>50%) of interstitial T cells, and occasional positive cells were seen penetrating the epidermis (Fig. 3*C*). Within perivascular infiltrates at 72 h, 50 to 60% of cells showed membrane staining, while a small percentage expressed cytoplasmic IL-2. Expression of IL-2 appeared maximal at 7 days, when both the numbers of T cells present and the extent of proliferation were at their highest levels. At that time point, >75% of interstitial cells and many perivascular cells expressed cytoplasmic IL-2 (Fig. 3*D*), and the majority of the remaining perivascular T cells expressed membrane-bound IL-2. By day 14 after intradermal PPD, only

Table II. *Proportions of perivascular cells expressing IL-15, IL-2, IL-6, and TNF-α at different time points in Mantoux reactions*

Cytokine	Days After Intradermal PPD				
	0	0.5	3	7	14
IL-15					
% perivascular expression[a]	1.9	28.4	77.6	84.6	7.9
Range (%)	0–5	15–50	70–85	75–90	5–10
Intensity of staining[b]	++	++	+++	++	+
IL-2					
% perivascular expression	0%	0%	59.5	65.7	3.5
Range (%)			50–70	60–75	0–5
Intensity of staining	−	−	++	+++	++
IL-6					
% perivascular expression	23.3	44.7	48.5	95.1	93.7
Range (%)	5–30	30–60	40–60	90–98	90–98
Intensity of staining	+++	++	++	+++	+++
TNF-α					
% perivascular expression	57.6	52.9	89	86.3	69.9
Range (%)	50–60	50–60	80–90	75–98	40–95
Intensity of staining	+++	++	+++	+++	+ to ++

[a] Both cells with membranous and cytoplasmic staining were taken into account when calculating the mean and range percentages of positive perivascular cells (in five perivascular infiltrates per section). A minimum of three subjects were investigated at each time point.

[b] Intensity of staining was graded as follows: −, none; +, weak; ++, moderate; +++, strong.

occasional (<5%) interstitial and perivascular T cells expressed cytoplasmic or membrane-bound IL-2 (Fig. 3*E*). This decrease in IL-2 at 14 days coincided with the decrease in Bcl-2 expression and cell numbers in the resolving Mantoux reactions.

In normal skin, epidermal KC showed moderate cytoplasmic staining with IL-15 (Fig. 8*A*, and Table II). Within the dermis, only occasional cells with cytoplasmic staining were seen. Twelve hours after intradermal PPD, staining intensity was greater in epidermal KC (Fig. 8*B*). Occasional strongly positive cells, with a dendritic morphology resembling Langerhans cells, were also present in this area. In the papillary dermis, intracytoplasmic IL-15 was present in numerous large, macrophage-like cells and occasional dendritic cells (DC), both within perivascular infiltrates in close proximity to lymphocytes, and in the interstitium (Fig. 8*B*). In addition, up to 50% of perivascular T cells showed membrane staining with IL-15. At 72 h, although fewer IL-15-positive KC were present, greater numbers of strongly positive epidermal DC were seen. In the papillary and upper reticular dermis, the distribution of IL-15 was the same as 12 h, but the staining intensity and number of positive cells were greater (Fig. 8*C*). In particular, >75% of perivascular lymphocytes expressed membrane-bound IL-15. At 7 days, when T cell proliferation and cell numbers were maximal, fewer dermal cells with intracytoplasmic staining were seen, but a majority (75–90%) of perivascular cells still expressed membrane-bound IL-15 (Fig. 8*D*). Epidermal KC IL-15, however, was markedly reduced by day 7 and undetectable at day 14, although occasional positive DC remained. By day 14, only very occasional perivascular macrophage-like cells were seen and minimal or no membranous IL-15 was present on perivascular lymphocytes (Fig. 8*E*). These results suggest that although IL-15 was present earlier than IL-2 during a Mantoux reaction, high levels of expression of both cytokines at day 7 coincided with increased cell numbers and proliferation. Conversely, the decrease in cell numbers on day 14 was associated with a marked reduction of expression of both IL-15 and IL-2. Thus, high levels of expression of these IL-2R γ-chain signaling cytokines were present at the height of the DTH response, whereas resolution was associated with markedly reduced levels, a situation favoring T cell apoptosis due to cytokine deprivation.

