Studies on the cellular interactions involved in experimental and chronic irritant dermatitis.

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I hereby declare that this thesis has been composed by myself and has neither been presented nor accepted in any previous application for a degree. This thesis is a record of work carried out by myself except where stated otherwise. All sources of information have been acknowledged by reference in the bibliography.

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CONTENTS

ABSTRACT

Chronic irritant contact dermatitis (ICD) is a common clinical problem, arising from contact with a diverse range of irritants, affecting people both in the workplace and the home. Previous studies have concentrated on the late acute irritant response (24 hours onwards) in normal volunteers. To date there is limited knowledge of acute irritant reactions in patients with established ICD. For this reason we have compared the effect of three chemically diverse common irritants on 100 patients with a history of chronic ICD for more than 6 weeks, and 31 normal volunteers. The irritants were titrated on normal skin to induce similar grades of erythema by 48 hours. The final concentrations, 80% nonanoic acid (NA), 5% sodium lauryl sulphate (SLS) and 0.01% dithranol (DL) were applied to the volar aspect of the forearm for various timepoints up to 48 hours. Samples were obtained from punch biopsies and suction blisters enabling us to study the cellular and molecular mechanisms associated with the onset of irritant reactions. In addition this has allowed comparisons to be made between acute reactions in ICD patients with previous data from normal volunteers.

Immunohistochemical methods were used to investigate a number of parameters, the findings of which were entered onto a database and statistically analysed. DL irritation evoked minimal histopathological changes. Epidermal damage however, was observed after both NA and SLS application. NA irritation profoundly affected the epidermal LC population, inducing redistribution, apoptosis and a dramatic decrease in LC numbers by 24 hours. Keratinocyte (KC) apoptosis and mild spongiosis was also evident. In contrast SLS irritation had a marked affect upon the epidermal KC population, inducing proliferation, parakeratosis, severe oedema and focal KC activation by 24 and 48 hours. KC activation, defined by MHC II and CD54 expression accompanied an extensive leucocyte exocytosis. SLS irritation also reduced epidermal LC numbers by 48 hours, but not as a result of apoptosis. These findings suggest that for the two irritants different mechanisms are involved in LC reduction and therefore have important implications for antigen presentation and immune responses. KC activation and the resultant leucocyte influx were probably triggered through cytokine production. We suggest that the potent T cell and neutrophil chemoattractant IL-8, present after SLS application, was responsible for the heavy dermal and subsequent epidermal leucocyte influx observed by 24 and 48 hours. In the case of NA irritation leucocyte infiltration was less significant, neutrophils were present in the papillary dermis infiltrate at 6 hours, although this influx was not sustained. In contrast, SLS irritation caused dermal accumulation of larger numbers of neutrophils at 24 and 48 hours. Western blotting studies revealed no evidence for the involvement of autoimmune mechanisms in the pathogenesis of either irritant reaction.

Our results clearly indicate that the pathological processes induced by NA or SLS are distinct despite comparable clinical reactions. Both irritants provoked epidermal damage, differentially affecting the epidermal architecture and cellular components, with important implications for immune reponses.

ABBREVIATIONS

Ab	antibody
ACD	allergic contact dermatitis
Ag	antigen
APC	antigen presenting cell
BC	benzalkonium chloiride
CLA	cutaneous lymphocyte associated antigen
CO	croton oil
DC	dendritic cell
DNA	deoxyribonucleic acid
DL	dithranol
DW	distilled water
EC	endothelial cell
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA	human leucocyte antigen
H&E	haematoxylin and eosin
ICAM-1	intercellular adhesion molecule-1
ICD	irritant contact dermatitis
IFNγ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
KC	keratinocyte
LC	Langerhans cell
LN	lymph node
MHC	major histocompatability complex
MLR	mixed lymphocyte reaction
NA	nonanoic acid
PCD	programmed cell death
SALT	skin associated lymphoid tissue
SC	stratum corneum
SIS	skin immune system
SLS	sodium lauryl sulphate
TCR	T cell receptor
TD	terminal differentiation
TEWL	transepidermal water loss
TNFα	tumour necrosis factor alpha
UVB	ultraviolet B
VCAM-1	vascular adhesion molecule-1

1.0. INTRODUCTION

Dermatitis is a widespread, social and economic problem. Its prognosis is often poor and to date there is little knowledge concerning cellular and molecular mechanisms underlying the disease process. This study investigated early irritant reactions in both patients and normal healthy volunteers to determine the mechanisms involved in initiation of chronic inflammation.

This introduction initially gives a brief description of the cutaneous environment. The cutaneous immune system is the main emphasis of the thesis; therefore two sections describe the cellular immunology in some detail. Then sections move onto chronic ICD itself and outline the irritants chosen for the study. Finally, a section on autoimmunity is also included as I propose that the chronicity of ICD may in some cases be the result of an autoimmune response.

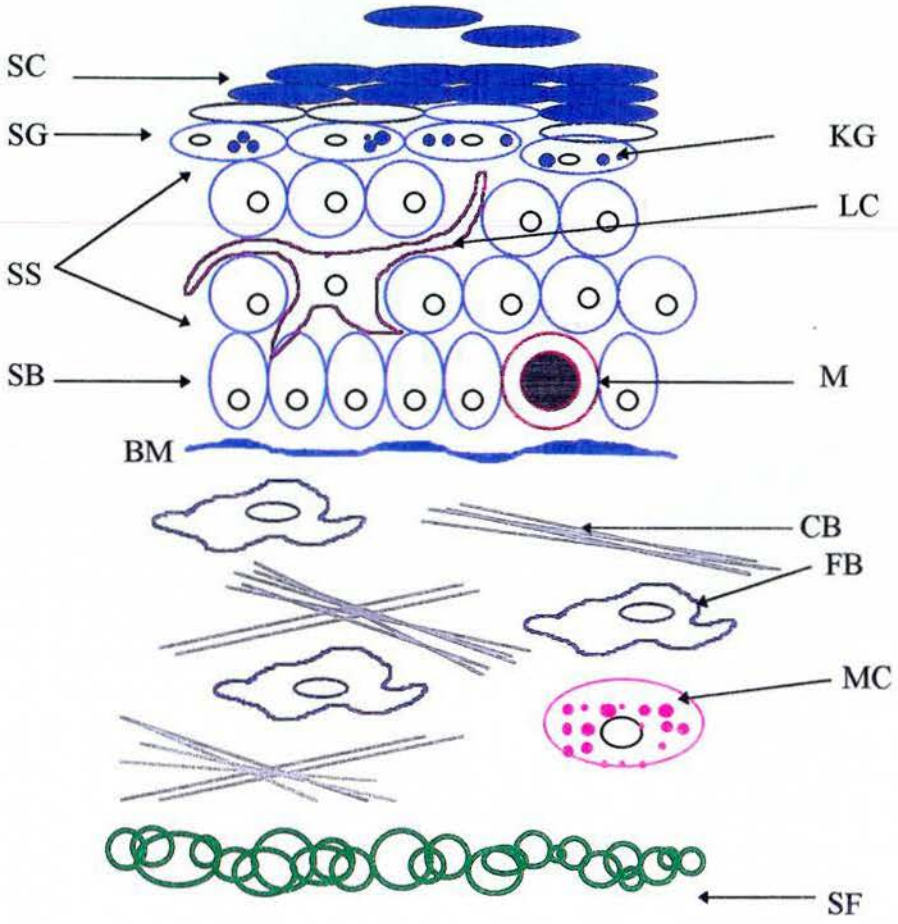
1.1. THE CUTANEOUS ENVIRONMENT

The skin is the largest organ in the body and functions as a protective barrier from the external environment. This physical barrier provides protection from sunlight, microorganisms, mechanical and chemical insults.

The skin comprises two distinct layers, the epidermis and dermis (**Figure 1.1**). These are separated by a semi-permeable basement membrane (dermal-epidermal junction) which permits the bi-directional movement of molecules across it. The epidermis is between 75-150μm thick over the whole body, except on the palms and soles where it is considerably thicker (400-600μm). It is composed predominantly of keratinocytes (KC) (90-95%), with smaller numbers of Langerhans cells (LC) (2-8%), melanocytes and Merkel cells. The underlying dermis is of varying thickness

FIGURE 1 : Schematic representation of the skin

(adapted from diagram by G.Priestley in The Molecular Aspects of Dermatology *John Wiley and sons, Ltd.*, 1993)



KEY :

SC	Stratum corneum	KG	Keratohyalin granules
SG	Stratum granulosum	LC	Langerhans cell
SS	Stratum spinosum	M	Melanocyte
SB	Stratum basale	FB	Fibroblast
BM	Basement membrane	CB	Collagen bundles
SF	Subdermal fat	MC	Mast cell

depending on the body region. This layer consists of a dense fibrous network of proteoglycans, fibronectin and other proteins, providing strength and elasticity to the skin as well as housing resident cells such as tissue macrophages and mast cells. The dermis also provides support to the vascular and nerve networks, and contains excretory and secretory glands, and keratinised appendageal structures such as hair. Based on evidence that both epidermal and dermal cells show immune function it has now become clear that the skin is an immunologically active organ capable of *eliciting* a local immune response (reviewed by Bos and Kapsenberg, 1986).

1.2. THE CUTANEOUS IMMUNE SYSTEM

Observations made by Silberberg and her colleagues in 1973, demonstrating the spatial apposition of epidermal dendritic cells and infiltrating T lymphocytes in allergic contact dermatitis, gave the first indication that the epidermis contained resident immunocompetent cells (Silberberg, 1973). These findings suggested that the epidermis is actively involved in immunological responses, providing more than just innate immunity. Early in this century, Alexander Besredka was one of the first people to suggest the existence of organ specific immunity (reviewed by Bos and Kapsenberg, 1993). Since then it has been hypothesised that the skin may function as a primary or secondary lymphoid organ although there has been no definitive evidence for these theories. The concept of the skin as an immune organ is supported by evidence of a cutaneous microenvironment capable of processing and presenting antigen (Ag), strategically located peripheral lymph nodes and subsets of 'skin homing' T lymphocytes. The phrase 'Skin Associated Lymphoid Tissue' (SALT) was coined in 1978 by Streilein to describe this cutaneous microenvironment. SALT is comprised of four functionally distinct cell types and a set of draining peripheral lymph nodes (reviewed by Streilein, 1983, Krueger and Stingl, 1989). The lymphatics start in the papillary dermis and drain the adjacent tissue in a one-way flow toward the regional lymph nodes, providing a regulatory mechanism for cell hydration, osmosis and immunological responses (Brand *et al*, 1992).

The cellular components of SALT include :

1. Langerhans Cells and dermal dendritic cells, the antigen presenting cells (APC) of the skin.
2. Keratinocytes, the largest cellular component creating a microenvironment suitable for Ag uptake.
3. Lymphocytes. B cells are seldom found in normal skin; the lymphocyte population is composed entirely of a distinct cutaneous subpopulation of T lymphocytes (Bos *et al*, 1987, reviewed by Krueger and Stingl, 1989).
4. Endothelial Cells (EC). Through receptor-ligand interaction the EC of dermal vessels promote the influx of lymphocytes into the dermis and eventually epidermis.

In 1986 Bos and Kapsenberg proposed the term 'Skin Immune System' (SIS) to further describe the complexity of cutaneous immune function. SIS describes the overall interactions of the cellular components of SALT with humoral factors and other cell types present in normal human skin. These include mast cells, macrophages and granulocytes (reviewed by Bos and Kapsenberg, 1986, 1993)

• LANGERHANS CELLS (LC)

In 1868, whilst working as student in Berlin, Paul Langerhans first described a population of epidermal dendritic cells in skin impregnated with gold chloride. Langerhans published elaborate drawings clearly showing the regularly spaced suprabasal dendritic cells within the epidermis, with branched and tapering processes extending far into the surrounding epithelium (reviewed by Hunter, 1983, Schuler, 1991). He suggested these cells were of neural origin, a concept unchallenged until the advent of the electron microscope and Birbeck's historic paper in 1961.

MORPHOLOGY AND DISTRIBUTION

Birbeck and colleagues first described LC ultrastructure using electron micrographs (Birbeck *et al*, 1961, reviewed by Choi and Sauder, 1986).

The main electron microscopic features include:

- an irregular surface membrane, lacking desmosomes.
- a lack of cytoplasmic tonofilaments and premelanosomes giving a 'clear' cytoplasm.
- a convoluted nucleus.
- a well developed Golgi zone with numerous mitochondria.
- the presence of unique rod-like trilaminar cytoplasmic organelles now known as Birbeck granules, found in association with small vesicles, forming a characteristic tennis racket-like structure, with the handle portion having a linear, striated lamella between its two membranes. The origin and function of these structures remain unclear.

LCs can also be characterised at the light microscope level by utilising immunological plasma membrane markers such as CD1a, MHC II and Fc receptors (Hanau *et al*, 1988).

There is a relatively even distribution of LCs throughout the skin. However, the cornea of normal eyes is devoid of LCs, except at its limbic attachment (Streilein, 1983). It was originally believed that LCs were confined to the epidermis but they have now been described in the dermis, lymph nodes and lymphatics draining the skin. Nevertheless, the largest number of LCs are found within the epidermis, comprising between 2-8% of epidermal cells (reviewed by Wolff and Stingl, 1983, Choi and Sauder, 1986)

ORIGIN AND FUNCTION

By the late 1970s two functional roles had been put forward for the LC. Firstly, involvement in regulation of KC proliferation and differentiation and secondly, performing macrophage-like functions in immune reactions. Little evidence exists to

prove that LCs play a role in KC regulation, although LCs constitutively produce IL-1, important for KC growth (reviewed by Sauder, 1984). Elaborate chimera experiments on mice, carried out by Katz and colleagues, provided indisputable evidence of the bone marrow (i.e. leucocyte) origin of the LC (reviewed by Katz *et* al, 1979), suggesting that LCs may perform macrophage-like functions. The origin of these cells was further corroborated by the recent finding that LCs uniformly express CD45, an Ag shared by all leucocytes (Schuler, 1991).

In the late seventies two independent groups showed LC expression of surface receptors already known to be present on the surface of cells involved in immune functions. LCs constitutively express Fc receptors (FcRs) and C3 receptors and are MHC II positive (Rowden *et al*, 1977, Klareskog *et* al 1977, Stingl *et* al, 1977). More recently it has been demonstrated that the FcRs expressed by normal epidermal LC are of the type FcγRII/CD32 (De la Salle *et al*, 1992). These receptors are believed to be important in endocytosis, phagocytosis and antibody (Ab) dependent cell mediated cytotoxicity. Since LCs have been shown to have a relatively poor phagocytic ability, expression of these receptors may be important in uptake of Ag, and may, for instance, play a role in allergic dermatitis (Schuler, 1991).

Shelley and Juhlin proposed that LCs form a reticuloepithelial trap for external Ag and then transport the Ag to the draining lymph node via the afferent lymphatics (Shelley and Juhlin, 1976). This was corroborated by Rowden and colleagues who demonstrated that LCs may bind to various allergens and then migrate to the draining lymph node (Rowden *et al*, 1977). Studies utilising skin-sensitising fluorochromes, for example, fluorescein-isothiocyanate (FITC) have demonstrated that a significant proportion of the DCs arriving in the draining lymph node (LN) after sensitisation bear the fluorochrome Ag (Macatonia *et al*, 1987). These data provide definitive evidence that the DCs found in the LN originate from the epidermis where they acquired the Ag.

Mixed lymphocyte reactions (MLR) have shown that LC enriched epidermal cell cultures can induce an Ag-specific T cell proliferative response comparable to that seen with macrophage cultures (Stingl *et al*, 1978). Antigen-presentation and MLR-stimulation of T cells by LCs is abrogated by the introduction of anti-Ia antibodies and complement, showing that LC-T cell interactions are MHC II restricted as would be expected from a professional APC (Stingl *et al*, 1978). Collectively these findings support the concept that LCs function as professional APCs forming a major part of the epidermal immune system.

Various groups have shown that freshly isolated epidermal LCs are very effective Ag processors but are relatively poor at Ag presentation. Cultured or dermal LCs on the other hand are inefficient Ag processors but are very good at Ag presentation (reviewed by Bos and Kapsenberg, 1993) The reports that LCs can perform a variety of functions depending on their cutaneous location indicate that LCs undergo some form of phenotypic and functional maturation during the migration from the epidermis allowing effective Ag presentation in the draining lymph nodes. The increase in LC APC function seen after cell culture is therefore thought to mimic the *in vivo* differentiation process occurring in LC migration to the draining lymph nodes (Symington *et al*, 1993, Shibaki *et al*, 1995).

LC MATURATION AND ACTIVATION **(Table 1.1, page 9)**

LC migration and ensuing maturational changes are mediated by cytokines. IL-1 (Heufler *et al*, 1988) and GM-CSF (Heufler *et al*, 1988, Kolenik, *et al*, 1990) have been implicated in maturational changes, while TNFα (Kimber and Cumberbatch, 1992) and GM-CSF (Rupec *et al*, 1996) are thought to be important signals for migration. IL-1 alone does not maintain LC viability but acts in synergy with GM-CSF in the enhancement of APC function (Heufler *et al*, 1988). GM-CSF is the major cytokine involved in the LC maturational process acting through decreased CD1a expression and an increase in the cell's ability to stimulate T cell proliferation (Kolenik *et al*, 1990). Further maturational changes occur during migration,

including an increase in MHC II expression (Cumberbatch *et al*, 1992), alteration in the patterns of adhesion molecules expressed and induction of B7 expression (Symington *et al*, 1993). TNFα is important for migration; various groups have shown that cutaneously injected recombinant TNF induces a time and dose dependent LC response (Kimber and Cumberbatch, 1992: Groves *et al*, 1995). GM-CSF and TNFα are also thought to be important in the generation of LCs from CD34+ haematopoietic progenitors (Caux *et al*, 1992).

Epidermal LCs express CD1a, CD1c and MHC II, once in the dermis they show a 10 fold lower expression of CD1a (Meunier *et al* , 1993), with CD1 expression now predominantly CD1c (Elder *et al*, 1993). Four CD1 proteins have been described (CD1a, b, c, and d). The CD1 isoforms are expressed on the cell surface in association with beta-2-microglobulin resulting in the formation of a novel, nonpolymorphic, Ag-presentation mechanism independent of MHC II (reviewed by Porcelli and Modlin, 1995). Preliminary data suggests that CD1 molecules may have evolved to present non-peptide lipid Ags to T cells in the skin (reviewed by Porcelli and Modlin, 1995).

Maturation not only involves downregulation of CD1a; LCs begin to express accessory molecules such as CD54 (ICAM-1) and CD58 (LFA-3) (**Table 1.1**). Mixed lymphocyte and time-course studies have revealed that interactions with CD54, and also CD58 are of prime importance during initiation of T cell activation (Marcel *et al*, 1994). LCs have also been shown to constitutively express CD50 (ICAM-3) which, as described for CD54, plays an important role in the initiation phase of T cell activation. Expression of these two LFA-1 ligands is differentially regulated (Teunissen *et al*, 1995). The cytoplasmic domains of the two molecules share little homology, resulting in the promotion of different signals upon LFA-1 (CD11a/CD18) ligation (Manara *et al*, 1996). It has been suggested that the initial T cell adhesion via CD50 (ICAM-3) may lead to an increase in CD54 (ICAM-1) expression (Manara *et al*, 1996). Members of the B7 family, expressed upon

TABLE 1.1: Adhesion molecules expressed on LC.

CD NUMBER	OTHER NAMES	CELLULAR DISTRIBUTION	CUTANEOUS FUNCTIONS
CD1a		immature LC	antigen presentation
CD1c		mature LC	antigen presentation
CD11c	α chain, β2 integrins	mature LC, leucocytes	adhesion and T cell activation
CD11a/CD18	LFA-1 (β2 integrins)	mature LC and other leucocytes	adhesion and T cell activation
CD49a/CD29	VLA-1	LC, activated T cells, monocytes	
CD49b/CD29	VLA-2	LC, KC, B cells, monocytes	LC migration
CD49c/CD29	VLA-3	LC, KC, B cells	LC migration
CD49d/CD29	VLA-4	LC, B cells	
CD49e/CD29	VLA-5	LC, memory T cells, monocytes	
CD49f/CD29	VLA-6	LC, memory T cells, monocytes	
CD50	ICAM-3	mature LC, leucocytes	adhesion and T cell activation
CD54	ICAM-1	mature LC, endothelium, activated cells	adhesion and T cell activation
CD62E	E-Selectin	endothelium	binds sialyl lewis-X on LC, aids migration
CD80	B7-1	mature LC, B cells	accessory molecule
CD86	B7-2	mature LC, B cells	accessory molecule

maturation, are also T cell costimulators, and may synergise with the effects of CD54 to initiate T cell activation (Symington *et al* , 1993).

Mature LCs have been shown to express both B7-1 (CD80) and B7-2 (CD86) which have recently been shown to be involved in the selective activation of TH1 and TH2 cell clones respectively (Nickoloff *et al*, 1995, Ozawa *et al*, 1996, table 1). In mice anti-B7-1 antibodies induce T cell production of IL-4 (TH2) whereas anti-B7-2 antibodies result in IFNγ production (TH1). Expression of adhesion molecules is known to be modulated by cytokines. In the case of B7-1, GM-CSF and IL-1 up-regulate LC expression whereas, IFNγ and IL-10 have been shown to suppress induction in cultured LC (Chang *et al*, 1995, Furue, 1996)). GM-CSF, can partially restore IFNγ induced suppression of B7-1 but the mechanism for IL-10 suppression is as yet unclear (Ozawa *et al*, 1996). The modulatory effects of these cytokines are seen only in early LC maturational stages, during initiation of B7 expression. This suggests that cytokines affect synthesis not degradation of already existing B7 molecules (Kawamura and Furue, 1995). IL-10 may therefore exert immunosuppressive effects by inhibiting B7 expression on LCs. IL-10 has also been shown to inhibit CD54 expression (Kawamura and Furue, 1995). KCs produce a wide spectrum of cytokines and it is possible that B7 expression may be reciprocally regulated in the epidermal microenviroment.