To determine whether this reduction in cytokine expression was specific to IL-2 and IL-15, we also investigated expression of IL-6 (Table II). This cytokine has previously been identified in blister fluid from PPD-induced DTH reactions (11), and in addition to its T cell costimulatory effects (35), may be involved in up-regulation of the cutaneous lymphocyte Ag on $CD4^+CD45RO^+$ responsible for this subset's ability to home to skin (36). Seven days after intradermal PPD, IL-6 expression was markedly up-regulated in epidermal KC, dermal DC, and perivascular areas (in which up to 98% of lymphocytes expressed cytoplasmic and/or membranous IL-6) compared with the 12- and 72-h time points (data not shown). However, in contrast to the marked reduction in IL-15 and IL-2 expression that occurred at day 14, membranous and cytoplasmic IL-6 expression remained high in perivascular lymphocytes at this time point (Fig. 8*F*). Collectively, these observations suggest that changes in expression of IL-2 and IL-15 may play a role in the generation and resolution of the Mantoux reaction.

Discussion

The generation and resolution of a localized immune response are governed by the migration of leukocytes into the site of injury, the proliferation of cells in situ, and the removal of these cells after antigenic clearance (37–39). Abnormal regulation of any of these phenomena may lead to chronic inflammation. We have investigated the kinetics of a Mantoux reaction to clarify factors that influence the generation and resolution of the response to better understand why chronic inflammation in cutaneous diseases such as atopic eczema persists (40).

Following the intradermal injection of PPD, T cells begin to accumulate perivascularly within the dermis by 12 h, reflecting increased transendothelial migration rather than proliferation, since no $Ki67^+$ T cells are present at this time. This increased migration has been attributed previously to the release of chemotactic factors such as IL-8 (12–14); however, other factors may also be involved. For example, naive and memory T cells show different recirculation pathways (41), and migration of skin-homing T cells is dependent on interactions between the lymphocyte homing receptors cutaneous lymphocyte Ag, VLA-4, and LFA-1, and their endothelial cell counter-receptors E-selectin, VCAM-1, and ICAM-1 (36, 42). Furthermore, highly differentiated $CD45RO^+CD45RB^{low}CD4^+$ T cells migrate preferentially (43, 44), and their rate of migration can be increased by endothelial cell activation, IL-15, and chemokines such as RANTES (44, 45). We have demonstrated that there was up-regulation of IL-15 as early as 12 h after PPD challenge. In addition to its effects on the rates of T cell transmigration, this cytokine has been shown to be an important chemoattractant for T cells (20–22). IL-15 may therefore also play a role in the early accumulation of T cells after PPD challenge.

There was an initial recruitment of both CD4 and CD8 cells into the lesions, followed later by a selective increase in $CD4^+$ T cell numbers. The increase of $CD4^+CD45RO^+$ T cells at the later time points is probably due to proliferation rather than migration, since 19% of this subset expressed Ki67 reactivity at 7 days after initiation of the reaction, coinciding with the peak in T cell numbers. Furthermore, this expansion of the $CD4^+$ T cell subset was selective, since no Ki67 reactivity was found in $CD8^+$ T cells in any of the samples tested. The proportion of Ag-specific T cells accumulating within DTH lesions is uncertain, as these have been variably reported to comprise either a majority or <1% of infiltrating T cells (46, 47), and T cell proliferation may therefore reflect a bystander (non-Ag-specific) response that may be driven by cytokines alone (48, 49). However, a recent observation in mice suggests that although Ag specificity does not influence migration into inflamed tissue, only Ag-specific cells are retained (50).

After antigenic stimulation, the induction of cell cycling is driven by cytokines such as IL-2 (51). In addition, it has recently been shown that IL-15 also triggers proliferation in activated T cells (22, 32–34). We showed that the substantial proliferative activity in T cells at 7 days after PPD challenge was associated with the presence of both of these cytokines, and confirmed that epidermal KC and DC, and dermal DC expressed IL-15 (52, 53). There was a marked reduction in staining intensity for IL-15 in both KC and monocytes/macrophages on day 7, when T cell numbers and T cell proliferation were maximal. This suggests that this cytokine may only contribute to the induction of the T cell proliferation during the early phases of the DTH response, and that other cytokines may then take over this role at later stages after PPD challenge. We found that in contrast to IL-15, IL-2 expression was low 12 h after intradermal PPD, and appreciable amounts were only observed at 72 h. At 7 days after challenge, when maximal proliferation and T cell numbers were evident, maximal IL-2 expression was observed. These data, although circumstantial, are compatible with the possibility that during the Mantoux reaction, IL-15, a non-T cell-derived cytokine, may promote the initial proliferative drive until T cells themselves synthesize IL-2, which maintains the proliferative activity.

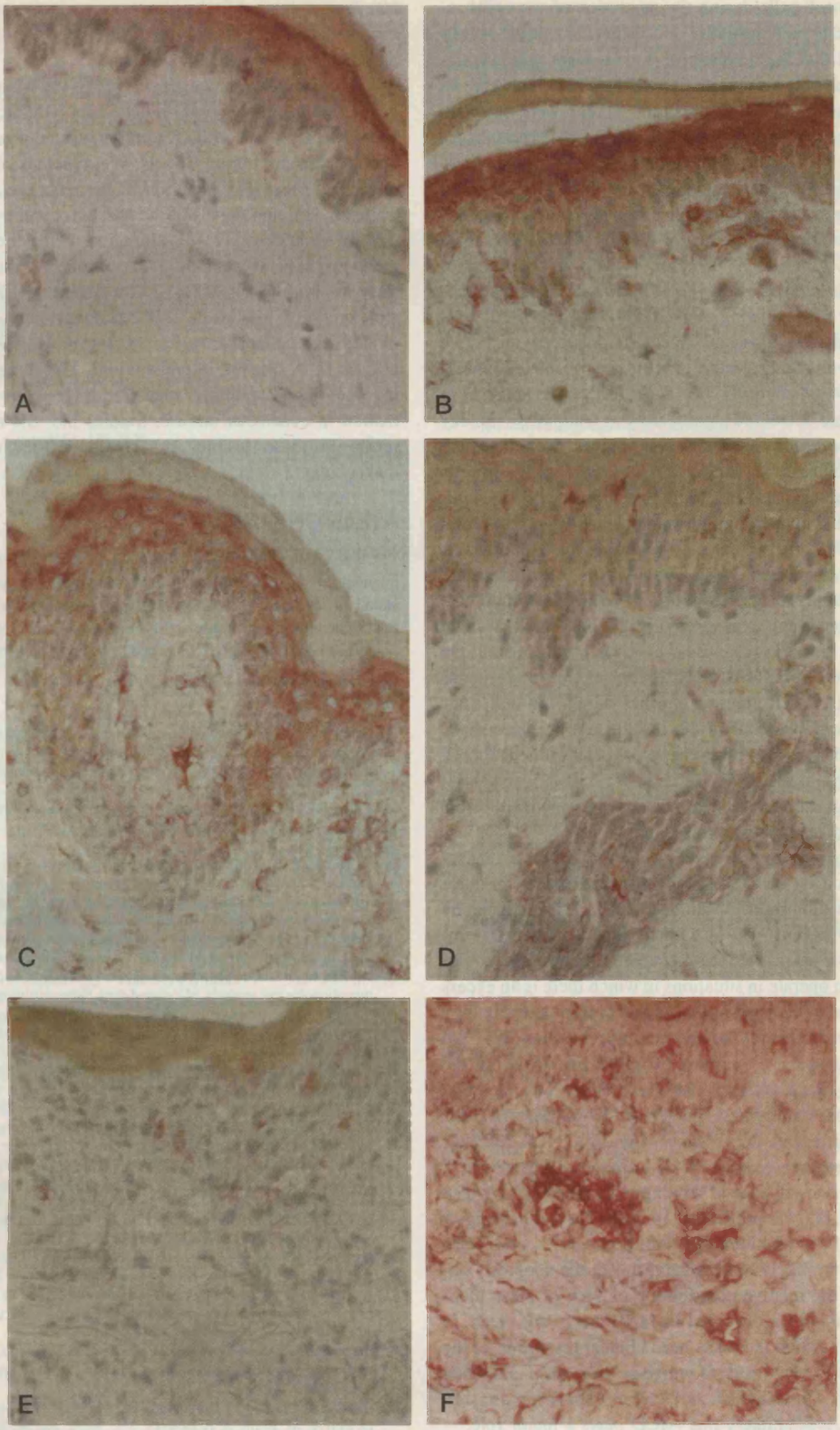

FIGURE 8. Cutaneous distribution of IL-15 (*A–E*) and IL-6 (*F*) after intradermal PPD were determined within skin sections using a biotin/streptavidin method. *A*, IL-15 was expressed in epidermal KC and occasional DC in normal skin. *B*, At 12 h, IL-15 expression was up-regulated in epidermal KC. In the papillary, dermis intracytoplasmic IL-15 was present in DC and large oval cells, both perivascularly and in the interstitium, and up to 50% of perivascular cells showed membrane staining. *C*, At 72 h, the distribution of IL-15 within the dermis was the same as at 12 h, but the staining intensity and number of positive cells were greater. *D*, At day 7, epidermal KC IL-15 was markedly reduced, but a majority (75–90%) of dermal perivascular cells still expressed membrane-bound IL-15. *E*, At day 14, epidermal KC and dermal IL-15 expression appeared down-regulated (magnification ×400). *F*, At 14 days, strong perivascular IL-6 expression remained (magnification ×250).