LCs weakly express the Very Late Antigen (VLA) adhesion molecules. These are made up of a common β chain (CD29) and variable α chains (the CD49 Ags). LCs express VLA-1/CD49a and VLA-3/CD49c and moderately express VLA-2/CD49b & VLA-5/CD49e (**Table 1.1**). The predominant VLA molecules expressed are 4 (CD49d) & 6 (CD49f), the function of which are still unclear. It has been suggested that the proportion of these VLA molecules expressed on the LC surface may vary during the maturation process. For example, CD49b and CD49c are also present on KCs, and may allow the formation of weak, transient bonds between the two cell types, allowing LC to either remain in the suprabasal area while their neighbouring KCs move upwards, or to migrate from the epidermis. LC motility is further aided because of the lack of firm cell-cell associations due to the absence of LC

desmosomes. On migration into the dermis CD49b and CD49c loose their importance and other VLA molecules take over (Le Varlet *et al*, 1991)

Ross and colleagues (1994) have shown expression of sialyl Lewis-x on epidermal LCs, which is enhanced upon activation. The ligand for sialyl Lewis-x, CD62E (E-selectin), is expressed on the dermal endothelium. Interaction between these molecules is therefore of significant importance in LC migration both into and out of the skin. LC expression of sialyl Lewis-x in normal skin is consistent with frequent turnover of the epidermal LC population, such that residual levels of adhesion molecules required for migration are present on resting cells. The increase in sialyl Lewis-x upon activation may therefore facilitate LC migration to the draining lymph nodes (Ross *et al*, 1994).

Recent data reveal that different phenotypic and functional stages of the LC may be present not only in the dermis and epidermis but within the epidermis itself. Flow cytometric analysis has revealed two populations of LCs within the epidermis. Firstly, a HLA-DR^{hi}, $CD11c^{hi}$ population. This may represent a recently activated group, having undergone maturational changes but without yet having migrated or a group of cells which has recently arrived in the epidermis. CD11c is the alpha chain of β2 integrins. β2 integrins are adhesion molecules responsible for cytoskeleton connection. Variation in expression may therefore regulate LC morphology, function or intraepidermal position. The second group of LC is the more traditional HLA-DR^{lo}, $CD11c^{lo}$ population of resident cells (Shibach *et al*, 1995).

LC-T LYMPHOCYTE INTERACTIONS

Activation of T cells requires two signals. The first must be a specific signal through MHC-Ag binding T cell receptor (TCR) and the second a costimulatory signal package via adhesion molecule binding and/or soluble cytokine/receptor ligation. LCs can interact with two distinct populations of T cells. They process and present Ag, not previously encountered by the immune system, to naive T cells in the draining lymph nodes. They may also function as professional APCs for the memory T cells present in the epidermis. Memory/effector T cells have less stringent requirements for

activation and may be stimulated by APCs resident in the epidermis. Adhesion molecule and cytokine expression are quantitatively and qualitatively more important in activation of naive T cells, initiated by mature LCs in the draining lymph nodes. These findings are supported from work carried out by Hauser and colleagues who have demonstrated that cultured LCs, pulsed with Ag, were able to activate unprimed T cells *in vitro* (reviewed by Streilein *et al*, 1990). This topic is covered in more detail in further sections.

• KERATINOCYTES (KC)

KC derive their name from the keratins that comprise between 30 and 50% of their total cellular protein. The keratin family encompasses more than 30 proteins forming 8nm intermediate cytoplasmic filaments, consisting of 2-5 polypeptides. Keratins are encoded by two separate gene families: one responsible for basic (type I) and the other for acidic (Type II) keratins. Individual keratins exist in pairs always consisting of one type I and one type II keratin. Keratin classes have been used in the literature to determine different stages in the terminal differentiation pathway (Woodcock-Mitchell *et al*, 1982). The main keratins in the basal cell and prickle cell layers are K5 (type II, 58kDa) and K14 (type I, 50kDa) (Fuchs and Green, 1980). As KC differentiation occurs these are replaced by K1 and K10 (reviewed by Fuchs, 1993 and Hunter *et al*, 1996).

TERMINAL DIFFERENTIATION **(Fig 1.2)**

The epidermis is a stratified squamous epithelium which undergoes a genetically programmed pathway of terminal differentiation or keratinisation (programmed cell death). Cells originate in the lower epidermal layers and undergo maturation, involving a series of morphological and biochemical changes, as they move up through the epidermal layers (reviewed by Montagna *et al*, 1992).

KCs arise in the basal layer, and move to the surface through the prickle layer which consists of several layers of polyhedral cells. This layer is so named because

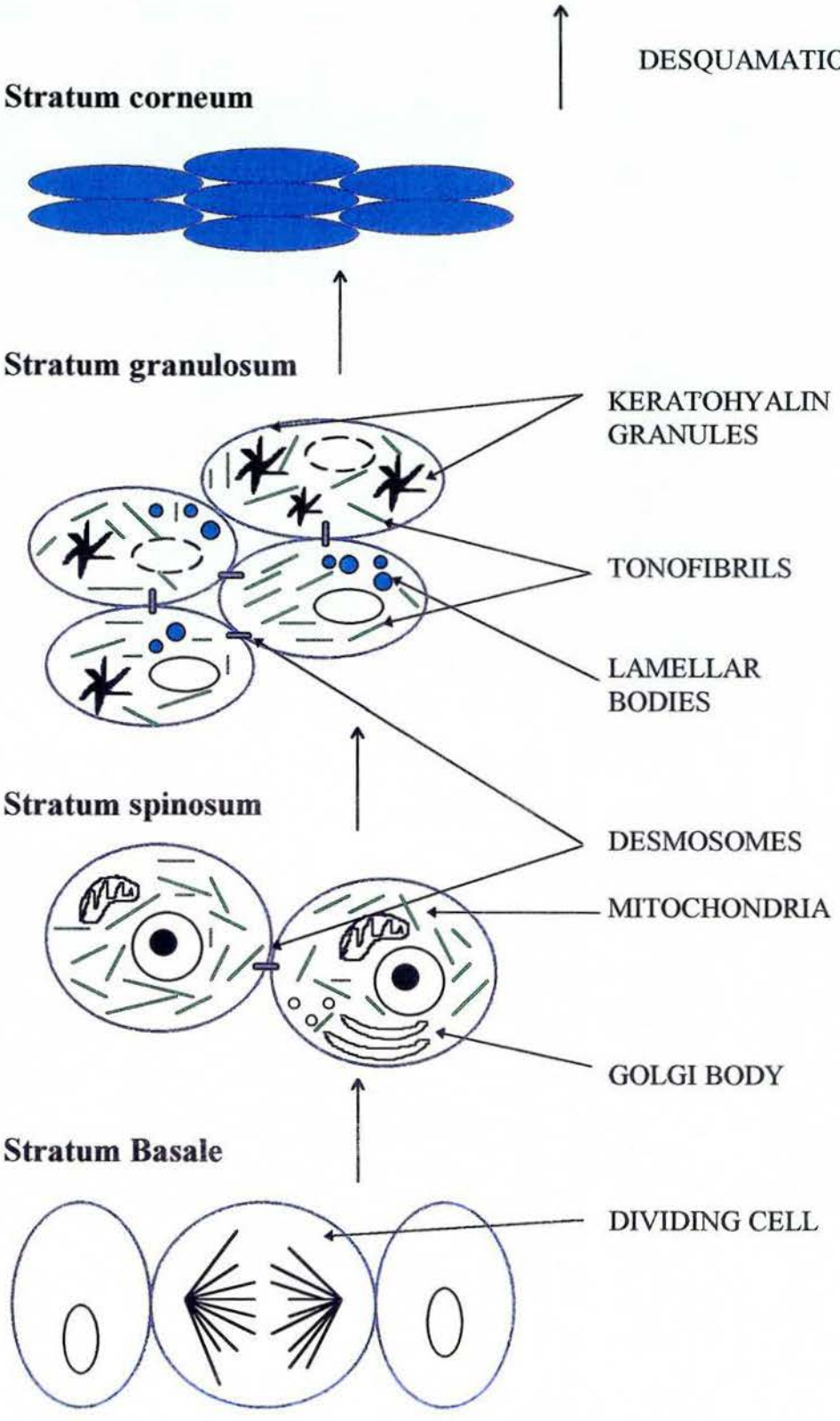
histologically the cells appear to be joined by 'spikes', bundles of keratin filaments which insert into the desmosomal plaques of opposing cells. Two or three layers of flattened KCs superficial to the prickle zone comprise the granular layer, characteristically defined by the presence of keratohyalin granules and physiological disintegration of cytoplasmic organelles, including the nucleus (Karasek *et al*, 1972). Keratohyalin and lamellar granules represent newly synthesised organelles containing a variety of lipids, complex carbohydrates and hydrolytic enzymes which contribute to the barrier function of the horny layer. An extension to the granular layer a compact transitional layer, may be observed in thickened skin .

The cells eventually reach the outermost or horny layer, which comprises several layers of flat anucleate cells which are ultimately sloughed from the skin. The characteristic feature of this layer is the formation of an insoluble cross-linked protein envelope, closely apposed to the cytoplasmic surface of the plasma membrane (reviewed by Watt, 1983). A complex mixture of polar and nonpolar lipids exists in the intercellular spaces of the SC which act together with keratohyalin granules to serve as a barrier against transcutaneous water loss (reviewed in Schurer, 1991).

Based on cellular kinetics the epidermis can be divided into a proliferative and a differentiating compartment (reviewed by Hamilton, 1972, Lavker and Sun, 1983) which are part of the terminal differentiation pathway. The proliferative compartment is comprised of basal and suprabasal cells which are responsible for steady cellular turnover within the skin. This is considered to be a mixed cell population containing both slow cycling stem cells and transient amplifying cells capable of undergoing a finite number of divisions. Differentiation occurs in the upper prickle and granular layers, where maturation, involving increased cell size (Sun and Green, 1976), altered keratin expression (Fuchs and Green, 1980, Woodcock-Mitchell *et al*, 1982) and adhesion molecule expression (reviewed by Haynes, 1987, Adams and Watt, 1990, Watt 1993) take place without cellular generation (reviewed by Lavker and Sun, 1983).

FIGURE 1.2: Terminal differentiation in the KC.

(adapted from diagram by Gawkrodger. In: An illustrated colur text. Dermatology. *Longman group UK, Ltd.,* 1992).



Programmed cell death (PCD) results in controlled cell deletion, avoiding induction of inflammation. PCD plays a complementary but opposite role to mitosis and is therefore of major importance in tissue homeostasis. The epidermis is an organ that undergoes terminal differentiation with the eventual death of KCs; this process has therefore been suggested to be a specialised form of programmed cell death (reviewed by Haake and Polakowska, 1993). The importance of the balance between cell death and proliferation is highlighted by the epidermal hyperplasia observed in many skin diseases, for example psoriasis. This hyperplasia may result not only from increased proliferation but may also be due to disruption of terminal differentiation.

IMMUNE FUNCTION

KCs are not solely programmed to synthesise keratin and undergo terminal differentiation, they are also capable of an immune/inflammatory function through the constitutive and inductive expression of surface molecules and cytokines (see section 1.2.6). In 1981 the potential role of the KC in immune functions was first proposed after two independent observations, the production of IL-1 by KCs (Luger *et al*, 1981) and KC expression of MHC II (Lampert *et al*, 1981).

MHC II expression is not constitutive but KC synthesis and expression of HLA-DR molecules has been described in diseased skin (Volc-Platzer *et al*, 1984; Lampert *et al*, 1981) or in culture after exposure to IFNγ (reviewed by Nickoloff *et al*, 1986). Expression of MHC II enables KCs to produce co-stimulatory signals for skin infiltrating T cells (reviewed by Nickoloff and Naidu, 1994). KCs are capable of phagocytosis (Mottaz and Zelikson, 1970) a function relevant to the concept of the KC acting as an APC through expression of HLA-DR, uptake of particulate Ag and presentation to LC. As LCs phagocytose poorly this may be an important early step in the initiation of an immune response against particulate Ag (reviewed by Streilein, 1990).

Constitutive production of KC cytokines is very low and, like many other cell types, KCs require an exogenous signal to initiate cytokine synthesis and/or secretion. KCs may become directly activated by environmental stimuli, such as mechanical irritation,

UVB irradiation or bacterial products (Nickoloff and Turka, 1994), responding through production of cytokines which influence both surrounding epidermal as well as dermal cells. Cytokine production results in KC activation and up-regulation of various surface accessory molecules including CD54 (Griffiths *et al*, 1989) and CD86 (Nickoloff *et al*, 1995). CD54/LFA-1 ligation is thought to be important in KC-T cell interactions as the presence of intraepidermal lymphocytes correlates well with KC CD54 expression in many skin diseases including psoriasis, atopic dermatitis and lichen planus. Cytokines also play a role in T lymphocyte migration into the epidermis. IL-1 and IL-8 are involved in T lymphocyte chemotaxis (Yoshimura, 1987; Dowd, 1988, reviewed by Barker *et al*, 1991) and are both produced by KCs (Luger *et al*, 1981; Larsen *et al*, 1989).

• APOPTOSIS

Apoptosis is a form of physiological PCD but may also occur as a co-ordinated cellular response to external stimuli which cause irreversible cell damage. The cellular triggers of apoptosis include DNA damage, intracellular receptor ligation (e.g. by steroid exposure) and ligand binding to specific cell surface receptors (for example CD95, TNFR). Further regulatory molecules may be required in addition to these triggers, for example various members of the bcl-2 family. This family will be discussed further as it is thought to be of particular importance in the skin. The apoptotic process itself depends on the activation of a group of cysteine proteases which appear to be directly responsible for the structural changes of apoptosis. Examples include Ced-3 in C.elegans (Ellis and Horntz*l*, 1986) and the IL-1β converting enzyme (ICE- like proteases) in man (Thornberry *et al*, 1992, Dost.B and Wyllie.A, 1996).

Apoptosis is characterised morphologically by two distinct stages (**Figure 1.3**). Firstly, nuclear and cytoplasmic condensation, cell shrinkage with compaction of the cytoplasmic organelles, rounding up of the cell and loss of cell-cell contact. This leads to the production of membrane bound apoptotic bodies of varying size. The

second stage is the rapid phagocytosis and degradation of these apoptotic bodies by macrophages or neighbouring cells (Kerr *et al*, 1972). Many factors, including cytokines, growth factors, non-physiological stimuli (such as UVB) and cell surface molecules (such as CD95/fas-Ligand) can initiate or suppress apoptosis.

The APO-1/fas antigen (CD95) is a member of the TNF receptor family, and can initiate apoptosis in many cell types through binding of fas ligand (fas-L) (Suda *et al*, 1993). It is thought that CD95 is involved in immune-related apoptosis, for instance in the development of T lymphocytes in the thymus and cytotoxic T lymphocyte mediated killing (Matsue *et al*, 1995).

Apoptosis occurs when an internally encoded suicide program, regulated by, among other molecules, members of the bcl-2 gene family. Bcl-2 functions as a repressor of PCD induced by a variety of stimuli, and therefore acts at a step common to many death pathways (reviewed by Cory, 1995). Bcl-2 has been shown to associate with another family member bax. Bax shows extensive homology with bcl-2 and forms homodimers and heterodimers with Bcl-2. Oltvai and co-workers have outlined a model to explain the interactions between these two closely related proteins (**Figure 1.4**). They have suggested that the ratio of bcl-2 to bax determines the susceptibility of a cell to death following an appropriate signal. When bcl-2 is in excess the cell is protected from death. In contrast, excess bax infers susceptibility to apoptosis following a death signal, i.e. bax itself will not trigger apoptosis (Oltiva *et al*, 1993).

Bcl-x is yet another member of the bcl-2 family. It exists as two splice variants, the larger of which, bcl-x_L, enhances cell survival. The smaller variant, bcl-x_S, antagonises the survival function of bcl-2 (Boise *et al*, 1993). Bcl-x may also play a central role in regulation of PCD, and may be important for regulation of apoptosis occurring through mechanisms that bypass or are independent of bcl-2 (Boise *et al*, 1993).

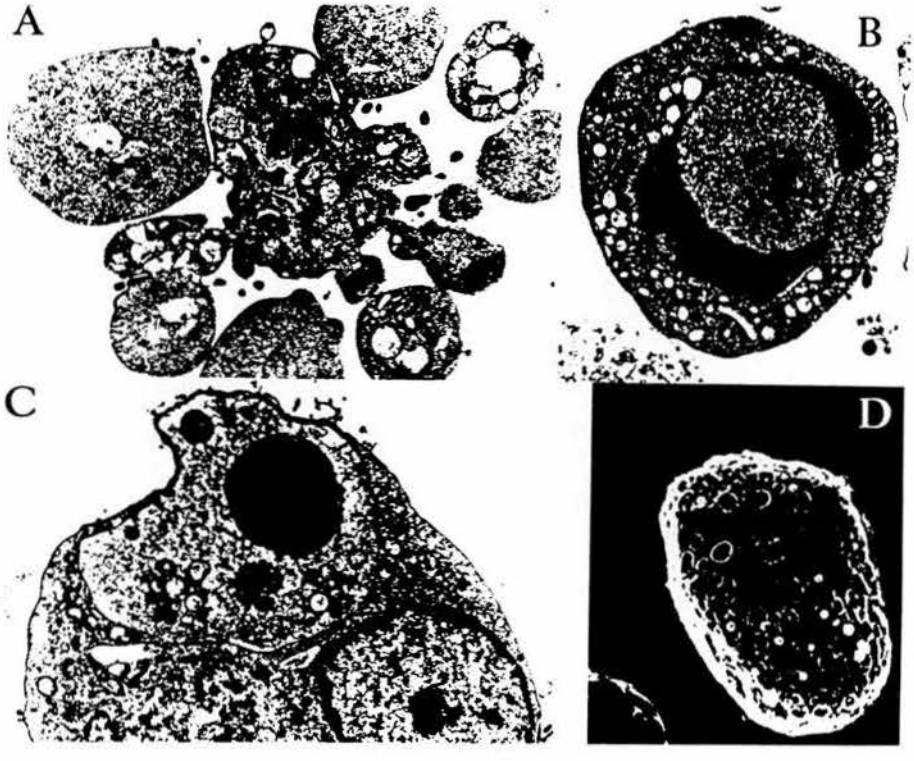
APOPTOSIS IN THE SKIN

Increased levels of apoptosis have been described in a variety of skin diseases. For

FIGURE 1.3:

The morphology of an apoptotic thymocyte. (Howie *et al*, 1994)

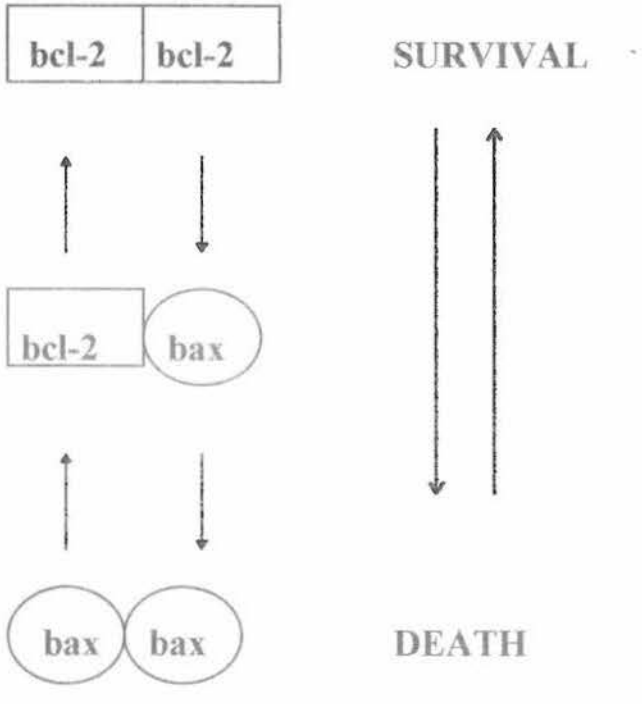
A). explosion to membrane-bound fragments B). nuclear, chromatin condensation. C). phagocytosis D). the pock-marked surface resulting from fusion of dilated endoplasmic reticulum with the cell membrane.



instance, high numbers of apoptotic bodies has been described in the epidermis of lichenoid diseases, graft versus host disease, fixed drug eruptions, regression of warts, UVB irradiation (sunburn cells) (reviewed by Haake and Polakowska, 1993) and more recently in psoriasis (Bianchi *et al*, 1994). This increased apoptosis suggests that the pathogenesis of many cutaneous diseases may involve an imbalance in the homeostatic mechanisms which determine whether KC death occurs through TD or apoptosis. UVB induction of KC TNFα production is thought to be involved in the induction of apoptosis, as anti-TNF antibodies can reduce DNA fragmentation (Schwarz *et al*, 1995). In psoriatic plaques downregulation of bcl-2 expression in the basal layer is proposed to be central to disease pathogenesis (Bianchi *et al*, 1994).

FIGURE 1.4:

Model of the interrelationships of bcl-2 and bax in the regulation of PCD.
(adapted from Oltiva *et al*, 1993)



Minimal bcl-2 expression has been demonstrated in normal KCs and in inflammatory skin diseases such as contact dermatitis. In contrast strong expression of bcl-2 has

been found in tumours such as basal cell carcinoma (Nakagawa *et al*, 1994). These findings suggest that cutaneously expressed bcl-2 may play a role in the increased proliferation found in malignant tumours but not in proliferation induced by inflammatory stimuli.

Sayama and co-workers have shown that normal KCs express CD95 (Sayama *et al*, 1994), and that this expression is constitutive (Matsue *et al*, 1995). CD95 expression is minimal in normal skin when compared with levels of expression observed in the lesional epidermis of a variety of inflammatory diseases, for example contact dermatitis (Sayama *et al*, 1994). CD95 mediated apoptosis in KCs has been shown to require KC activation, through IFNγ or IL-2 signalling (Matsue *et al*, 1995). In fact IFNγ exposure has been shown to result in a 4 fold increase in the number of cultured KCs expressing CD95 (Sayama *et al*, 1994). These findings suggest that CD95 may not be involved in promoting terminal differentiation but rather in induction of apoptosis in inflammatory skin diseases.

Dendritic cells undergo apoptosis unless they are appropriately stimulated either via cell-cell contact or through cytokines. Mature LCs have been shown to be rescued from apoptosis by TNFα or CD40-CD40L interaction. Conversely, IL-10 has been shown to reduce LC viability (Ludewig *et al*, 1995). Apoptosis of LCs, and other effector cells, may play an important role in the control or resolution of skin inflammation.

• SKIN LYMPHOCYTES

Lymphocytes are mobile cells which normally circulate in the peripheral blood. However at specific sites these cells migrate into organs including spleen, lung, lymph nodes and skin. This is not a random migration and is dependent upon a variety of factors including whether the cell is a T or B lymphocyte, if the cell is resting or activated and where the Ag specificity of the lymphocyte was acquired. Under these specific conditions lymphocytes adhere to and migrate through the vessel wall into the

tissue. These migratory processes are directed by highly specific receptor/ligand interactions (reviewed by Nickoloff, 1990).

The existence of organ-specific migration pathways has been demonstrated by various groups by showing, for example, that some T lymphocytes generated within the peripheral lymph nodes will preferentially infiltrate the skin. Further examples include mesenteric T lymphocytes which when injected back into the host will home to the gut (reviewed by Streilein, 1983). The migratory properties of these cells are independent of Ag-specificity .

Specific migration is thought, in some cases, to be determined by selective recognition of organ-specific determinants on the endothelial cells of the specialised high endothelial venules (HEV) (Stevens *et al*, 1992). In the case of skin, memory T lymphocytes expressing the cutaneous lymphocyte antigen (CLA) specifically migrate into the skin through CLA - E-selectin (CD62E) interaction (reviewed by Bos and Kapsenberg, 1993).

Small numbers of lymphocytes are present in normal skin, suggesting that lymphocyte trafficking occurs as part of the normal homeostatic mechanism. Ninety percent of cutaneous T lymphocytes are seen to be clustered around postcapillary venules, with an even distribution of CD4 and CD8 positive cells, the majority of which are activated. Less than 10% of the lymphocytes are observed subepidermally or as single cells, this small population is mainly CD8 positive suppresser/cytotoxic cells. The presence of intraepidermal lymphocytes was probably first described by Kondon in 1922. Since that time it has been established that less than 2% of the total lymphocyte population in normal skin is intraepidermal (reviewed by Bos *et al*, 1987) and of the CD2/CD3/CD8 phenotype (reviewed by Krueger and Stingl, 1989).

The skin homing T lymphocyte plays an important role in disease and is prominent in many dermatological disorders ranging from the cutaneous lymphomas to common inflammatory skin diseases such as psoriasis.

LYMPHOCYTE MIGRATION

Adhesion of lymphocytes to the vascular endothelium is the first step in the migration (diapedesis) of cells from blood into tissue. Adhesion molecules have been described as playing an important role in this process, for example CD54 (ICAM-1), CD106 (VCAM-1) and CD62E (E-Selectin) (reviewed by Barker, 1992). Many of the EC adhesion molecules important in diapedesis are not expressed or expressed only in low levels in normal skin. CD54, for example, is expressed only in low levels on the vascular endothelium of normal skin (Griffiths *et al*, 1989). This low level expression of adhesion molecules is probably important for T lymphocyte migration involved in normal homeostatic control. Work carried out by Haskard *et al* suggested that the mechanisms involved in the initial binding of the lymphocyte to the endothelium may differ depending on the activation state of the endothelium. LFA-1 appears to be important for binding to unstimulated rather than stimulated endothelium where CD54 is important (Haskard *et al*, 1986). The intensity of EC CD54 expression correlates with the degree of dermal infiltration and is pivotal in the migration process (Griffiths *et al*, 1989).

The early immune events following any type of skin injury, be it antigen driven, chemical, or simply mechanical, are very similar. Firstly, the dermal endothelium becomes activated by primary cytokines, particularly IL-1 and TNFα. Primary cytokines are those cytokines released immediately upon skin injury. Stored IL-1 released from the stratum corneum and in concert with IL-1 and TNFα production from subsequently activated KC results in EC activation and expression of CD62E, VCAM-1 and CD54 (Butcher *et al*, 1979). IFNγ and lipopolysaccharide have also been shown to increase the avidity and density of adhesion molecules expressed on the EC membrane (Haskard *et al*, 1987). IFNγ in combination with TNFα induced expression of mRNA for IL-8 and CD54 in epidermal KC (Barker *et al*, 1990). The importance of IL-1 and TNFα in inflammation have been highlighted in experiments where recombinant cytokine was injected into the dermis. IL-1 resulted in the generation of a local inflammatory infiltrate (Butcher *et al*, 1979, Dowd *et al*, 1988). Injection of recombinant TNFα resulted in induction of EC CD62E, CD54 and VCAM-1 expression; KC CD54 expression was also evident (Groves *et al*, 1995).

Upon EC activation circulating lymphocytes bind to adhesion molecules and migrate through the endothelium into the injured site. The infiltrating cells then migrate through the dermis and into the epidermis along a gradient of increasing concentration of chemotactic factors. IL-1, released by epidermal KC, has been shown to be a potent T cell chemoattractant (Sauder *et al*, 1988) which in concert with many other factors plays a key role in cutaneous inflammation. Once in the epidermis T lymphocytes interact with the epidermal KC resulting in further KC activation (**see KC section**).

• GRANULOCYTES

Polymorphonuclear leucocytes contain a multilobed nucleus and many granules and are classified as neutrophils, eosinophils or basophils. These cells play an important role in acute inflammation through phagocytosis of microorganisms (Fehr and Dahinden, 1979).

Neutrophils contain two major types of granules; primary or azophilic granules which are lysosomes containing acid hydrolases, myeloperoxidase and lysozyme. Secondary granules which contain lactofein in addition to lysozyme. Neutrophils are phagocytes which engulf and destroy harmful organisms entering the body. Extracellular release of granules and cytotoxic substances may also occur, usually through ligation of FcγR on the neutrophil cell surface. Neutrophils are thus an important element of the natural immune system defences.

Neutrophils are particularly important in skin defence due to their rapid migratory ability. Neutrophils have been shown to bind E-selectin on endothelial cells (Rohde *et al*, 1992) and migrate into the skin. Recently a novel transcellular mode of neutrophil diapedesis has been put forward. The endothelial cell is observed to engulf the neutrophil and subsequently release it at the abluminal side (Shubert *et al*, 1989). Once in the dermis the cells respond to chemoattractants, released upon acute injury (Rees *et al*, 1994), in particular IL-8 (Swensson *et al*, 1991). Exposure of

granulocytes to chemoattractants increases adhesion molecule expression and induces metabolic stimulation of these cells (reviewed by Csato *et al*, 1985).

• MAST CELLS

Mast cells are large, mononuclear cells filled with membrane-bound secretory granules. Mature mast cells are not usually found in the peripheral blood stream but are located in extravascular areas, adjacent to blood vessels, nerves or beneath epithelial surfaces. Mast cells undergo a characteristic pattern of biochemical and morphological changes during degranulation. A multitude of biologically active mediators, including histamine, proteases and acid hydrolases are released from preformed secretory granules resulting in acute inflammation (reviewed in Galli, 1993, Dvorak, 1995).

The tissue distribution of these cells enables them to provide a degree of natural immunity, giving some protection against parasites, microbes and environmental Ags. The precise nature of the mast cell and the mediators which they produce vary with anatomical location (reviewed in Galli, 1993, Dvorak, 1995).

• CYTOKINES IN THE SKIN

Cytokines are several different family types of glycoproteins with immunoregulatory capabilities. These molecules bind specific receptors through which they regulate activation, proliferation, differentiation, migration and immune function of many cells. Most cytokines have pleiotropic effects and may act both locally and systemically. They may act synergistically or as antagonists, and can reciprocally up or downregulate their own production and activity in an autocrine fashion. Cytokines have been separated into several groups including :-

CHEMOKINES e.g. IL-8

GROWTH FACTORS e.g. GM-CSF

INTERFERONS (α,β,γ)

TUMOUR NECROSIS FACTOR (α and β)

SUPPRESSIVE FACTORS e.g. IL-10

A number of cells within the skin, including dermal leucocytes, KCs, LCs and melanocytes are capable of cytokine synthesis. KCs produce significant amounts of a wide variety of cytokines (**Table 1.2, page 29**) capable of interaction with high affinity receptors on their own and other cell membranes. Production of cytokines is modulated by a plethora of factors including cell cycle, cell-differentiation and biological or physiochemical agents such as UV irradiation, bacterial cell products, toxins and cytokines themselves. This chapter will summarise the cytokines pertinent to this thesis.

INTERLEUKIN 1 (IL-1)

Most cells in the body are capable of responding as a target for IL-1, including LCswhich constitutively produce IL-1 (Choi and Sauder, 1986) and KCs which can also synthesise and secrete IL-1 (Luger *et al*, 1981; Gahring *et al*, 1985; Didierjean *et al*, 1989). In contrast to macrophages , IL-1α is the predominant active form of IL-1 released from KCs; IL-1β forms are inactive. This can be explained by the observation that pro-IL-1β can only be cleaved by a specific protease present in macrophages but not KCs (reviewed by Luger, 1989). This may be biologically irrelevant as both IL-1 peptides bind the same receptors with comparable although not identical affinities (Kupper *et al*, 1988). There are two distinct forms of IL-1 receptor (type 1 - IL-1RtI [80kD]and type 2 - IL-1RtII [68kD]), type 1 being expressed on T cells, fibroblasts, KCs and endothelial cells and type 2 on B cells, neutrophils and bone marrow cells. The two forms of IL-1 bind distinct sites on the receptors; in general IL-1α binds more effectively to type 1 and IL-1β to type 2. KCs are able to regulate the number of IL-1R expressed on the cell surface and can therefore regulate their own sensitivity to exogenous IL-1. For instance, activated KCs show heightened receptor expression and IL-1 production and can therefore respond to IL-1 in an autocrine or paracrine fashion. The absence of inflammation in normal skin

suggests that the interaction of IL-1 with its receptor on KCs is somehow blocked and/or that KCs may decrease the numbers of receptors expressed on the cell surface. An IL-1 receptor antagonist (IL-1ra) has recently been described as a competitive inhibitor of IL-1, without resulting in cell activation (reviewed by Kupper, 1990). The fact that KCs express IL-1R suggests an autocrine function for IL-1 within the skin. IL-1, may activate KCs to express new surface molecules including ICAM-1 (CD54) or to initiate synthesis or secretion of various cytokines including IL-1, IL-6, IL-8, GM-CSF and TNFα (reviewed by Luger, 1989, Kupper, 1990, Larsen *et al*, 1993). IL-1 has been shown to induce KC proliferation (Sauder *et al*, 1988) and may be of importance together with many other KC cytokines in maintenance of cell turnover in normal skin or in induction of increased proliferation in the case of some skin diseases.

Several groups have reported that large quantities of IL-1 can be found in the SC (Gahring *et al*, 1985) and outer layers of the epidermis (Didierjean *et al*, 1989). IL-1 is synthesised in the basal layer forming large stores of cytoplasmic IL-1 in cells of the upper epidermal layers which are not capable of IL-1 production (reviewed by Ansel, 1990). This may make pathological sense as basal proliferating KC are involved in wound healing and inflammation where KC cytokines most probably play a vital role (reviewed by Ansel, 1990). Epidermal KC also secrete IL-1ra (Hammerberg *et al*, 1992). This finding highlights the complexity of cytokine networks, suggesting that the balance between IL-ra and IL-1 may be critical in KC growth as well as inflammatory processes.

TUMOUR NECROSIS FACTORα (TNFα)

This cytokine mediates a variety of cellular functions including induction of KC CD54 expression, IL-8 secretion (reviewed by Nickoloff *et al*, 1991; Barker *et al*, 1991) and increased production of IL-6 and IL-1 (Kirstensen *et al*, 1993). Various exogenous agents, including endotoxin, UV radiation and urushiol have been shown to induce TNFα production in cultured KCs (Barker *et al*, 1991).

TABLE 1.2:

Epidermal cytokines described in this section.

Cytokine	Synthesised by:		M.Wt.	Main Functions	Reference
	KC	LC			
IL-1α	Yes	Yes	17 kD (biologically active form)	pro-inflammatory, KC proliferation	Luger, 1981
IL-1β	Yes	Yes	17 kD (biologically active form)	pro-inflammatory, KC proliferation	Luger, 1981
TNFα	Yes	No	51 kD homotrimer	pro-inflammatory, LC viability	Ansel, 1990
IL-8	Yes	No	8.4 kD	Neutrophil and T cell recruitment	Larsen, 1989
GM-CSF	Yes	No	22 kD dimer	KC growth factor, LC maturation	Kupper, 1988
IL-10	Yes	No	30 -40 kD	inhibition of cytokine synthesis	Enk, 1995

Although the role of TNFα in the epidermis is still unclear, there is evidence that along with other epidermal cytokines such as IL-1 and GM-CSF, TNFα controls LC viability. TNFα has also been shown to influence LC migration during cutaneous

immune responses (Cumberbatch *et al*, 1992). TNFα induces KC CD54 expression and endothelial expression of E-selectin, VCAM-1 and CD54 leading to the recruitment of leucocytes into the site of injection (Groves *et al*, 1995).

INTERLEUKIN 8

IL-8 is a member of the supergene family of pro-inflammatory and chemotactic cytokines known as the chemokines. Twelve recently cloned cytokines have been assigned to this group, on the basis of containing a conserved motif of four cysteine residues (reviewed by Taub *et al*, 1993). Intradermally injected IL-8 results in neutrophil and monocyte recruitment (Leonard *et al*, 1991, Swensson *et al*, 1991). IL-8 itself is not a monocyte chemoattractant, therefore induction of other chemokines must occur after IL-8 receptor ligation.

IL-8 is not found in normal skin (Paludan and Thestrup-Pedersen, 1992) but KC production is upregulated upon barrier perturbation (Garner *et al*, 1994) or in various diseases, particularly psoriasis. IL-8 along with monocyte chemoattractant and activating factor (MCAF) can be upregulated by various cytokines, including IL-1, TNFα and IFNγ (Larsen *et al*, 1989).

GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF)

Results from Braunstein's group corroborated previous *in vitro* studies (Kupper *et al*, 1988, Kaplan *et al*, 1992) by showing that intradermal injection of GM-CSF induces KC proliferation as well as regenerative differentiation of the epidermis (Braunstein *et al*, 1994). Production of GM-CSF by epidermal KC may therefore provide a potential autocrine or paracrine regulator of epidermal growth.

Other studies indicate that GM-CSF may also activate mature granulocytes, eosinophils, T cells (Griffin *et al*, 1990, Burgess *et al*, 1987) and KC (Kupper *et al*, 1988) and play a role in LC maturation (Heuffler *et al*, 1988; Kimber *et al*, 1992).

GM-CSF synthesis is upregulated by IL-1 (Kupper *et al*, 1998) and both cytokines act synergistically in the activation of granulocytes and macrophages and in enhancement of LC function, thereby transiently enhancing local antimicrobial defences at the site of tissue injury.

INTERLEUKIN-10

IL-10 inhibits the production of IL-1, IL-6, IL-8, TNFα and GM-CSF at the transcriptional level in monocytes, and has also been shown to inhibit its own synthesis (De Waal-Malefyt *et al*, 1991). Some of the effects of IL-10 in the skin have been described in the previous sections, including downregulation of both ICAM-1 and B7 molecule expression (Kawamura *et al*, 1995). IL-10 has also been shown to play a role in induction of apoptosis or decreased viability in cultured LC (Ludewig *et al*, 1995). All the functions of IL-10 in the skin appear anti-inflammatory.

1.3. IRRITANT CONTACT DERMATITIS

Skin diseases are the commonest occupational illness, of which dermatitis is the most prevalent. Contact dermatitis may be allergic (ACD) or irritant (ICD), with irritant considered to be the commoner of the two. ACD is associated with immunological memory, resulting from sensitization to a specific antigen. In contrast, ICD is defined as a localised non-immunological inflammation due to the direct influence of single or repeated exposure to one or more external factors (Malten, 1981). Irritant dermatitis is characterised clinically by erythema, oedema and dryness at the affected site and, too often, has a poor prognosis.

Contact allergens initiate inflammation by immunological reactions, whereas, irritants induce damage through direct cytotoxic action, without prior sensitisation (Kligman and Wooding, 1967). Both irritant and allergic dermatitis, although induced by different initiating pathways, result in very similar end stage inflammatory responses, making the diagnosis problematic (Brasch *et al*, 1992). They are usually distinguished by examination and patch testing. Diagnostic confusion means that knowledge of disease prevalence is inadequate and more accurate diagnostic aids are urgently required.

Differentiation between many allergic and irritant reactions can be made with epicutaneous patch tests (Kligman and Epstein, 1975). This method is used routinely for diagnosis of ACD in the clinic and for research purposes. The chemical is usually applied to the skin for 24 - 48 hours and the resulting reaction assessed:

1. ALLERGIC REACTION

This evolves slowly, peaking 2-3 days after exposure. The reaction radiates out from the patch site and takes between 7 and 10 days to resolve.

2. IRRITANT REACTION

This often starts with burning and discomfort, seen a few hours after exposure. The reaction often peaks after only 24 hours, staying sharply confined to the patch area, and generally heals within a few days.

Two major types of ICD have been defined, acute and chronic (reviewed by Maltem, 1981, Berardesca and Distante, 1994):

1. ACUTE

Caused by a single exposure to an irritant. This results in inflammation which may be clinically and histologically indistinguishable from allergic contact dermatitis. The reaction is restricted to the site of exposure, is dose dependent and may result in a wide spectrum of clinical responses. Acute ICD may be the result of an occupational accident, for example chemical burns.

2. CHRONIC

This is more complex than acute ICD and may involve exposure to multiple causative factors over a considerable period of time. These factors on their own would not be sufficient to induce ICD but the cumulative effect results in disease. The chronicity of ICD has therefore been largely attributed to cumulative 'irritant effect', usually associated with impaired stratum corneum function. The SC forms the outermost protective epidermal layer, damage of which enhances irritant penetration into the skin. The condition will not always resolve with avoidance of all irritant exposure and the molecular and cellular basis of its persistence have yet to be determined.

A wide variety of structurally unrelated chemical irritants may be encountered in daily life affecting people in a great many occupations, including builders, caterers, cleaners and hairdressers. These irritants have been separated into 4 major classes (shown in **Table 1.3**) and affect the skin through many different mechanisms. SC damage is a mechanism common to many irritants and is an important feature of irritant reactions.

Organic solvents and detergents are the most important groups. Organic solvents are believed to be responsible for between 6 and 20% of ICD cases, with detergents accounting for almost as many cases (Bruze and Emmett, 1990).

The response to contact with any given irritant is unpredictable and dependent on many factors, some of which are outlined in **Table 1.4**. Susceptibility varies among

Table 1.3: Major categories of irritation inducing substances. (Adapted from tables by Bruze and Emmett. In: Irritant contact dermatitis. *Marcel Dekker, Inc.* 1990)

IRRITANT CLASSES	EXAMPLES	MODE of ACTION
DESICCANTS	acrylic dust	increase SC brittleness, resulting in increased permeability
ABRASIVES	fibre-glass	mechanical irritation
ACIDS/ALKALIS	H_2SO_4/NaOH	increase TEWL, protein denaturation
ORGANIC SOLVENTS & *DETERGENTS	SLS benzalkonium chloride	decrease barrier function, denature keratins and alter skin lipids

*group of most importance in induction of ICD.

TABLE 1.4: Factors which influence irritant reactions. (Adapted from tables by Bruze and Emmett. In: Irritant contact dermatitis. *Marcel Dekker, Inc.* 1990)

IRRITANT	physical nature chemical properties presence of reactive groups
EXPOSURE	concentration exposure time occlusion
PATIENT	anatomical region age genetic background skin condition race prior irritant exposure
ENVIRONMENTAL	humidity temperature

populations (Kligman and Wooding, 1967), with some individuals even showing reactions to water if exposed frequently enough. Water can cause dry skin through solubilisation and removal of hygroscopic substances from the epidermis. In contrast, frequent contact may also result in increased hydration of the skin, facilitating penetration of water soluble irritants (Kligman and Wooding, 1967, reviewed in Bruze and Emmet, 1990) Dry skin, a result of increased transepidermal water loss (TEWL) (reviewed by Berardesca and Distante, 1994) is more susceptible to irritation. Therefore irritation is more common in the winter months when outdoor temperatures are low with low relative humidity, resulting in skin dryness (Tupker *et al*, 1990). Patients with atopic dermatitis also show an increased risk of developing ICD, possibly because of dry skin, and therefore lower barrier function (Tupker *et al*, 1990; Lee *et al*, 1995).

Young children have been shown to be susceptible to irritants; this is believed to be due to their high TEWL. TEWL decreases with age and maybe associated with increased barrier function. Therefore elderly people have a decreased susceptibility to irritation (reviewed by Berardesca and Distante, 1994). Race and gender may also be contributory factors but, so far, studies have been contradictory.

EXPERIMENTALLY INDUCED IRRITANT REACTIONS

As already stated the mechanisms involved in induction of ICD and the resultant immunological consequences are poorly understood. In order to shed some light on the process several groups have investigated experimentally induced irritant and allergic reactions in healthy volunteers. It is from these experimental studies that the majority of information concerning cellular infiltration and activation in ICD has been obtained. **Table 1.5** outlines experimental methodologies previously employed in studies of experimental ICD.

Some experimentally induced allergic and irritant reactions produced 48 hours after patch testing show similar inflammatory response in biopsies taken at 72 hours. This infiltrate consists mainly of memory T cells and macrophages (Brasch *et al*, 1992).

TABLE 1.5:
Summary of experimental designs used in the investigation of experimentally induce irritant reactions.