The clearance of T cells during the resolution of the DTH response may be due to both the efflux of cells or to the death of cells in situ. We found that the numbers of T cells were reduced significantly at 14 days after the initiation of the PPD challenge. At this time, significant numbers of apoptotic T cells were detected. These apoptotic cells could also be detected inside macrophages (data not shown). It is likely that the numbers of apoptotic cells detected represent a substantial underestimate of the total extent of apoptosis taking place (54). It is well recognized that activated T cells require the continued presence of certain cytokines, such as those that signal via the IL-2R common γ-chain, to prevent apoptosis (3–6, 16). It has been shown that these cytokines may prevent apoptosis by up-regulating Bcl-2 relative to Bax in T cells (15, 16, 18). For mature activated T cells, IL-2 and IL-15 are the most efficient at preventing death (15–19). It is of interest, therefore, that on day 14, when most of the apoptosis was detected, both IL-2 and IL-15 were decreased significantly as compared with day 7, when maximal proliferation and T cell numbers were detected. The strikingly decreased Bcl-2 that was observed at this time is compatible with previous observations that these cytokines regulate apoptosis via the induction of this molecule. This suggests that when maximal levels of IL-2 and IL-15 are present, T cell proliferation may occur. Conversely, when levels of these cytokines are limiting, T cells undergo apoptosis due to cytokine withdrawal. We also investigated T cell expression of Bcl-2 and Bax in a single subject, in which the PPD response was investigated at multiple time points. The reduction of Bcl-2 after the peak of the response preceded the fall in T cell numbers, further suggesting that cytokine deprivation was involved in the resolution of the response. However, apoptosis was also observed at the peak of the response on day 7, when high levels of proliferation, IL-2, IL-15, and Bcl-2 were observed, suggesting that other mechanisms were responsible for the induction of apoptosis at this time.

The apoptosis of mature activated T cells may occur as consequence of religation of the TCR in cells that are already in cycle (55–57). This activation-induced cell death (AICD) is mediated by interaction of CD95 (Fas/Apo-1) with its ligand, which is transiently expressed on activated T cells (6, 57). The phenomenon of AICD is thought to operate in situations in which there is an excess of Ag, and may be a mechanism that prevents immunopathology resulting from overactivation of the immune system (58). We therefore investigated the expression of CD95 ligand during the Mantoux reaction. The kinetics of CD95 expression indicated that maximal expression was found in the perivascular infiltrates at the peak of the response, but was reduced significantly at 14 days. Furthermore, the expression of TNF-α, which can also induce T cell apoptosis as a consequence of binding to its receptor, was also maximal at 7 days, but was reduced at 14 days. These data suggest that at the peak of the Mantoux reaction, both CD95- and TNF-α-mediated death are likely to be involved with the apoptosis observed. However, the relative contribution of each of these pathways to the overall death observed at this time is not clear. In contrast, at 14 days, when there has been clinical resolution of the response, presumably as a result of antigenic clearance, and when T cell proliferation, CD95 ligand, and TNF-α expression are substantially reduced, it is unlikely that AICD plays a major role.

These results collectively suggest that, while apoptosis occurring during the induction phase of the PPD response may involve the interactions of either CD95 or TNFR with their ligands, apoptosis during the resolution phase may be controlled by the regulation of Bcl-2/Bax levels by cytokines (6, 57, 58). Apart from the γ-chain cytokines, stromal cell factors have also been shown in vivo to rescue activated T cells from apoptosis via a mechanism involving up-regulation of Bcl-x_L independently of Bcl-2 (2, 59, 60). It would therefore be of interest to determine whether stromal cell-mediated mechanisms are also involved, both in the induction and resolution of the PPD response.

In summary, we have shown that in the Mantoux reaction, the generation of the response involves not only recruitment, but also T cell proliferation, while resolution occurs in part by induction of apoptosis in infiltrating T cells. We hypothesize that the proliferative phase and the resolution of the response appear to be controlled by different levels of the same group of cytokines, the presence of which promotes proliferation, while the absence of these mediators leads to apoptosis. Our recent observations suggest that there are high levels of Bcl-2, low levels of CD95 ligand expression, and only low levels of T cell apoptosis in cutaneous lesions of atopic eczema patients (C. H. Orteu, A. N. Akbar, L. W. Poulter, and M. H. A. Rustin, in preparation). This suggests that dysregulation of T cell apoptosis may contribute to chronicity of inflammation in cutaneous disease. Studies into the regulation of apoptosis in these lesions are clearly pertinent to their future management.

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