	EXPERIMENTAL DETAILS				
REFERENCE	Subjects	Irritants (vehicle)	Skin site	Application	Biopsy (B) Blister (SB)
Fisher and Maibach, 1975	normal volunteers	0.5%, 1% or 2% SLS (DW)	upper back	24, 48, 96 hrs (replaced every 24 hrs)	B
Kanerva *et al*, 1984	normal volunteers	0.2% DL (petrolatum)	back	30 mins 3 hrs	B - 48 hrs B - 24 hrs
Ferguson *et al*, 1985	ACD patients, uninvolved skin	2%, 5% or 10% SLS (yellow soft paraffin)	upper back	48 hrs	B - 72 hrs
Lindberg and Emtestam, 1986	normal volunteers	0.5% SLS (DW)	outer thigh	6 or 24 hrs	B - 6, 24, 48 & 96 hrs
Willis *et al*, 1986	normal volunteers	0.5% BC	volar forearm	3, 6, 12, 24 or 48 hrs	B - 3, 6, 12, 24, 48, 98, 196 hrs
Avnstorp *et al*, 1987	normal volunteers	5% SLS (DW) 1% CO (petrolatum)	lower back	4, 8, 24, 48 hrs	B - 72 hrs

TABLE 1.5 (contd.):

	EXPERIMENTAL DETAILS				
REFERENCE	Subjects	Irritants (vehicle)	Skin site	Application	Biopsy (B) Blister (SB)
Lindberg *et al*, 1990	normal volunteers	2% or 4% SLS (DW) 20% or 80% NA (propan-ol)	gluteal	24 hrs	B - 48 hrs
Willis *et al*, 1989, 1992 and 1993	normal volunteers	5% SLS(DW), 0.5% BC(DW), 0.8% CO(yellow soft paraffin), 0.02% DL(yellow soft paraffin) 80% NA (propan-ol), 100% PG	volar forearm	48 hrs	B - 1 hr later
Kanerva, 1990	normal volunteers	0.1% DL (petrolatum)	back	30 mins or 3 hrs	B - 24 or 48 hrs
Brasch *et al*, 1992	ACD patients	1%, 2% or 5% SLS (DW)	upper back	24 hrs	B - 72 hrs
Mikulowska and Falck, 1994	normal volunteers	1% SLS (DW)	forearm	48 hrs (replaced every 24 hrs)	B - 2, 5, 7 & 14 days
Gerberick *et al*, 1994	normal volunteers	0.25%, 0.5% or 0.1% SLS (PBS), 13.9% NA (prop-an-ol)	forearm	48 hrs	SB
Le *et al*, 1995	ACD or ICD uninvolved	3% SLS (DW)	upper back	24 or 48 hrs	B - 1 - 5days

These similar inflammatory responses, result through different initiating pathways driven either by allergen or irritant. Investigation into early irritant reactions, taking place prior to 72 hours, may be helpful in understanding the pathogenesis of ICD.

Experimentally induced irritation may affect the epidermis in different ways. The type and severity of damage caused is dependent on the type of irritant, its concentration and the time of application used. A wide variety of histopathological changes; including spongiosis, vesiculation, intracellular oedema, necrosis and parakeratosis have been observed after irritant exposure (**Table 1.6**). For example, dithranol (DL) irritation results in mild spongiosis (Willis *et* al, 1989). DL has also been shown to damage KC, LC and melanocytes (Kanerva *et al*, 1984, Kanerva, 1990). Induction of mitochondrial damage, often observed in the outer epidermal layers (Willis *et al*, 1989), is characteristic of DL irritation (Kanerva *et al*, 1984, Kanerva, 1990). DL application may also result in KC swelling (Willis *et* al, 1989) and occasionally apoptosis (Kanerva, 1990).

Epidermal oedema and spongiosis appear to be relatively non-specific occurring after application of many irritants, for example DL, benzalchonium chloride (BC), NA and SLS (Willis *et al*, 1986, Mikulowska and Falck, 1994). The degree of oedema produced probably reflects the intensity of inflammation.

Experimentally induced SLS irritation results in acanthosis, parakeratosis, spongiosis and occasionally vesiculation (Willis *et al*, 1989, Mikulowska and Falck, 1994). In contrast, NA application has a profound affect upon the epidermal KC population. The nuclei are affected becoming shrunken, with condensed, darkly staining marginated heterochromatin (Willis *et al*, 1989).

Studies have also shown that some irritants may increase epidermal proliferation (**Table 1.7, page 40**), suggesting that alteration in KC kinetics may be partly responsible for the parakeratosis often seen in ICD reactions with common irritants such as SLS, DL and NA (Willis *et al*, 1992). Changes in epidermal proliferation

TABLE 1.6:

Histopathalogical changes induced in experimental irritant reactions. Summary of experimental designs are outlined in **Table 1.5**

REFERENCE	Irritant	SUMMARY OF FINDINGS
Kanerva *et al*, 1984	DL	LC mitochondria damaged
Willis *et al*, 1986	BC	Spongiosis and oedema observed at 12 hrs.
Willis *et al*, 1989	SLS	Parakeratosis and patchy spongiosis observed. Keratohyalin granules were absent.
	NA	No parakeratosis and minimal spongiosis. "Tongues of dense, irregularly shaped eosinophilic KC containing shrunken nuclei that extended downwards from the stratum granulosum" were also observed.
	PG	Basketweave stratum corneum. Mild, patchy spongiosis .
	BC	Mild, patchy spongiosis. Occasional areas of necrotic damage in the upper prickle layer.
	DL	Some spongiosis, particularly in basal layers. Mitochondrial damage present in KC, particularly in the stratum granulosum and upper prickle layer.
Kanerva, 1990	DL	LC damage- swollen mitochondria and broken cristae. KC were also affected. They became cytolytic with oedemic cytoplasm. A few scattered KC underwent dyskeratosis (apoptosis). KC and LC damage was evident for more than 10 days.
Mikulowska and Falck, 1994	SLS	Acanthosis, focal spongiosis and intraepidermal vesiculation were common.

TABLE 1.7:

Epidermal cellular infiltration and KC response to experimentally induced irritant reactions. Summary of experimental designs are outlined in **Table 1.5**.

REFERENCE	Irritant	SUMMARY OF FINDINGS
Willis *et al*, 1986	BC	Epidermal lymphocyte influx begining at 6 hrs, increasing until maximal at 24 - 96 hrs.
Lindberg *et al*, 1990	SLS	CD3 +ve cell infiltration into epidermis. KC CD54 expression.
	NA	CD3 +ve cell infiltration into epidermis. Infiltration was comparable to that seen with SLS irritation. No KC CD54 expression.
Kanerva, 1990	DL	Epidermal T cell and macrophage infiltration.
Brasch *et al*, 1992	SLS	Epidermal T cell infiltration. Cells were helper/memory phenotype. Focal KC CD54 expression but no MHC II expression.
Willis *et al*, 1993	CO	Epidermal vesicles containing large numbers of infiltrating cells of varying phenotypes (including CD4+ve and CD8+ve T cells, macrophages and neutrophils). Increased levels of HLA-DR +ve cells.
	NA	Very few inflammatory cells observed, decreased levels of HLA-DR +ve epidermal cells.
	DL	Single cells infiltrating epidermis, macrophages CD4+ve T cells, and neutrophils. Increased levels of HLA-DR +ve cells.
	SLS	Macrophage and CD4+ve T cell epidermal infiltration. Epidermal neutrophils also observed. Increased levels of HLA-DR +ve cells.
Gerberick *et al*, 1994	SLS or NA	Epidermal macrophage infiltration.

are also common in other types of skin damage such as allergic dermatitis, UV and tape striping and are not specific.

Exposure to irritants may result in the expression of immune associated antigens, such as CD54 and MHC II, on KCs (**Table 1.7, page 40**). These findings imply that KCs play an important regulatory role in the irritant response. KCs can also synthesise and secrete a wide spectrum of cytokines (see previous section) enabling them to play an active role in irritant responses

One interesting feature of irritation is the migration of LCs from the epidermis (Mikulowska and Falck, 1994) to the regional draining lymph node (Brand *et al*, 1993). Experimentally induced irritant reactions have provided contradictory results (**Table 1.8, page 42**), with groups showing increased (Scheynius *et al*, 1984), unchanged (Kanerva *et al*, 1984) and decreased (Ferguson *et al*, 1985, Scheynius *at al*, 1992) epidermal LC numbers. These findings may reflect differences in the concentration, application time and irritant used. For instance, 80% NA has been shown to result in a significant decrease in epidermal LC number (Lindberg *et* al, 1990, Scheynius *et* al, 1992). In contrast, 5% SLS induced an increase in LC number (Lindberg and Emtestam, 1986, Willis *et al*, 1993).

Epidermal and dermal infiltration occur in experimentally induced irritant reactions (**Table 1.7, page 40** and **Table 1.9, page 43**) This infiltrate is predominantly mononuclear, composed of T cells and macrophages (Ferguson *et al*, 1985, Willis *et al*, 1993). The T cell population comprises both CD4+ve and CD8+ve cells, although the CD4+ve phenotype accounts for the majority of cells (Avnstorp *et al*, 1987). It is of interest that the pattern, and density of cellular infiltration may differ depending on the irritant used, for instance, neutrophils have been observed in SLS and croton oil (CO) reactions (Willis *et al*, 1993).

For the study we chose three well characterised common irritants, nonanoic acid, sodium lauryl sulphate and dithranol, in order to explore the mechanisms of action of

TABLE 1.8:

Epidermal LC response to experimentally induced irritant reactions. Summary of experimental designs are outlined in **Table 1.5**.

REFERENCE	Irritant	SUMMARY OF FINDINGS
Kanerva *et al*, 1984	DL	Normal epidermal CD1a+ve number, mitochondrial damage.
Ferguson *et al*, 1985	SLS	Decreased number of epidermal CD1a+ve cells.
Lindberg and Emtestam, 1986	SLS	Increased number of epidermal CD1a+ve cells at 48 and 96 hrs. Redistribution of LC with a polarisation towards the outer layers.
Willis *et al*, 1986	BC	Increased numbers of basal and suprabasal CD1a+ve cells by 6 hrs, occassional LC-Lymphocyte aposition within the epidermis. At 12 hrs, CD1a+ve epidermal numbers are normal.
Avnstorp *et al*, 1987	SLS or CO	No effect on epidermal CD1a+ve number.
Lindberg *et al*, 1990	SLS	Minor diferences in epidermal CD1a+ve cell number.
	NA	Decreased numbers of epidermal CD1a+ve cells.
Scheynius *et al*, 1992	SLS	Minor differences in epidermal CD1a+ve cell number.
	NA	Decreased number of epidermal CD1a+ve cells.
Brasch *et al*, 1992	SLS	Decreased numbers of epidermal CD1a+ve cells.
Mikulowska and Falck, 1994	SLS	Decreased numbers of epidermal CD1a+ve cells at 48 hrs. They suggested that LC are migrating to the dermis.
Willis *et al*, 1993	SLS	Increased numbers of epidermal CD1a+ve cells.
Gerberick *et al*, 1994	SLS or NA	Decreased numbers of epidermal CD1a+ve cells.

TABLE 1.9:

Dermal infiltration in response to experimentally induced irritant reactions. Summary of experimental designs are outlined in **Table 1.5.**

REFERENCE	Irritant	SUMMARY OF FINDINGS
Ferguson *et al*, 1985	SLS	Dermal infiltration was predominantly mononuclear but neutrophils were also observed scattered throughout the dermis. CD4+ve and CD8+ve T cells were present, with CD4+ve predominant.
Avnstorp *et al*, 1987	SLS or CO	Macrophage and T cell infiltration was observed. CD4+ve and CD8+ve T cells were present, with CD4+ve predominant.
Willis *et al*, 1993	Table 1.5	Macrophage, neutrophil and T cell infiltration were observed. CD4+ve and CD8+ve T cells were present, with CD4+ve predominant.
Mikulowska and Falck, 1994	SLS	Mononuclear dermal infiltration.

these different classes of irritants. The next section explores the current literature available on these three irritants.

• DITHRANOL (DL)

In 1878, Squires first used a crude extract of Goa powder to treat psoriasis. The anti-psoriatic component of this powder was later shown to be an anthracene derivative, chrysarobine. By 1916 a synthetic analogue, 1,8-dihydroxy-9-anthrone (anthralin, dithranol, cignolin) had been produced which is still used for psoriasis treatment (reviewed by Ashton *et al*, 1983). This synthetic product caused considerably less irritation than the original powder, but skin staining, as first described by Squires, still occurred. Even today skin irritation and discolouration remain the major therapeutic drawbacks to this psoriasis treatment (reviewed by Kanerva *et al*, 1990, Ashton *et al*, 1983). Psoriasis is characterised by hyperproliferation, parakeratosis, epidermal accumulation of T cells and neutrophils and dermal inflammation. These features are resolved following topical therapy with DL. The exact mechanism of DL's action has still to be determined.

DL inhibits of KC hyperproliferation (Willis *et al*, 1992), granulocyte function and may also exert an immunosuppressive effect (reviewed by Kemeny *et al*, 1990, Van de Kerkhof, 1991). Decreased epidermal T cell numbers are also observed after DL application (Baker *et al*, 1985). Anti-chemotactic activity of DL has been described in psoriatic skin (Ternowitz, 1987), which could account for the decreased T cell and neutrophil infiltration observed after DL treatment. DL is thought to influence KCs and lymphocytes by inhibition of DNA synthesis and repair, cellular respiration and key enzymes of the metabolic pathway. *In vitro* treatment of neutrophils with DL results in a dose dependent inhibition of migration, superoxide anion generation and leukotrine B4 production (reviewed by Kemeny *et al* 1990); accounting for decreased neutrophil function.

Irritation, resulting in a dose and concentration dependent inflammatory response, has been observed in many studies (Mustakallio, 1979; Goransson, 1987). At a concentration of 0.02%, DL caused mild to moderate erythema, which induces non-specific spongiosis and marked swelling of KCs in the upper epidermis (Wills *et al*, 1989).

DL is strongly lipophilic, becoming associated with the cell membrane within minutes of epicutaneous application. Here DL is rapidly oxidised, causing production of superoxide which are thought to be responsible for the skin discolouration anions (reviewed by Kanerva *et al*, 1990, Ashton *et al*, 1983). These free radicals along with other mediators, such as histamine and platelet activating factor(PAF), induce inflammation. DL may also affect lipid metabolism through interference with enzymic activity (reviewed by Kanerva *et al*, 1990). DL penetration may be influenced by the vehicle in which it is applied, for instance, the hydrophilic vehicle propylene glycol enhances penetration into the dermis(reviewed by Ashton *et al*, 1983).

There is still some confusion as to whether DL affects epidermal LC density. Results have shown this to be unaffected (Kanerva *et al*, 1984) or decreased after irritation (Gawkrodger *et al*, 1986). Electron microscopy has shown that LC are sensitive to DL, with application resulting in swollen mitochondria with broken cristae, indicative of degeneration and therefore reduced function (Kanerva, 1990, Morliere *et al*, 1985). Mitochondrial inhibition is thought to arise through binding of free radicals to the cell membrane (reviewed by Ashton, 1983), resulting in decreased respiration and subsequently decreased energy production. KCs may also be affected by DL, becoming cytolytic with oedematous cytoplasm and sometimes undergoing apoptosis (reviewed by Kanerva, 1990). The diminished energy available for cell division and differentiation could, in part, explain some of the therapeutic effects, such as decreased proliferation, in DL treatment of psoriasis. This powerful anti-mitotic effect may also be mediated through inhibition of DNA replication and/or cytosolic enzymes (reviewed by Kanerva, 1990).

• NONANOIC ACID (NA)

Nonanoic or pelargonic acid is a C9 free fatty acid, found in lacquers, plastics, synthetic flavours and odours, turbojet lubricants and gasoline. NA causes irritation after occluded and repeated exposure (Stillman *et al*, 1975). Forty eight hour patch testing on normal skin caused KC damage in the upper layers of the epidermis. Examination of these KCs revealed the presence of dense aggregates of keratin filaments in the cytoplasm, indicative of disturbances in KC metabolism and differentiation (Willis *et al*, 1989). Studies also showed an increase in basal KC proliferation which may explain the presence of parakeratosis (Willis *et al*, 1989; Willis *et al*, 1992). For these reasons NA is commonly used in studies investigating irritancy and also as an experimental model for parakeratosis (Wahlberg *et al*, 1985).

NA irritation fails to cause leucocyte exocytosis or epidermal ICAM-1 expression (Lindberg *et al*, 1990). This lack of response may be the result of induction of localised immuno-inhibitory factors. If present in large enough concentrations these factors could suppress the affects of pro-inflammatory mediators (Willis *et al*, 1993).

NA irritation has also been shown to affect LCs. Various groups have shown that NA application results in a dramatic decrease in epidermal LC density (Willis *et al*, 1990, Lindberg *et al*, 1990), coupled with a decrease in dendrite length. Electron microscopy revealed that the NA induced morphological signs of LC injury (Willis *et al*, 1990).

• SODIUM LAURYL SULFATE (SLS)

Sodium lauryl sulfate is an anionic detergent commonly used in cleaning products and cosmetics and as an emulsifier in many pharmaceutical products. This detergent has been shown to induce considerable changes in skin, including increased TEWL, blood flow, SC turnover time and pH (reviewed in Wilhelm *et al*, 1994; Lee and

Maibach, 1995). The intensity and duration of the resultant inflammatory response is related to the number of applications and concentration (Novak and Francom, 1984).

SLS irritation results in KC damage, including induction of parakeratosis (Willis *et al*, 1991, 1992). Parakeratosis may have a number of causes, including increased KC proliferation, incomplete keratinization or direct cytotoxic injury. SLS induced irritation is accompanied by increased levels of IL-6 (Berardesca and Distante, 1994) and TNFα (Hunziker *et al*, 1992). IL-6 and IL-8 are potent KC growth factors (Grossman *et al*, 1989, Tuschil *et al*, 1992) and may be responsible for the hyperproliferation and parakeratosis often observed (Willis *et al*, 1989). SLS has also been shown to influence LC numbers. Groups have shown increased epidermal cell numbers (Lindberg *et al*, 1990), decreased (reviewed by Falck, 1981) or minor differences (Scheynius *et al*, 1992), or unaltered numbers but showing evidence of LC degeneration and decreased dendrite length (Willis *et al*, 1990). Brand and colleagues have demonstrated in human volunteers that LC migration from the skin to the regional lymph nodes takes place during experimentally induced SLS irritation (Brand *et al*, 1992, 1993, 1995). They developed a method of microsurgical lymph cannulation in order to investigate the phenotype and numbers of cells present in the lymph before and after experimental irritation. 10% SLS in distilled water was applied to the foot 48 hours after cannulation. Application of SLS was repeated after 24 hours and remained on the skin for a total of 36 hours (Brand *et al*, 1993). Leucocyte numbers were investigated from 2 - 8 days after irritation. Results showed increased numbers of LCs, T and B cells even at 8 days when the clinical reaction had resolved (Brand *et al*, 1992, 1993, 1995). LCs may migrate into the skin soon after irritation and then as the reaction progresses they leave via the afferent lymph.

Several mechanisms have been put forward to explain the effect of SLS on the skin, including :

1. removal of epidermal lipid through the solubilizing action of surfactants.
2. denaturation of epidermal proteins.

Penetration of SLS through the outermost epidermal layers allows the irritant to reach areas where tissue function may be affected. SLS has been shown to solubilize cholesterol, free fatty acids and the esters of these compounds (Froebe *et al*, 1990). Despite this, only very small amounts of lipid are actually removed from the SC. Froebe and co-workers have shown that application of 2% SLS removes only 4% of the total SC lipid. These findings are of interest and suggest that lipid removal alone cannot be responsible for irritation (Froebe *et al*, 1990). The quantity of SC lipid is therefore not affected by irritation; however, significant changes in lipid classes, for instance ceramides, have been observed. Changes in the distribution of certain ceramide species have been observed in SLS irritation (Fulmer *et al*, 1986). These changes are thought to reflect altered keratinization associated with abnormal ceramide biosynthesis.

SLS may bind to and reversibly denature epidermal keratin (Harrold.S, 1959, Froebe *et al*, 1990). Binding to SC proteins may alter barrier function (Fullerton, 1994), one mechanism through which SLS may exert its irritant effect. Binding of proteins may be defensive, preventing the irritant from penetrating further into the epidermis (Blank and Gould, 1959).

Radioactively labelled SLS has been shown to penetrate the skin in a concentration and temperature dependent manner (Emilson *et al*, 1993). Small amounts of SLS penetrate through the epidermis into the dermis, but the majority remains on the skin surface or in the SC (Fullerton *et al*, 1994).

1.4. AUTOIMMUNITY

This thesis proposes that chronic ICD may in some patients have an autoimmune component. The inflammatory response to certain irritants results in epidermal damage and could result in the release of a normally sequestered self Ag. An immune response raised against this self Ag will result in autoimmunity.

The term autoimmune disease refers to a wide spectrum of systemic and organ specific diseases, characterised by damage to tissues caused by humoral and/or cell mediated immune responses. These are complex disease processes involving many components including, environmental, genetic and immunologic factors.

Several mechanisms have been put forward to explain the induction of pathologic autoimmune responses (reviewed in Bona *et al*, 1993, Stites *et al*, 1994).

For example:

- release of sequestered Ag
- molecular mimicry and cross-reactivity
- polyclonal T cell activation - superantigens (sAg)
- generation of neo-self determinants

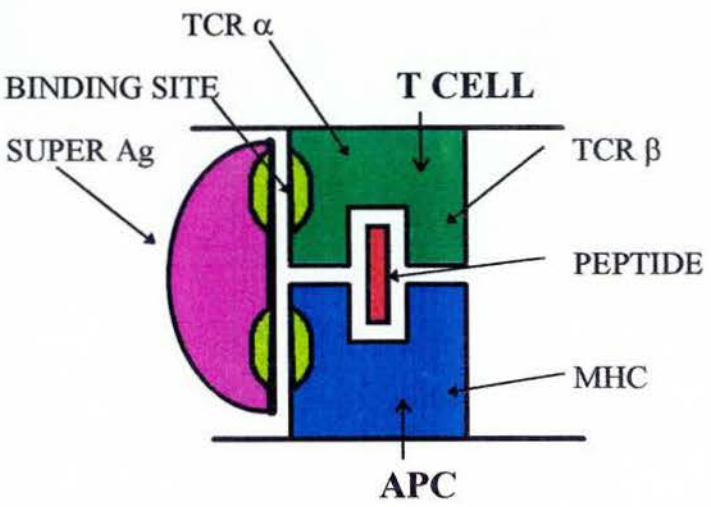
A sequestered or cryptic Ag is normally unavailable to the immune system and therefore avoids tolerance induction. Sequestered Ags can be found in privileged sites, such as the cornea of the eye (anatomical sequestration) (reviewed by Gery and Strelein, 1994), or may simply be unavailable to the immune system because they are inside cells (molecular sequestration). Release of such Ags may occur as a result of trauma or inflammation, providing an opportunity for development of autoimmunity. It has been suggested that if the availability of a sequestered Ag increases beyond a certain threshold a specific T cell response will occur resulting in autoimmunuity. Increased threshold may also arise as a result of molecular mimicry or interaction with sAg. (Lipham *et al*, 1991, Appelmelk *et al*, 1996)

Induction of autoimmunity can also occur through immunisation with an Ag that cross-reacts with or has identical antigenic sites (molecular mimicry) to a self-Ag (Appelmelk *et al*, 1996). The resultant immune response will therefore recognise both self and foreign Ag. Anti-idiotype auto-Abs may also be the consequence of a natural immune response. With these initiation methods persistence of the stimulus would ensure continued autoimmunity.

Polyclonal T cell activation has also been considered as a contributing mechanism, particularly in systemic diseases, such as Systemic lupus erythematosus. Polyclonal activation involves proliferation of many T cell clones of different specificity. For example, superantigens are capable of stimulating T cells through binding MHC II and the Vβ domains of the T cell receptor irrespective of T cell Ag specificity (**Figure 1.5**).

FIGURE 1.5:

Polyclonal T cell activation through superantigen binding. Super Ag binds more conserved regions of the MHC, compared to conventional Ag.



Autoimmune responses may be directed against newly created self determinants, neo-Ag. Neo-Ags may be generated through drug or other haptenic groups binding to self Ag or through recombination, conversion or mutation following viral infection. The resultant auto-Abs may recognise not only the neo-protein but also the native protein and may therefore persist after the stimulus has been withdrawn (Kubicka-Muranyi *et al*, 1995).

• EXAMPLES OF CUTANEOUS AUTOIMMUNITY

PEMPHIGUS AND PEMPHIGOID

Pemphigus refers to a large group of bullous diseases characterised by epidermal cell- cell detachment (acantholysis), leading to blister formation. These diseases are considered to have an autoimmune component due to the presence of circulating and skin auto-Abs directed against KCs (reviewed by Thivolet, 1994).

Pemphigus vulgaris (PV) is a potentially lethal disease in which blister formation occurs just above the basal layer. The epidermal blisters occur as a result of the loss of cell-cell adhesion caused by the action of auto-Abs against a KC cell surface glycoprotein, the PV antigen (PVA) (Thivolet, 1994, Amagon, 1995). PVA is a 130kDa molecule belonging to the desmoglein subfamily of cadherin adhesion molecules (Karpati *et al*, 1994).

Bullous pemphigoid is another blistering disease, characterised by auto-Abs directed against molecules in the lamina lucida of the basement membrane which are thought to be involved in epidermal-dermal adhesion. The target Ags have been identified as two molecules of molecular weight 230kDa and 180kDa (reviewed by Giudice *et al*1995, Fairley *et al*, 1995).

GUTTATE PSORIASIS

Guttate psoriasis is a T-cell mediated disease known to be associated with streptococcal throat infections. The observations that T lymphocytes with certain Vβ TCR families predominate in lesions of this type of psoriasis, suggests that streptococcal sAg and induction of autoimmunity may be involved in disease pathogenesis. Valdimarsson and colleagues have recently proposed an autoimmune model for the onset and maintenance of guttate psoriasis (reviewed by Vladimarsson *et al*, 1995).

SCLERODERMA (SYSTEMIC SCLEROSIS)

The term scleroderma includes a heterogeneous group of localised and systemic conditions causing thickening and hardening of the skin. Sera from patients with scleroderma contain a wide range of auto-Abs, including various anti-nuclear Abs (Parodi *et al*, 1995), Abs to polynucleotides and Abs that react with collagen (reviewed by Maddison, 1988). Anti-nuclear Abs for example can be detected in 95% of patients. Anti-nuclear Abs and Abs directed against nuclear antigens such as topisomerase 1 are capable of complement fixation. This suggests that these auto-Abs may be directly involved in disease pathogenesis through initiation of complement-mediated tissue injury (reviewed by Maddison, 1988).

1.5. AIMS OF THESIS

This chapter has reviewed relevant information to date concerning the three irritants chosen for this study. DL, an alcohol, has been studied in respect to psoriasis treatment, the success of which appears to effect the anti-mitotic potential of DL. NA represents the acid group of irritants and SLS is a member of the detergents class, one of the largest groups of chemicals with irritant capabilities. All three irritants can produce inflammatory skin responses, but the histological components of these reactions seem very different. The cellular and molecular mechanisms involved in these different patterns of skin damage have yet to be fully investigated.

A unique approach was undertaken for this investigation of chronic ICD, involving comparison of patients with chronic ICD and normal healthy volunteers. The study aimed to determine whether the intrinsic nature of the skin from patients with chronic ICD contributes to disease pathogenesis and if this method of experimental irritation is a good model for chronic ICD.

The central purpose of this project was to define the role(s) of the inflammatory and immunological responses in the pathogenesis of chronic ICD, and to determine the nature of the early inflammatory/ immunological events in experimentally induced irritant reactions in healthy volunteers and patients with chronic ICD.

The experimental aims of the study were:-

- to compare the light histological appearance of biopsies taken from skin treated with different irritants (NA, SLS and DL) to induce a similar degree of erythema.

- to compare changes in the epidermal Langerhans cell population after irritation. To investigate any differences between normal volunteer skin and the uninvolved skin of patients with chronic ICD.

- to compare sequential changes in the cellular infiltrate, accessory/adhesion molecule expression and cytokine profiles after irritation.

- to compare 1 and 6 hour with 24 and 48 hour reactions. This investigation aimed to define initiating or early events taking place in an irritant reaction. The later time-points of 24 and 48 hours allowed this study to investigate the more established phase of the reaction.

- to compare atopics with non-atopics, males with females and patients with and without irritant dermatitis lesions at the time of experimentation. This study aimed to determine if any particular group of individuals were more susceptible to experimental irritation.

- to investigate whether the blood of patients with ICD contained antibodies against skin antigens which might indicate that some cases of chronic ICD represent a form of autoimmune disease.

2.0. MATERIALS AND METHODS

All reagents were purchased from SIGMA unless otherwise stated.

2.1. CLINICAL DETAILS

Ethical approval for taking biopsies and suction blisters from human subjects was obtained from the Lothian Research Ethics Committee. All patients involved with the study gave informed, written consent.

• PATIENT DETAILS

Patients were recruited from the Dermatology out patient clinic at The Royal Infirmary, Edinburgh. All patients had a history of chronic irritant dermatitis for a period of at least six weeks, a minimum of one criteria outlined in **Table 2.1** and had attended the patch test clinic showing no relevant positive allergic reactions. A summary of patient details, including atopic status, age and sex, are shown in **Table 2.2.** Full patient details are outlined in appendix I.

• IRRITANTS AND GRADING OF ERYTHEMA

Optimal concentrations of the three irritants (NA, SLS & DL) were determined by titration on normal healthy volunteers to produce similar grades of moderate but distinct erythema, assessed visually (**Table 2.3**) and with a Minolta Chroma Meter CR-300, by 48 hrs. Subsequently the concentrations used for the final study were 80% nonanoic acid in propan-1-ol (w/w) and (w/v) 5% aqueous sodium lauryl sulphate and 0.01% dithranol in yellow soft paraffin (w/w). The vehicle controls used were propan-1-ol, distilled water and yellow soft paraffin respectively.

TABLE 2.1:

Criteria used for the diagnosis of chronic irritant dermatitis.

HISTORY	• occupational exposure • affected areas relatively static
CLINICAL	• erythema, hyperkeratosis or fissuring • glazed, scaled appearance of skin • if hand dermatitis * the above plus ring pattern pulpitis dorsa of the hands
PATCH TESTS	Negative (or non relevant positive reaction)

* as the majority of patients in the studies had hand dermatitis, important clinical patterns (summarised below) which suggest an irritant aetiology were used for patient assessment.

Ring pattern - i.e. underneath ring

Pulpitis - the pulps of the fingers

TABLE 2.2:

Summary of patient details.

	MALE (n = 35)	**FEMALE (n = 31)**
ATOPIC	12	13
NON-ATOPIC	23	18
ACTIVE ICD	28	15
NO ACTIVE ICD	7	16
AGE -RANGE	20 - 58	18 - 66

TABLE 2.3:

Visual clinical grading scale.

GRADE	DEFINITION OF REACTION
0	No reaction
1	Barely perceptible erythema
2	Moderate but distinct erythema with no induration
3	Indurated erythema
4	Bullae formation/ crusting

• IRRITANT APPLICATION AND TISSUE BIOPSIES

Tissue biopsies were taken, under 1% lignocaine local anesthesia, from clinically unaffected skin on the volar surface of the forearm. A normal control biopsy was taken at time zero. Three standard 8mm Finn chambers^{R} were then applied, two containing 15µl of the irritant and one containing the same volume of the vehicle for that irritant (vehicle control). Patches were removed after 1, 6, 24 or 48 hours; one hour later the degree of erythema was assessed visually and using the Chroma Meter. Three 6mm punch biopsies were then taken from each patient (1 vehicle control, 2 experimental). Half of each biopsy was snap frozen in liquid nitrogen and the remainder formalin fixed and paraffin embedded.

• SUCTION BLISTERS (Plate 2.1)

A total of 120µl) was applied to the volar aspect of the forearm, for 1 , 6 or 24 hrs prior to raising blisters, using standard four 8mm Finn Chambers^{R} (30µl of irritant in

each chamber). Irritant was Suction blisters were then raised under pressure at the site of the irritant reactions using plastic chambers based on the Dermovac^{R} (Kiistala, 1968). Suction blister fluid was removed from each blister using a sterile needle, pooled and stored at -70^{0}C (40 -200µl). The epidermal roofs were then carefully excised, snap frozen in liquid N_2, and stored at -193^{0}C.

2.2. HAEMATOXYLIN AND EOSIN (H & E) SECTIONS

• H & E staining

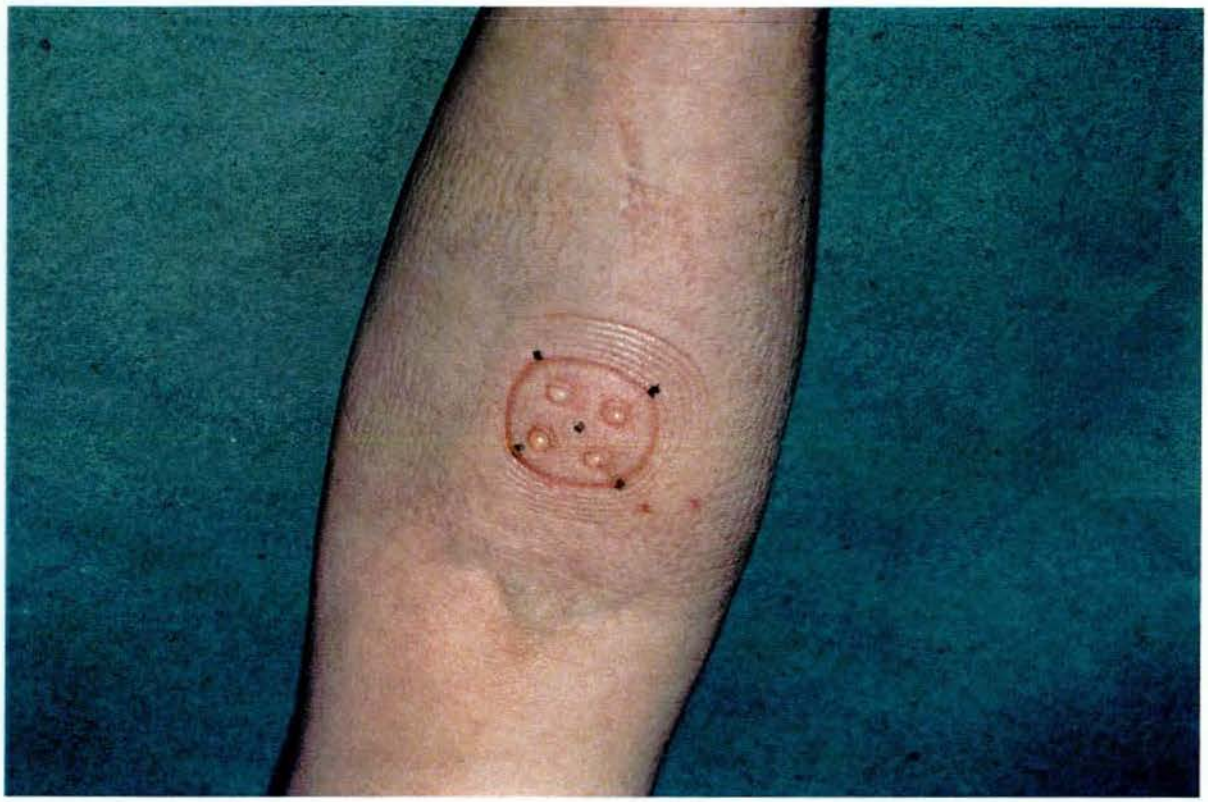
Paraffin sections (3µm) were cut from ice-cooled paraffin blocks using a microtome and floated on warm water onto poly-L-lysine coated glass slides. The slides were dried overnight in a 37^{0}C incubator. The paraffin sections were then dewaxed in xylene and passed down through an alcohol gradient (100% ethanol, 74% ethanol, 64% ethanol) to water (rehydration). Sections were immersed in Harris' Haematoxylin (BDH chemicals Ltd.), 5 mins, in order to stain the nuclei. After washing in water, sections were differentiated using 1% acid alcohol (5-10 seconds) and washed again. Following differentiation sections were placed in Scotts Tap water (1-2 mins.), washed and stained with alcoholic/aqueous Eosin (1 min). The sections were washed and dehydrated by moving back up through the alcohol gradient to xylene. Finally the sections were mounted in Pertex (Cell path Ltd.) and examined under a light microscope.

• Analysis of H & E sections

Each of the sections stained using the above method was assessed microscopically by three independent observers (Dr. Shahidullah, C. Sands and myself) and the results recorded on the proforma shown in **Table 2.4a,b,c**. Sections were assessed for a variety of parameters, firstly the presence or absence of appendages was noted (e.g.

PLATE 2.1:

Suction blisters raised on site of experimental irritation.



hair follicles, sweat ducts), and then assessment was continued as outlined in **Table 2.4a**, 2.**4b** and 2.**4c**.

Table 2.4a: Analysis of stratum corneum.

Stratum corneum - 0 - compact		parakeratosis (yes) - 1	
	1 - basketweave		(none) - 0

Starting with the Stratum corneum, this was categorised as either compact or basketweave in structure. The presence or absence of parakeratosis was also noted.

Table 2.4b: Analysis of epidermis.

KEY:

I.C. - intracellular oedema	Ves. - vesiculation	Spon. - spongiosis
N - neutrophil	E - eosinophil	

Oedema 0 - nil	**Infiltrate** - overall 0 - negligible	**Cellular Infiltrate**	
1 - mild (<25%)	1 - mild	0 - nil 1 - present	
2 - mod (25-75%)	2 - heavy	Granulocyte (G)	
3 - heavy (>75%)		Mononuclear (M)	

	Apoptosis	**Oedema**	**Infiltrate**	**Cells in infiltrate**	
		I.C. Ves Spon		**G**	**M**
Granular Layer					
Prickle cell Layer					
Basal Layer					

Biopsies were scored for the presence or absence of apoptosis in the prickle and basal layer only. The granular layer was not analysed as the dark keratohyalin granules

present in this area present technical difficulties for scoring. The total number of apoptotic cells per mm/basement membrane was also recorded (see section 2.4.).

The three epidermal layers (granular, prickle and basal) were assessed for oedema and cellular infiltration. Three types of oedema were analysed: intracellular, spongiosis (intercellular) and vesiculation. All three were scored on an arbitrary scale according to the severity of oedema, 0(nil), 1(mild), 2(moderate) and 3(heavy). Cellular infiltration was scored according to severity, 0(negligible), 1(mild) and 2(heavy), and cell type, G(granulocytic) and M(mononuclear). The granulocytic group was then further divided into neutrophils and eosinophils using morphological criteria.

Table 2.4c: Analysis of dermis.

Oedema 0 - nil
1 - mild (<25%)
2 - mod (25-75%)
3 - heavy (>75%)

Infiltrate(overall) 0 - negligible
1 - mild
2 - heavy

Cellular Infiltrate
0 - nil 1 - present
Granulocyte (G)
Mononuclear (M)

		Oedema	**Infiltrate**	**Cells in infiltrate**	
				G	**M**
Localised	papillary				
	reticular				
	perivascular				
	peri-appendage				
Diffuse					

The dermis was also assessed for the presence of oedema and cellular infiltration. The dermis was divided into separate compartments for the purpose of analysis. These sections included the papillary dermis, reticular dermis, perivascular areas, peri-appendage areas (hair follicle and/or sweat duct). The arbitrary scoring scale was the same as that employed for the epidermis.

All the categorised data was entered onto a Microsoft Access(v2.0) database and analysed using the Minitab statistical analysis package. The methods of statistical analysis are described in section 2.5.

2.3. IMMUNOCYTOCHEMICAL STAINING

All immunocytochemical staining was carried out in a semi-automated, "Sequenza" (Shandon) wet chamber (**Plate 2.2**). This system allows many slides to be stained simultaneously, significant reduction in background staining and reproducible results over time. The Sequenza uses plastic clips to hold the slides in a rack. A fine space exists between the slide and the plastic clip allowing 100µl of liquid to be held in an even film against the slide by capillary action. Placing more fluid into the reservoir at the top of the plastic clip displaces the fluid held against the section allowing standardisation of volumes and incubation times. Sections were analysed using the HOME microscope as outlined in section 2.4.

All antibodies were titrated on skin tissue to determine optimum dilution and incubation conditions (see **Tables 2.5a and b**).

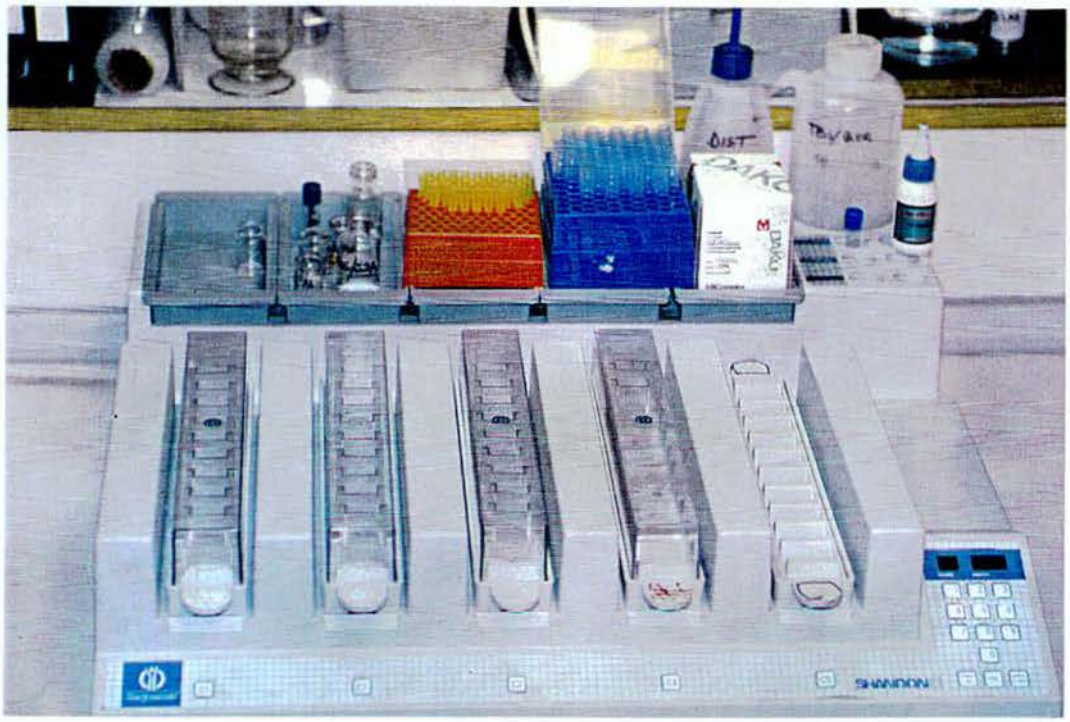
• PARAFFIN SECTIONS (Table 2.5a)

Paraffin embedded sections (3µm) were deparaffinised and rehydrated by passing through an alcohol gradient (see H&E section). When using the chromagen 3,3-diaminobenzidine for visualisation, endogenous peroxidase was blocked prior to staining (3% Hydrogen Peroxide (H_20_2), Fisons) in distilled water (DW) for 15 mins).

Antigen retrieval was carried out for certain antigens on paraffin sections only. Crosslinks caused by the paraffin embedding process may be broken using trypsin (ICN Biomedicals Inc.) or microwaving to reveal the antigen under investigation. Trypsin treatment (0.1% trypsin, 0.1% $CaCl_2$ pH7.8) was carried out for 30 mins at

PLATE 2.2:

The "Sequenza" (Shandon).



37^0C or sections were microwaved in citrate buffer (1.05g sodium citrate in 500ml DW, pH 6.0) for 2x 5mins then allowed to cool for at least 20 mins.

Before staining sections were washed in running tap water, clipped into the Sequenza and washed with buffer (0.1% BSA/Tris buffered saline (TBS), pH7.6). Sections were pre-incubated for 10 mins with 100ml of blocking buffer, either 20% normal rabbit serum (NRS (SAPU), diluted in TBS), 20% normal swine serum (NSS, Seralab) or 20% normal goat serum (NGS, SAPU) depending on the species in which the secondary antibody was raised. Pre-incubation in serum helps to prevent non-specific binding of antibodies to Fc receptors. After blocking, sections were incubated in 100µl of primary antibody diluted optimally in blocking buffer (1 hour, room temp.). For some antibodies, optimal incubation times were found to be overnight at 4^0C or for 2 hours at 37^0C (**Table 2.4a**). Sections were then washed twice in TBS (reservoir filled twice, 2x 5 mins) before incubation (30 mins, room temp) in biotinylated secondary antibody, again diluted in blocking serum. Washing was repeated as before and sections incubated (30 mins, room temp) in ABC- streptavidin HRP complex (for DAB chromagen) or ABC-AP complex (for Fast red visualisation) (ABC kits, DAKO). Sections were washed as before and the antibody staining visualised.

DAB VISUALISATION

Each section was incubated with 100µl of DAB (3,3-diaminobenzidine)/hydrogen peroxide substrate solution. DAB was applied to each section at a concentration of 0.5mg/ml of 1% hydrogen peroxide for 5 minutes and sections washed in water. The slides were then removed from the Sequenza racks and counterstained in haematoxylin (1 min) before 'bluing up' in Scott's Tap water. Finally the sections were dehydrated by moving up through an alcohol gradient before mounting in pertex.

FAST RED VISUALISATION

All sections were incubated with 500µl of alkaline phosphates substrate (Appendix II) (30 mins, in the dark), then washed off with water. Sections were then counterstained

Table 2.5a :

Antibodies used on paraffin tissue sections

Antibody	Specificity	Optimal Dilution	Trypsin (T) Microwaving (M) No treatment (N)	Temperature	Detection : Fast Red (FR) DAB
Mast cell tryptase	Mast cells	1/1000	T	room temp.	FR
MIB-1/Ki-67	Proliferating cells, Ki-67 Ag	1/30	M	37°C, 2 hours	FR
CD68	Macrophages	½00	T	room temp.	FR
CD1a	Langerhans cells	Neat	T	room temp.	FR
Bcl-2	All nucleated cells	1/10	M	room temp	DAB
Bax	All nucleated cells	1/50	T	room temp	DAB
Bcl-x	All nucleated cells	1/50	T	room temp	DAB
CD95/fas	Leucocytes, epithelial cells	1/300	T	room temp	DAB

** SEE APPENDIX II FOR DETAILS OF ANTIBODY SOURCE

as with DAB visualisation but were mounted, without dehydration, using an aqueous mountant (BDH) as Fast red substrate is dissolved by xylene.

- **FROZEN SECTIONS (Table 2.5b)**

Snap-frozen skin biopsies were partially thawed and then transferred onto a cool metal chuck. The biopsy was then frozen in OCT (Bayer Diagnostics) using liquid nitrogen and 6μm sections were cut at -20^{0}C using a cryostat. The frozen sections were air dried for a minimum of 3 hours and then fixed in dry/distilled acetone (Fisons) for 10 mins. As with paraffin staining, sections were washed in running tap water, clipped into the Sequenza and then washed with TBS buffer. Sections were blocked with serum and stained following the same procedure as for paraffin sections. Fast red visualisation was the only method employed for frozen sections.

2.4. The H.O.M.E microscope

(Highly Optimised Microscope Environment)

The Zeiss HOME microscope allows quantitative image analysis by linking a mouse driven personal computer directly to the microscope field of view. This system allows the viewer to count cells and delineate areas using the mouse while looking down the microscope. This HOME was used to delineate both epidermis and dermis, eliminating any appendageal areas (**Plate 2.3**), and allowing the density of positive cells to be calculated for both dermal and epidermal areas. The length of basement membrane and average epidermal thickness were also measured. All data were saved onto the computer hard disc and could be checked by other members of the team at a later time. All numerical data obtained from the HOME microscope was entered onto Microsoft Access (v2.0) relational databases and analysed using the Minitab statistical package.

** **Table 2.5b :**

Antibodies used on frozen tissue sections

Antibody	Specificity	Optimal Dilution	Temperature (ON - overnight)	Detection : Fast Red (FR) DAB
CD3	T cells	1/10	4°C ON	FR
CD4	T cells : CD4	1/10	4°C ON	FR
CD8	T cells : CD8	1/10	4°C ON	FR
CD15	Neutrophils	1/10	room temp	FR
CD54	ICAM-1	1/50	room temp.	FR
IFNγ	IFNγ	1/300	room temp.	FR
L243	MHC II	1/100	room temp	FR
CD1a	Langerhans cells	1/400	room temp.	FR
CD62 E	E-selectin	1/100	room temp.	FR

** SEE APPENDIX II FOR DETAILS OF ANTIBODY SOURCE

• DATABASE DESIGN DETAILS

Relational databases were used, in which the data were represented as tables. Each row in a table has the same number of labelled columns (Field), describing the nature of the data in that column (e.g. patient code). The design of a table included the definition of field names, the order in which fields should appear in the table, the type of data that each field contained, and the size and format of the fields. A primary key denotes a unique index on the table which cannot be repeated. In the case of our data the primary key chosen was the patient code, which allowed all data associated with the patient code to be linked.

2.5. STATISTICAL ANALYSIS

Non-parametric statistics were chosen after taking advice from Professor Leonard in the STATLAB at The Kings buildings, Edinburgh University and Dr. Robin Prescott in The Department of Public Health, Edinburgh University.

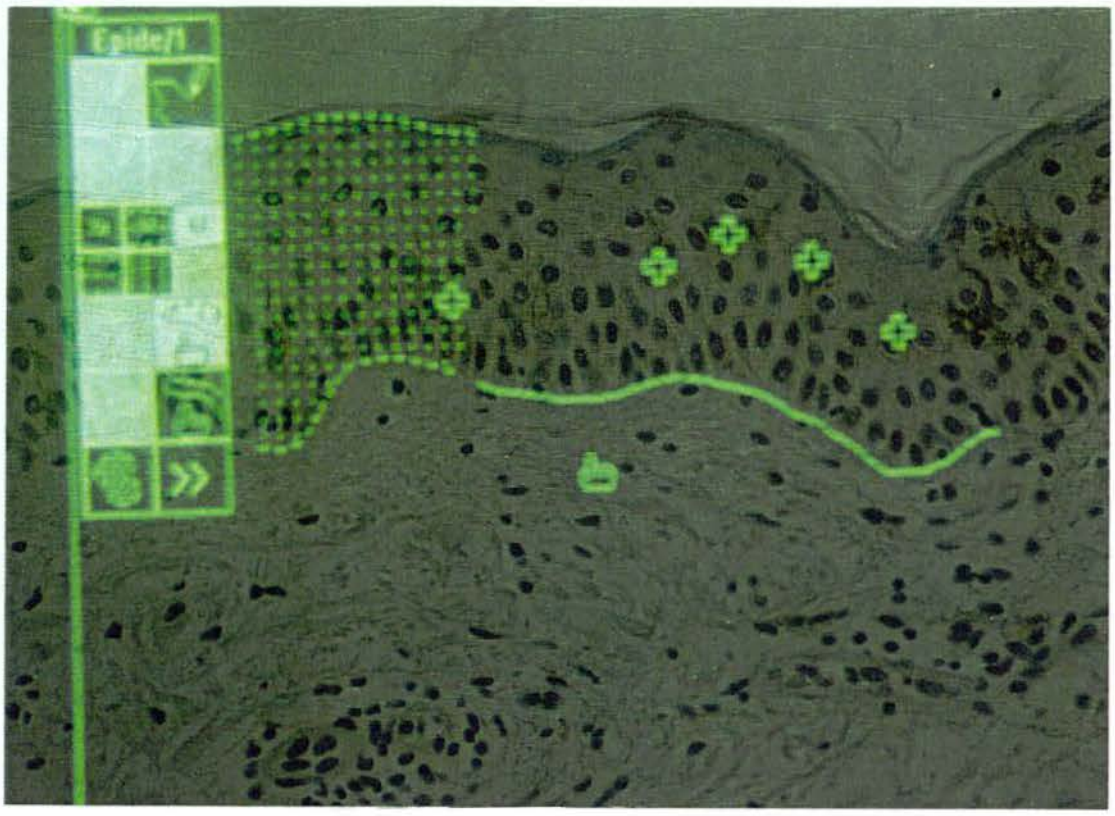
This group of tests were chosen for two main reasons :

1. Inferences are not made about the population from which the sample was taken.
2. These tests enable analysis of numerically graded data (ordinal), which make up a large proportion of the results obtained in this study.

Overall changes within each group, i.e. 0, 1 and 6 hours or 0, 24 and 48 hours, were investigated using the Friedman test. Any significant differences were then further qualified by comparison of paired samples, using the McNemar/Sign test. Analysis of results (numerical) between groups were carried out using either a Wilcoxon signed ranks sum test (paired data) or a Mann Whitney-U test (non-paired data); exact versions of these tests were used because of the small sample sizes.

PLATE 2.3:

Analysis of CD1a+ve cells using the HOME microscope. The computer overlay is shown in green. Crosses mark positive cell. The basement membrane (BM) can be measured and epidermal area calculated (green shaded area, A)



2.6. IN SITU CELL DEATH DETECTION

The in situ cell death detection kit supplied by Boehringer and Manheim (Cat No. 1684809) was used following the manufacturers instructions briefly outlined here. Paraffin sections (3μm) were cut and rehydrated as for immunocytochemistry. Sections were then incubated in proteinase K (20mg/ml, 15mins room temperature), to remove crosslinking and to allow fluroscein labelled nucleotide binding of DNA strand breaks. Sections were then incubated with 50 μl negative control (FITC labelled nucleotide mixture) or with 50 μl enzyme reaction mixture (terminal deoxynucleotidyl transferase) for 60mins at 37°C. Slides were then washed in PBS, incubated with an anti-fluroscein antibody (30 mins, 37°C), washed in PBS and visualised using a standard Fast Red procedure. Slides were counterstained with haematoxylin and mounted in aquamount.

2.7. CYTOKINE ASSAYS

• INTERLEUKIN-1 (IL-1α) & INTERLEUKIN-8 (IL-8)

Commercial assay kits produced by R&D systems (IL-1, code: DLA50 and IL-8, code: D8000) were used to determine the amount of cytokine present in the suction blister fluid. The assays employed the quantitative "sandwich" enzyme immunoassay technique (ELISA). A 96 well microtiter plate was provided in the kit, this was pre-coated with monoclonal antibody specific for the cytokine under investigation. Serial dilutions of standards and samples were pipetted into the wells and allowed to incubate for 2 hours at room temperature. Any cytokine present in the samples bind to the antibody attached to the plate. After washing away any unbound proteins, an enzyme-linked polyclonal antibody specific for the cytokine being measured was added to the wells to "sandwich" the cytokine immobilised during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, the substrate solution was

added to each well. The intensity of the coloured product produced was proportional to the amount of cytokine present in the sample. The colour development was stopped after 3-5 mins. and the optical density of each well read on a Dynatech MR5000 plate reader. A standard curve was plotted, using optical density versus the concentration of the known standards. By comparing the optical densities of the unknown samples with the standard curve, the concentration of cytokine present was determined. The lowest limit of detection was 3.9pg/ml for the IL-1α ELISA and 93.8pg/ml for the IL-8 ELISA.

• INTERFERON GAMMA (IFNγ)

A commercially available ELISA kit produced by Genzyme (code: 1556-00) was used to determine the amount of IFNγ present in the suction blister fluid. A 96 well microtiter plate was coated overnight with monoclonal anti-human IFNγ antibody (4^{o}C) and then washed to remove any unbound antibody. Serial dilution's of standards and samples were then pipetted into the wells and incubated at 37^{o}C for 2 hours. After washing, the plate was incubated with a polyclonal goat anti-human IFNγ antibody, washed and incubated with a donkey anti-goat biotinylated antibody. The streptavidin-peroxidase solution was then added to the plate before the final incubation with the substrate reagent, resulting in a colour change. The colour development was stopped after 4-6 mins. and the plate read at 492 nm on the MR5000 plate reader. A standard curve was plotted as for the other assays and the concentration of IFNγ present in the samples calculated from the standard curve. The lowest limit of detection with this kit was 3.0 pg/ml.

2.8. WESTERN BLOTTING

• SKIN LYSATES

Skin biopsies (6mm) from lesional sites of patients with ICD or normal skin biopsies from breast reductions were used for Western Blotting. The epidermis was separated from the dermis by incubation (37°C for 4 hours) with Dispase (Gibco BRL) and then washed in Phosphate Buffered Saline (PBS) (UNIPATH Ltd.). The epidermis and dermis were then lysed separately in 0.5ml of lysis buffer. The lysis buffer used was based on a method by Peterson and Wuepper (1984) (Appendix II). The lysis process involved freeze thawing the samples three times using liquid nitrogen, followed by ultrasonication for 3 mins. The lysates were spun in a microcentrifuge (1,000rpm, 2 mins) and the supernatant collected. The amount of protein in each sample was determined using the Biorad DC assay following the manufacturers instructions. Known protein standards (Bio-Rad, serial dilution's of γ-globulin, 1.37 mg/ml-0.17mg/ml) were run along with the test samples, all samples were run in triplicate. The concentration of protein present in each sample was then calculated from the standard curve.

• CASTING GELS

The Biorad minigel system (**Plate 2.4**) was used for Western Blotting analysis of the protein lysates. The glass plate sandwiches were assembled into the clamp assembly, as per the manufacturer's instructions. A single toothed comb was placed in the assembled glass sandwich and a mark made on the glass plate to indicate the pouring level of the separating gel. The combs were then set aside for use later.

10 ml of 10% acrylamide separating gel (Appendix II) was prepared, and after swirling gently, was pipetted between the glass plates up to the previously marked

level. The gel was then overlaid with water to ensure even setting. After allowing the gel to polymerise for 40 mins, the water was drained off and the stacking gel prepared (Appendix II). The single toothed comb was inserted into the glass sandwich and the stacking gel pipetted over it. The stacking gel was then left to set for 30 mins.

• RUNNING THE GEL

The clamp assemblies holding the glass plates were removed from the casting stand and transferred to the inner core. Running buffer (Appendix II) was poured into the upper buffer chamber up to the level of the larger glass plate and the lower buffer chamber was filled to a level at least 1 cm above the bottom of the glass plates, so as to maintain the electric circuit.

Lysates (1:2 dilution) and pre-stained molecular weight markers (Bio-Rad) were boiled for 5 mins. in reducing sample buffer (Appendix II) and then loaded onto the gel. The gel was then run at 200V until the leading edge of the Bromophenol blue reached the edge of the gel.

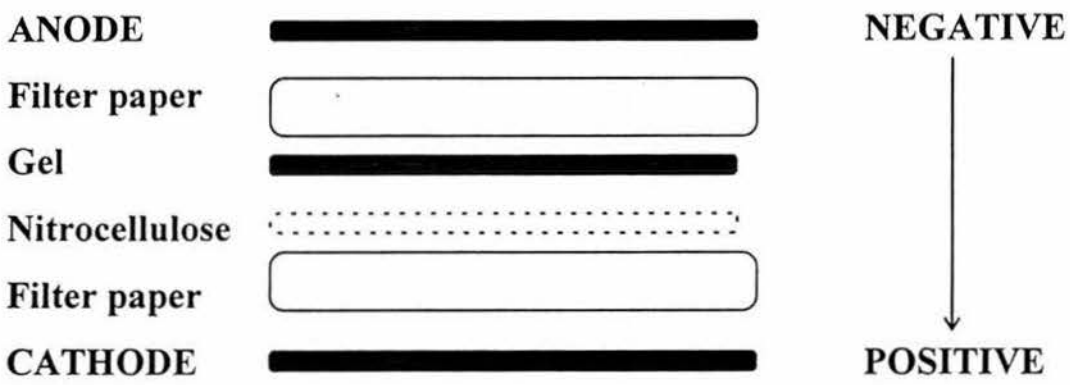
• ELECTROPHORETIC TRANSFER

The power supply was disconnected, the inner core removed and the running buffer poured off. The gels were placed in transfer buffer (Appendix II) to equilibrate before transfer. Nitrocellulose (Amersham Life Science) and filter paper (x2) pieces were cut to the size of each gel and were also soaked in transfer buffer. The gels were then sandwiched between nitrocellulose and filter paper, as shown in **Fig 2.1** and any trapped air was removed by rolling a pipette gently across the sandwich before it was clipped into the transfer apparatus. Transfer was carried out for 1 hour at 100V. After transfer the strip of nitrocellulose with the molecular weight markers was removed

and dried and the rest of the nitrocellulose blocked overnight (4°C in 5% Marvel/PBS) to prevent non-specific binding in the detection stage.

Figure 2.1

Transfer of a gel onto a nitrocellulose membrane



- **IMMUNOBLOTTING.**

The method used was adapted from work by Wallace and colleagues (Wallace *et al*, 1994). Test serum taken from the patient at the same time as the biopsy was optimally diluted (1/50) in Marvel/TBS/tween and incubated with the nitrocellulose (1hr, room temperature). The nitrocellulose was then washed thoroughly in TBS/tween and then incubated with mouse anti-human IgG (DAKO)(1:1000, 60 mins.). After washing the nitrocellulose was then incubated with a biotinylated rabbit anti-mouse antibody (Dakopatts) (1:1000, 30 mins), washed and incubated with ABC-AP (1:30) for30 mins. Detection of bands was carried out using nitro blue tetrazolium/ 5-bromo-4-chloro-3 indolyl phosphate (NBT/BCIP) (Appendix II).

3.0. RESULTS

(Appendix IV for raw data)

A pilot study, based on previous work from C.Willis and colleagues (Willis *et* al, 1988 and 1989), was carried out by Dr. Shahidullah (HS) to determine the optimal concentrations of irritant to be used in the study. Irritants were titrated on normal healthy volunteers to produce similar grades of erythema by 48 hours (**Plate 3.1**, data shown in appendix III). Subsequently the concentrations used were 80% NA in propan-1-ol (w/w), 5% aqueous SLS (w/v) and 0.01% DL in yellow soft paraffin (w/v).

Colorimeteric analysis showed that NA produced a time dependent increase in erythema, whereas significant erythema was not observed until 6 hours after SLS application (appendix 111). Despite these differences both SLS and NA resulted in comparable erythema by 48 hours. Erythema was measured using a Minolta Chroma Meter CR-300 which graded the redness of the skin. DL irritation resulted in yellow/brown staining of the skin. This staining interfered with the Minolta readings making it impossible to quantitatively assess erythema. For this reason DL was dropped from the study, hence the limited data relating to DL irritation.

No differences were observed, in any of the parameters tested, between atopics and non-atopics, males and females or patients with or without active disease. Data relating to these categories has therefore been pooled and all further results refer to pooled data.

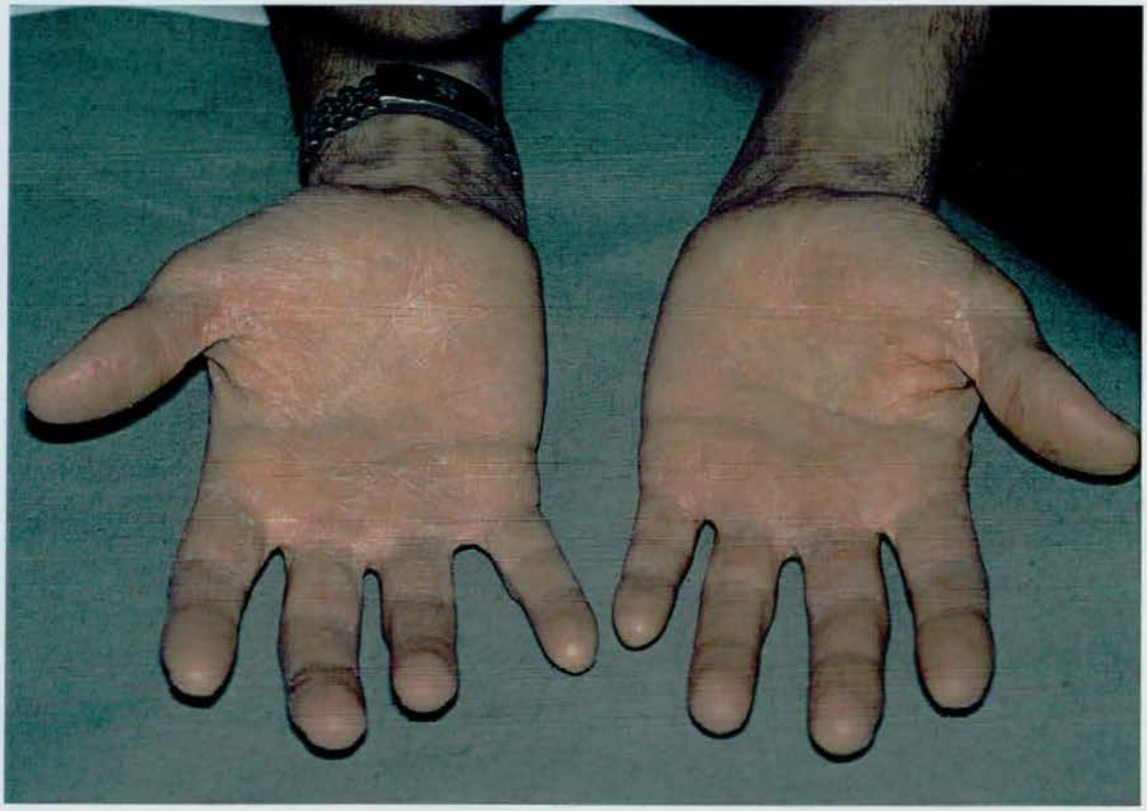
3.1. DITHRANOL

EPIDERMAL CHANGES

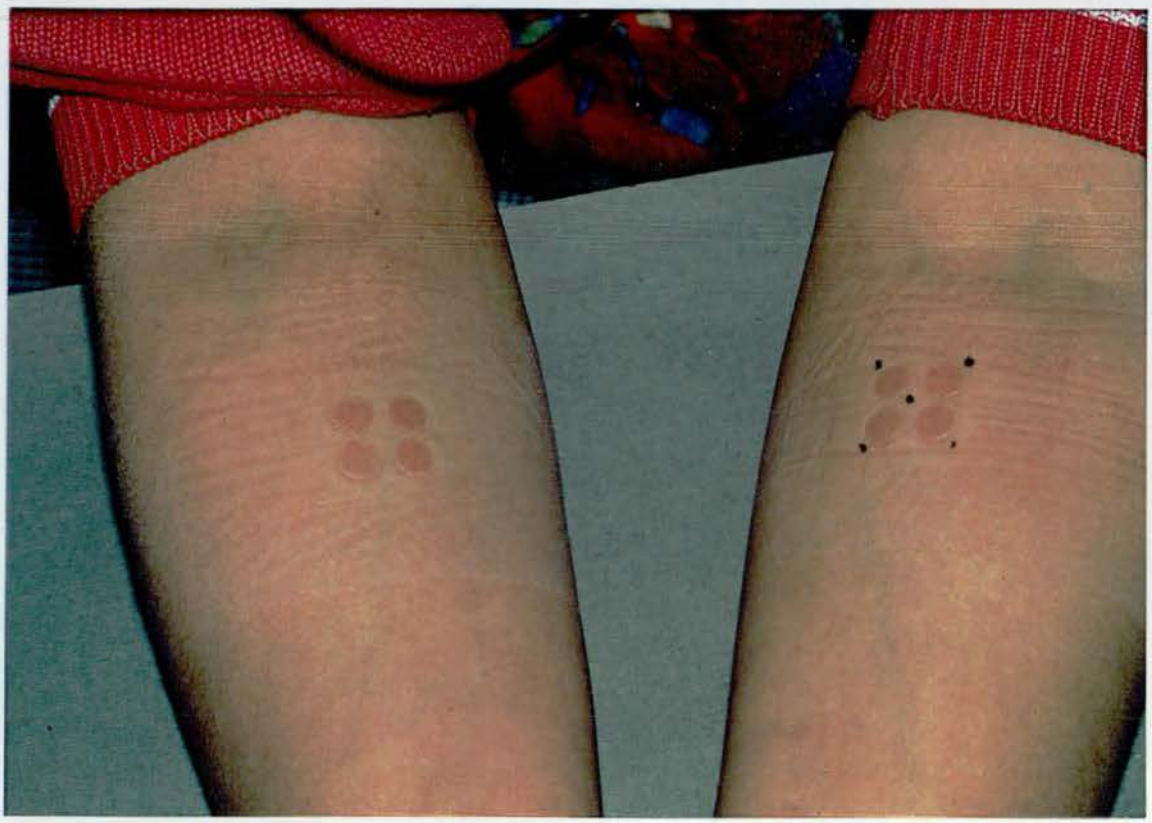
DL irritation resulted in minimal epidermal damage; with no parakeratosis, mild spongiosis in less than 15% of biopsies (**Table 3.1**, **Plate 3.2**) and no vesicultaion.

PLATE 3.1:

a) Irritant contact dermatitis.



b) Experimentally induced erythema 24 hours after irritant application.



Intracellular oedema was observed in both control and irritant biopsies. Significant basal layer oedema occurred at 1 and 6 hours after DL application ($p<0.05$, Friedman test).

EPIDERMAL INFILTRATION AND ACCESSORY MOLECULE EXPRESSION

Epidermal leucocyte infiltration was absent, with all biopsies showing normal epidermal leucocyte numbers, i.e. less than 4 leucocytes/mm basement membrane (BM). Epidermal granulocytes were absent. Focal KC CD54 expression was observed in irritant treated biopsies.

DERMAL INFILTRATION

DL application had no effect on dermal leucocyte infiltration with mild papillary dermal and perivascular infiltration, comprised predominantly of mononuclear cells observed in approximately 75% of both control and irritant treated biopsies. Significant reticular dermis leucocyte infiltration was not observed. A few scattered granulocytes were observed.

TABLE 3.1:

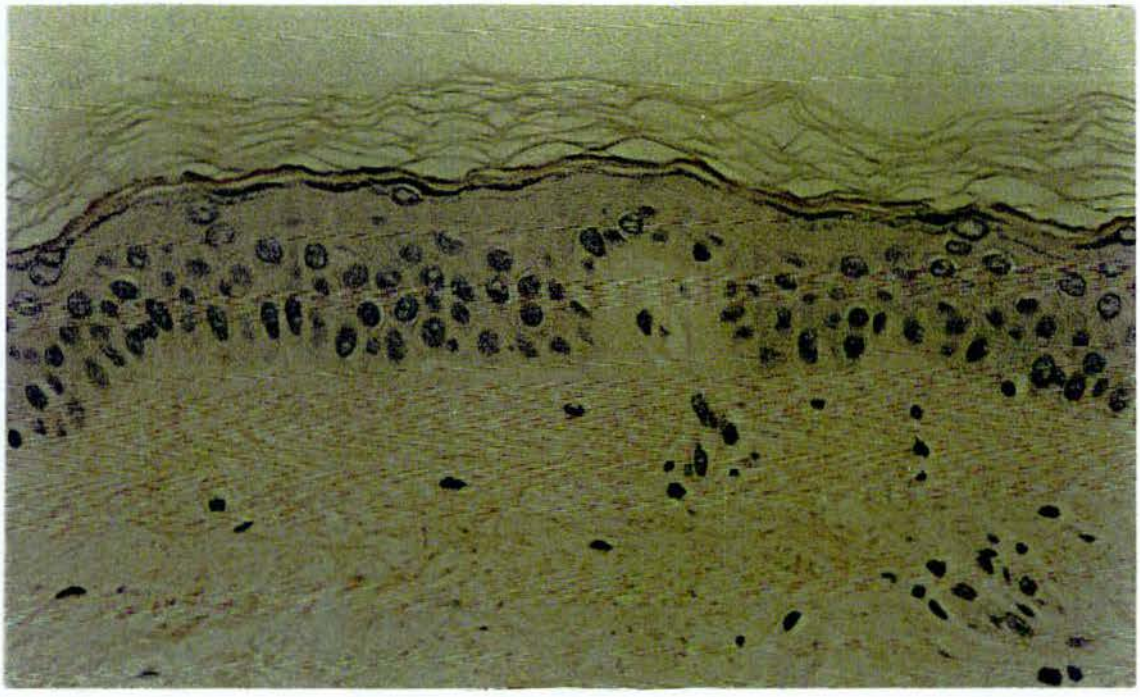
H & E analysis of data relating to epidermal damage 1 and 6 hours after DL application. The results shown indicate the percentage of positive biopsies for each of the parameters investigated.

% of POSITIVE BIOPSIES			
PARAMETER	Control (n = 16)	DL (n = 16)	
	0 hours	1 hour	6 hours
Parakeratosis	0	0	0
Intracellular Oedema (basal) - mild	63	44	44
Intracellular Oedema (basal) - moderate	0	19	13
Intracellular Oedema (basal) - severe	0	0	13
Spongiosis (prickle)- mild	6.3	12.5	12.5
Spongiosis (prickle)- moderate	0	0	6
Spongiosis (prickle)- severe	0	0	0
Vesiculation	0	0	0

PLATE 3.2:

H & E section 6 hours after DL application (mag. x20).

Minimal epidermal changes compared with control (0 hour).



• SUMMARY

DL irritation evoked only mild erythema even 48 hours after application, a result reflected by the minimal changes reported in the histological studies. A small amount of focal KC CD54 expression was observed in the two 24 and 48 hour biopsies examined but conclusions can not be drawn from such a small sample of patients. In summary, intracellular oedema was the extent of the epidermal damage resulting from DL application, with no apparent affect on the dermis.

Since the erythema produced by DL irritation was not comparable to that resulting from NA and SLS it seems likely that the poor inflammatory response to DL simply reflects an inadequate irritant concentration.

3.2. SODIUM LAURYL SULFATE & NONANOIC ACID

3.3. EPIDERMAL CHANGES

- **H&E ANALYSIS (Tables 3.2a and b)**

1). SLS

Parakeratosis was observed at 24 and 48 hours after irritation (**Plate 3.3**), but, because of small sample size, this was not significantly different from control groups. In comparison with control biopsies significant spongiosis was observed at 1, 6, 24 and 48 hours ($p<0.01$, Friedman Test), and severe spongiosis by 48 hours ($p<0.02$, McNemar Test). By the later timepoints of 24 and 48 hours spongiosis became extensive enough to cause vesiculation (2/8 and 3/8 biopsies respectively, **Plate 3.4**). Mild basal layer intracellular oedema was observed in both control and irritant treated biopsies.

2). NA

Parakeratosis was observed in 43% (3/7) of biopsies at 48 hours, but this was not significant. Although spongiosis and vesiculation were evident at both early and late timepoints, this was not significantly greater than in untreated samples. Mild to moderate intracellular oedema was observed at 24 hours. At 48 hours basal layer oedema had increased with 57% of biopsies showing moderate and 14% severe intracellular oedema.

- **SUMMARY**

Investigation of H & E stained sections revealed that NA and SLS application differentially affect the epidermis although the reactions are clinically similar **(plate 3.5).**

PLATE 3.3:

H & E section showing parakeratosis (P) 48 hours after SLS application (mag. x20)

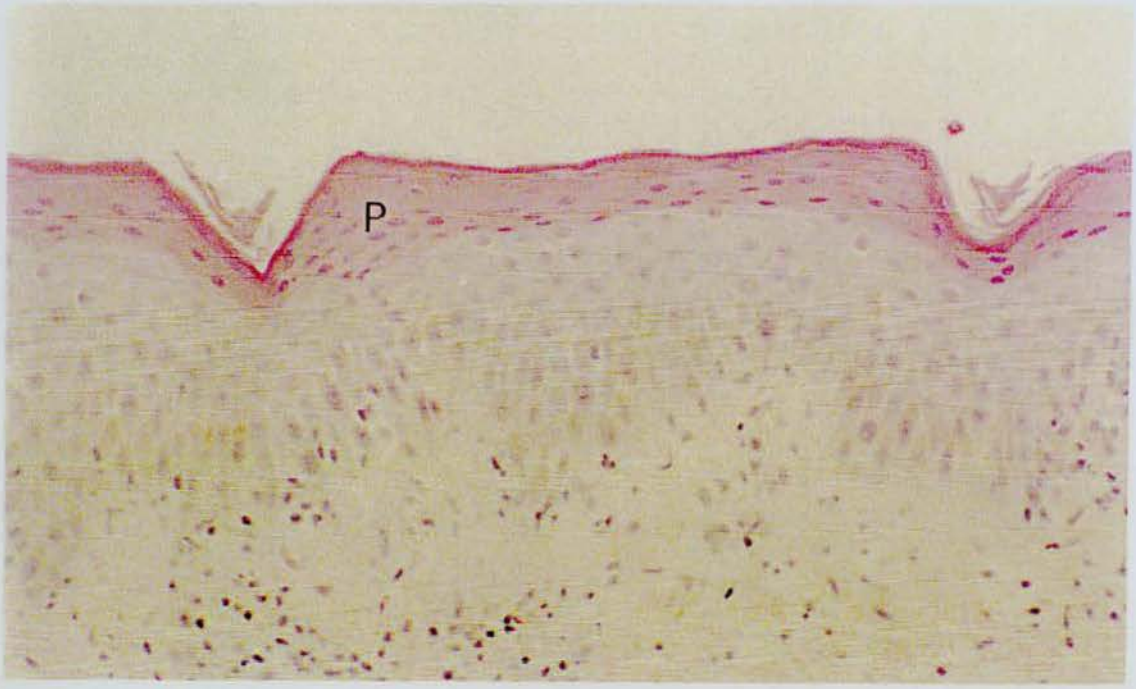
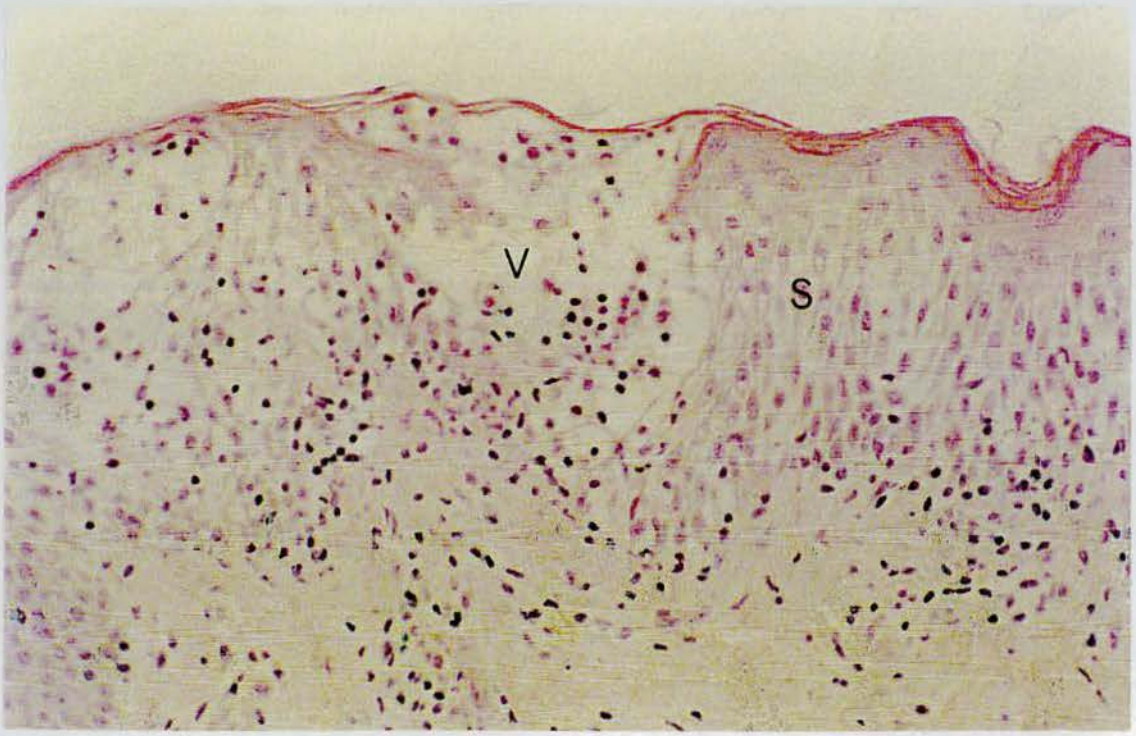


PLATE 3.4:

H& E section showing spongiosis (S) and vesiculation (V) 48 hours after SLS application. (mag. x20)

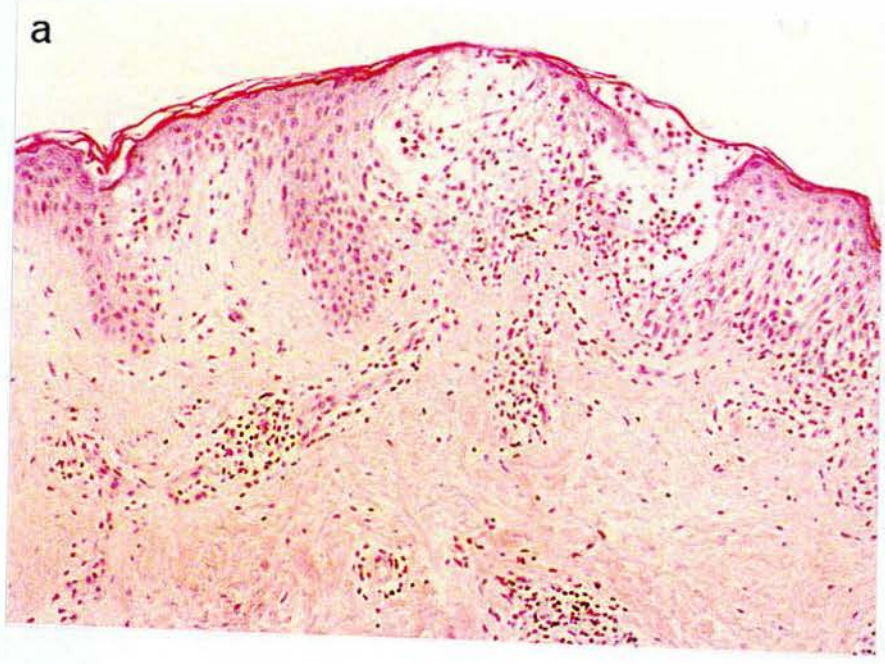


The data indicate that NA irritation leads to mild spongiosis, whereas SLS results in severe spongiosis, often progressing to vesiculation by 24 and 48 hours.

Parakeratosis was observed with both irritants by 24 and 48 hours, although this was a more prominent feature after SLS irritation. NA irritation resulted in basal layer intracellular oedema.

PLATE 3.5:

a) H & E section showing epidermal changes 48 hours after a) SLS application and b) NA (mag. x10)



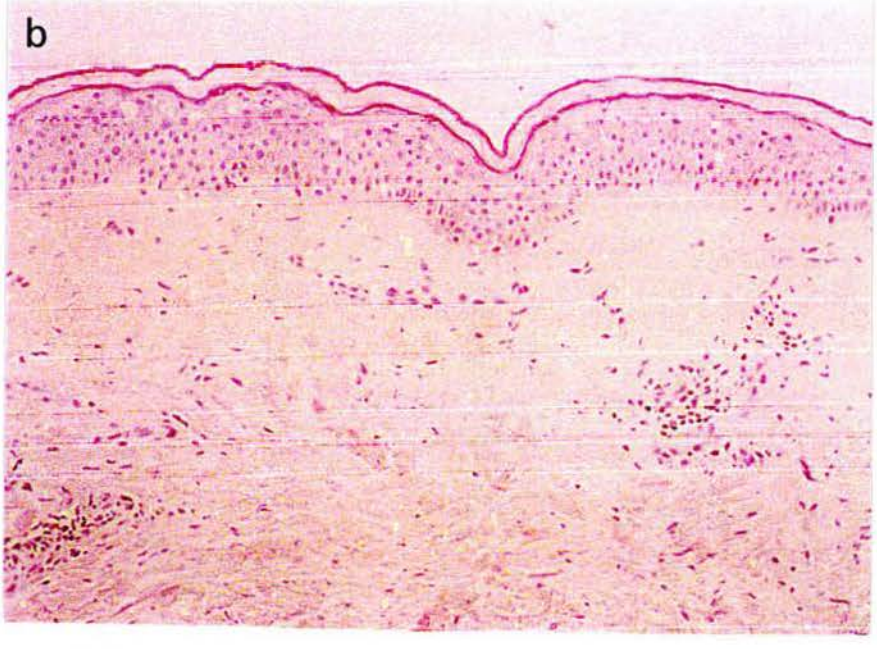


TABLE 3.2a:

H & E analysis of data relating to epidermal damage 1and 6 hours after NA and SLS application. The results shown indicate the percentage of positive biopsies for each of the parameters investigated

	% of POSITIVE BIOPSIES				
PARAMETER	Control (n =33)	NA (n = 17)		SLS (n = 16)	
	0 hrs	1 hr	6 hrs	1 hr	6 hrs
Parakeratosis	0	6	12	0	6
Intracellular Oedema (basal) - mild	49	59	47	57	63
Intracellular Oedema (basal) - moderate	12	18	12	19	12.5
Intracellular Oedema (basal) - severe	0	0	14	0	0
Spongiosis (prickle)- mild	21	24	29	19	31
Spongiosis (prickle)- moderate	0	6	12	0	6
Spongiosis (prickle)- severe	0	6	6	0	0
Vesiculation	6	6	24	6	12.5

TABLE 3.2b:

H & E analysis of data relating to epidermal damage 24 and 48 hours after NA and SLS application. The results shown indicate the percentage of positive biopsies for each of the parameters investigated.

	% of POSITIVE BIOPSIES				
PARAMETER	Control (n = 15)	NA (n = 7)		SLS (n = 8)	
	0 hrs	24 hs	48 hrs	24 hrs	48 hrs
Parakeratosis	0	14	43	38	50
Intracellular Oedema (basal) - mild	67	57	14	50	50
Intracellular Oedema (basal) - moderate	0	29	57	0	0
Intracellular Oedema (basal) - severe	0	0	14	0	0
Spongiosis (prickle)- mild	13	14	14	0	0
Spongiosis (prickle)- moderate	0	14	43	25	13
Spongiosis (prickle)- severe	0	14	14	13	75
Vesiculation	0	14	0	25	38

• CELLULAR INFILTRATION AND CD54/ICAM-1 MOLECULE EXPRESSION (Table 3.3)

1). SLS

Control and irritant biopsies taken up to 6 hours showed normal numbers of epidermal CD3+ve lymphocytes , i.e. <4 cells/mm basement membrane. However by 24 ($p<0.04$) and 48 ($p<0.02$) hours epidermal CD3+ve lymphocyte infiltration had occurred (**Plate 3.6**). Further investigation of the CD3+ve subset of lymphocyte revealed that after 24 hours the ratio of CD4+ve/CD8+ve lymphocytes was 3:1 (ratios were calculated using mean values). By 48 hours the numbers of CD8+ve lymphocytes had increased and the number of CD4+ve lymphocytes decreased reducing the ratio to 2:1 (**Table 3.4**). Significant neutrophil, eosinophil and mast cell exocytosis were not observed. However, epidermal macrophage (CD68) infiltration was observed at 48 hours ($p<0.04$).

Epidermal CD54 expression was not observed in control biopsies, although 13/39 (33%) of SLS treated biopsies showed focal CD54 expression. Intense focal CD54 expression correlated (100%) with sites of epidermal CD3+ve lymphocyte infiltration (**plate 3.6**). However CD3+ve cells were observed in epidermal areas without CD54 expression; 15% (5/34) of biopsies with epidermal CD3+ve infiltration lacked epidermal CD54 expression.

2). NA

Epidermal lymphocyte infiltration was absent, with all biopsies showing normal CD3+ve cell numbers within the epidermis, i.e. all groups showing less than 4 lymphocytes/mm BM. Neutrophil, eosinophil and mast cell infiltration were also absent in the epidermis. Statistically significant but mild epidermal macrophage (CD68) infiltration however, was observed at 24 ($p<0.04$) and 48 ($p<0.04$) hours (**Table 3.3**). KC CD54 expression was not observed.

PLATE 3.6:

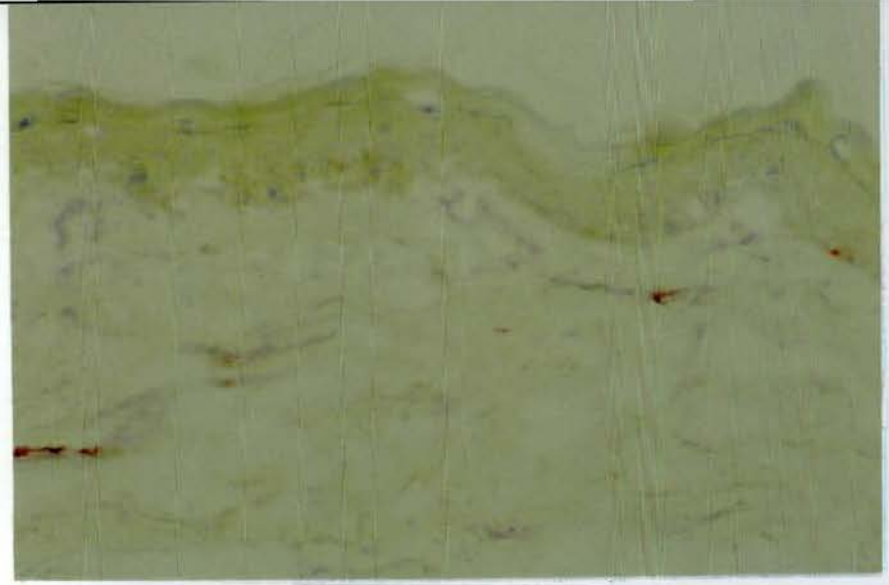
a) uninvolved skin, < 4 CD3+ve cells/mm basement membrane

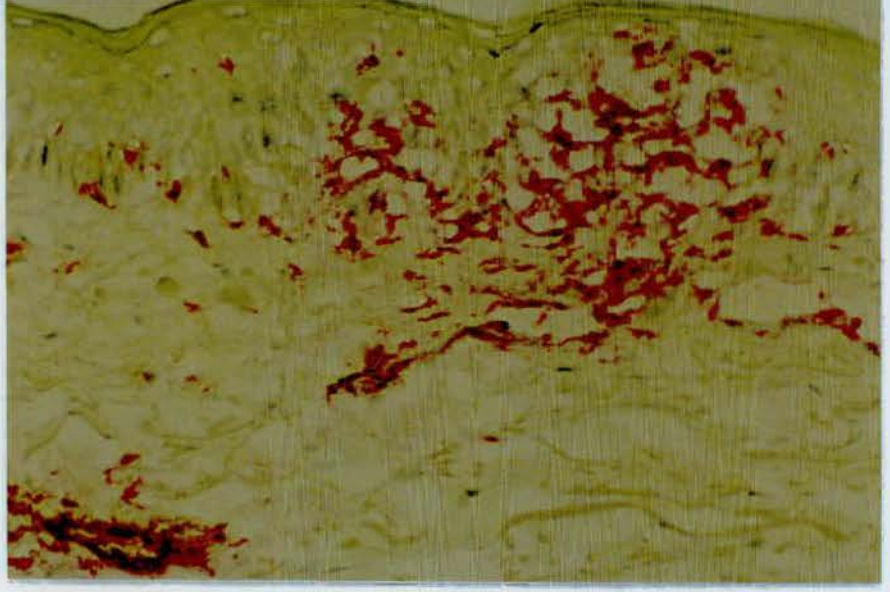
b) SLS irritation, 48 hours, CD3+ve epidermal infiltration

c) uninvolved skin, ICAM-1 expressed on endothelium

d) SLS irritation, 48 hours, focal epidermal ICAM-1 expression

(frozen sections , mag. x20).



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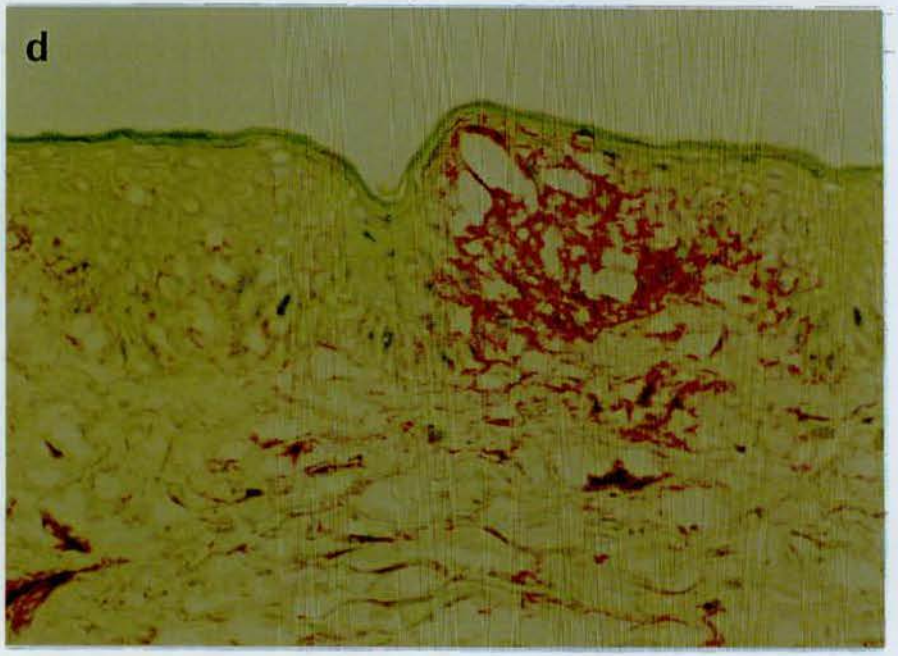
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• SUMMARY

SLS and NA irritation result in very different patterns of epidermal cellular infiltration. SLS induced epidermal lymphocyte infiltration, with a CD3+ve lymphocyte influx evident by 24 hours. Investigation of the epidermal T lymphocyte subset present after SLS irritation showed infiltration of both CD4+ve and CD8+ve cells. The CD4/CD8 ratio changed with time of irritant exposure, decreasing from 3:1 at 24 hours to 2:1 by 48 hours. NA did not induce epidermal lymphocyte infiltration. Epidermal macrophages were observed after both NA and SLS irritation.

Following SLS irritation, the resultant focal KC CD54 expression correlated with the extensive lymphocyte exocytosis. Infiltration was however also observed in areas lacking CD54 expression.

TABLE 3.3:

Median number (and range) of CD3+ve and CD68+ve cell per mm basement membrane at 0, 24 and 48 hours after **SLS** and **NA** application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test).

TIME (hrs)	CD3+ve (T lymphocytes)		CD68+ve (macrophages)	
	MEDIAN	RANGE	MEDIAN	RANGE
SLS	n = 7		n = 8	
0	0	0 - 9	0	0 - 0.4
24	14.4*(p<0.04)	0 - 50.5	1.8	0 -16.7
48	12.9*(p<0.02)	3.2 - 37.2	10.3*(p<0.04)	0 -26.4
NA	n =6		n = 7	
0	0	0 -0	0	0 - 0
24	0	0 - 0	1.9*(p<0.04)	0 -18.8
48	0	0 - 5.3	2.3*(p<0.04)	1.1 -10.6

TABLE 3.4:

Median number (and range) of CD4+ve and CD8+ve cell per mm basement membrane at 0, 24 and 48 hours after **SLS** application.

TIME (hrs) (n)	CD4+ve (T lymphocytes)		CD8+ve (lymphocytes)	
	MEDIAN	RANGE	MEDIAN	RANGE
0 (n=13)	0	0 - 0	0	0 - 0
24 (n=7)	13.6	0 - 37.5	0.2	0 - 12.4
48 (n=6)	10.5	0 - 54.1	8.6	0 - 13.5

• MHC II EXPRESSION & EPIDERMAL CD1a+ve LC

(Plates 3.7 and 3.8 and Tables 3.5, 3.6 and 3.7)

Epidermal LC number was investigated in normal skin from volunteers. Comparison of LC number between volunteer biopsies and those taken from clinically normal sites in patients with chronic ICD revealed that patients have intrinsically higher levels of epidermal LC ($p<0.03$, Mann Whitney-U test, **Table 3.5**, **Plate 3.8a**).

TABLE 3.5:

Median number (and range) of epidermal CD1a+ve cells per mm basement membrane in clinically normal skin from patients with chronic ICD and normal healthy volunteers. The two groups are significantly different ($p <0.03$, Mann-Whitney U test).

	N	MEDIAN	RANGE
VOLUNTEERS	8	9.1	4.8 - 15.5
ICD PATIENTS	45	12.2	1.3 - 30

1). SLS

A normal epidermal distribution of dendritic MHC II+ve cells (i.e. LC) was observed in control and irritant treated biopsies. However, by 6 hours after SLS application 3/9 biopsies showed evidence of focal KC MHC II expression (**Plate 3.7a**). At 24 and 48 hours KC MHC II expression was more prominent, with 8/10 biopsies showing at least one area of focal KC expression (**Plate 3.7b**).

A significant (Friedman Test, $p<0.05$) change in LC density was observed between control, 1 and 6 hour biopsies and also between control, 24 and 48 hour biopsies

($p<0.05$). The decrease was significant at 48 hours ($p<0.04$) when assessed using the Wilcoxon-signed ranks sum test (**Table 3.6**).

2). NA

A normal epidermal distribution of dendritic MHC II+ve (i.e. LC) cells was observed in control and irritant treated biopsies. At 6 hours the distribution of MHC II expression was again normal, with 2/5 biopsies showing focal KC expression. The majority of biopsies (9/11) at 24 and 48 hours showed basal expression of MHC II, MHC II+ve infiltrating cells were observed in one biopsy.

Control biopsies showed a normal epidermal suprabasal distribution of LC (**Plate 3.8b)** however by 6 hours after NA application the cells had moved into a predominantly basal position in 12/16 biopsies (**Plate 3.8c**). Basal layer distribution remained prominent at 24 (4/5 biopsies) and 48 (4/5 biopsies) hours (**Plate 3.8d**). Comparison of control, 24 and 48 hour biopsies revealed a significant ($p<0.01$, Friedman test) change in LC density, represented by a decrease in epidermal LC numbers at 24 hours ($p<0.04$) and 48 hours ($p<0.002$).

Comparison of SLS with NA revealed that even though SLS resulted in decreased epidermal LC numbers the results after NA application were considerably more dramatic. Significantly larger decreases in LC number were shown by 24 ($p<0.01$) and 48 ($p<0.008$) hours after NA application.

CONFOCAL LASER SCANNING MICROSCOPY (CSLM) - NA IRRITATION

CSLM data confirmed the immunohistochemical results discussed in the previous paragraph, showing a reduction in LC number (**Table 3.7**) after NA application with cells becoming rounded by 24 hours.

Normal healthy volunteers had a median of 5.6 dendrites/LC with a median length of 14.5μm prior to NA application. Patients with chronic ICD had a median of 4.5 dendrites/LC with a median length of 11.5μm. Neither of these parameters was significantly different between the two groups (Mann Whitney U-Test).

PLATE 3.8:

a) normal, unirritated skin (normal volunteer), CD1a+ve cells

b) normal, unirritated skin (ICD patient), CD1a+ve cells

c) NA irritation, 6 hours, CD1a+ve cells

d) NA irritation, 48 hours, CD1a+ve cells

(frozen sections, mag. X20).

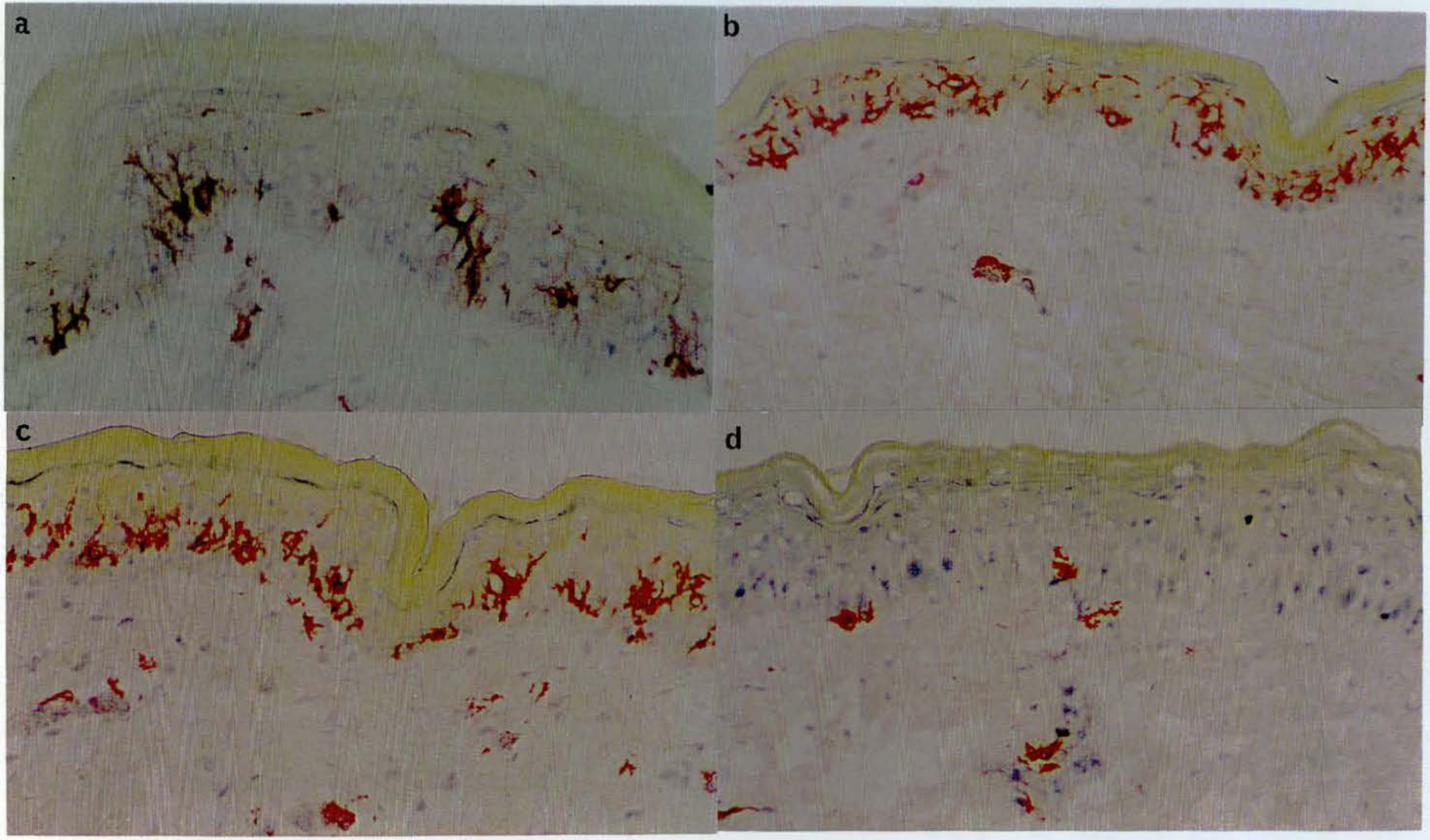
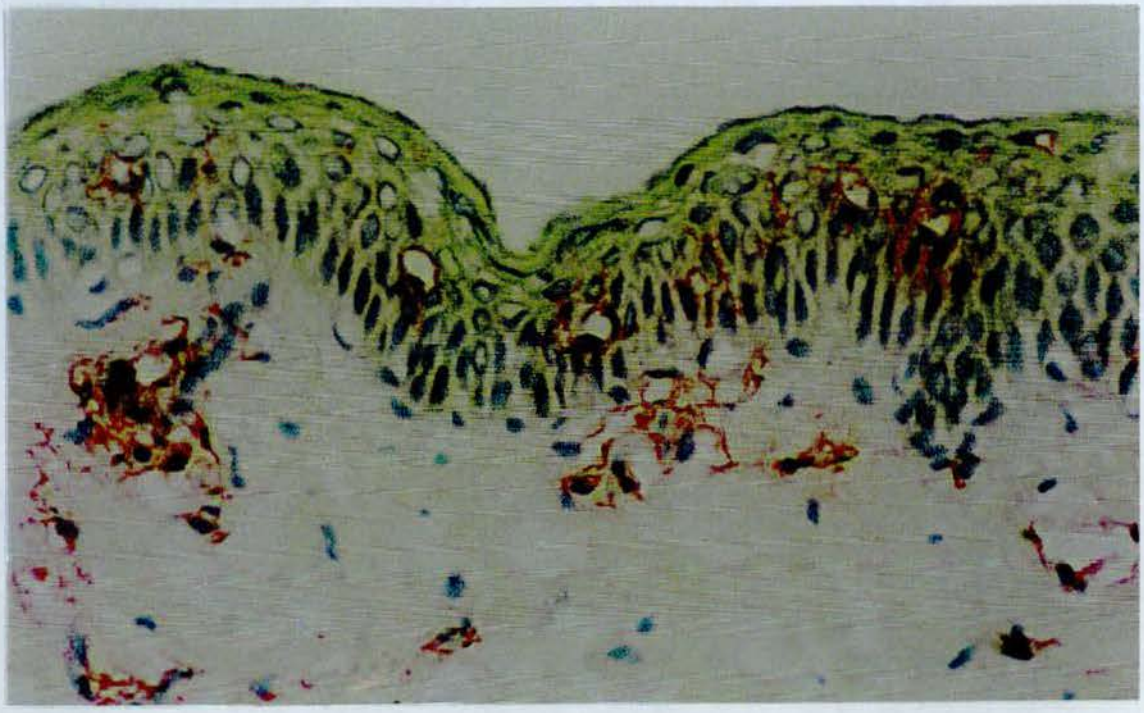
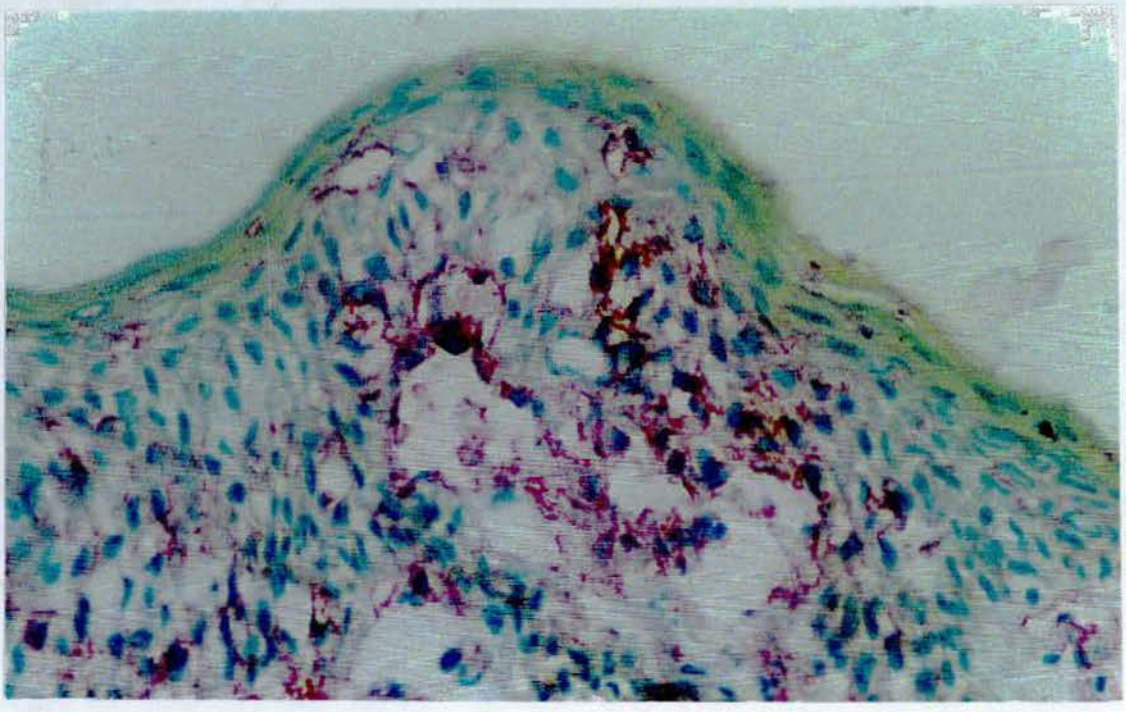

a
b
c
d

PLATE 3.7:

a) MHC II expression 6 hours after SLS irritation. (mag. x20).



b) MHC II expression 48 hours after SLS application. (mag. x20)



Irritant application resulted in a change in both dendrite number and length. By 24 hours volunteers showed significantly decreased numbers of dendrites compared to time 0 controls (median 3.5 dendrites/LC, $p<0.03$). Volunteers also showed significantly decreased dendrite length at 6 (median 4.5μm, $p<0.001$) and 24 hours (median 5.0μm, $p<0.001$) after NA application. Patients did not show a significant decrease in the number of dendrites when compared to time 0 controls. However, dendrite length was significantly decreased at 24 hours (median 5.0μm, $p<0.001$).

Comparison between patients and volunteers revealed that at 6 hours the decrease in dendrite length was significantly greater in volunteers than patients ($p<0.001$). At 1 and 24 hours no differences were observed between the two groups.

• SUMMARY

Clinically normal skin from patients with chronic ICD have a higher number of epidermal LC than normal skin from healthy volunteers.

Epidermal LC responded to cutaneous irritant application, with a more prominent response occurring after NA irritation. In contrast, KC response to irritation was only observed with SLS application.

SLS caused focal KC activation, manifested by MHC II expression, from 6 hours, and a decrease in epidermal LC density at 48 hours.

NA application failed to induce KC activation; the LC population however was dramatically affected. Irritation led to a redistribution of the epidermal LC, resulting in a predominantly basal distribution by 6 hours. A dramatic reduction in epidermal LC number occurred by 24 hours, combined with qualitative cellular changes resulting in more rounded cell bodies with fewer, shorter dendrites.

TABLE 3.6:

Median number (and range) of epidermal CD1a+ve cells per mm basement membrane at 0, 1, 6 ,24 and 48 hours after a). SLS and b). NA application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test).

a) median number and range of CD1a+ve LC after SLS application.

TIME (hours)	N	MEDIAN	RANGE
0	12	12.2	5.6 - 16.3
1	12	9.2	5 - 25.6
6	12	8.0	2.5 - 18.8
0	6	19.0	8.6 - 30
24	6	11.3	9.6 - 31
48	6	9.8*($p<0.04$)	5.5 - 11.2

b) median number and range of CD1a+ve LC after NA application.

TIME (hours)	N	MEDIAN	RANGE
0	15	12.7	6.2 - 17.8
1	15	12.1	4.2 - 21.3
6	15	10.8	2.6 - 27.5
0	6	12.5	9.2 - 21.3
24	6	7.4*($p<0.04$)	2.9 - 10.2
48	5	3.0*($p<0.002$)	0.5 - 5.0

TABLE 3.7:

Median (and range) number of LC per square mm at 0, 1, 6 and 24 hours after NA application (CSLM data).

TIME (hrs)	N	MEDIAN	RANGE
VOLUNTEERS			
0	10	383.5	162 - 652
1	9	450.0	247 - 597
6	6	342.5	285 - 371
24	5	144.0	94 - 241
PATIENTS			
0	3	418	418 - 427
1	9	326	224 - 407
6	6	218	153 - 318

3.2.7. CELL PROLIFERATION AND DEATH

PROLIFERATION (**Table 3.8**)

1). SLS

Cells undergoing DNA synthesis were detected using an anti-Ki-67 Ab. Ki-67 reacts with a nuclear Ag expressed on all human proliferating cells. Expression of this Ag occurs preferentially during late G_1, S, M and G_2 phases of the cell cycle. Basal layer Ki-67+ve cells were not significantly different from control levels at 1 and 6 hours after irritation. Comparison (Friedman Test) of control and irritant treated biopsies at 24 and 48 hours, revealed a significant difference in the number of Ki-67+ve cells/mm basement membrane ($p<0.05$). When assessed using a Wilcoxon-signed ranks sum test a significant increase in the numbers of proliferating cells occurred at 48 hours ($p<0.04$, **Plate 3.9**). This increase in cellular proliferation corresponded with increased epidermal thickness (48 hours, $p<0.02$, **Table 3.9**).

2). NA

The number of Ki-67+ve basal cells was not significantly different from control levels at 1 and 6 hours after irritation. Comparison (Friedman Test) of control and irritant treated biopsies at 24 and 48 hours, revealed a significant difference in the number of Ki-67+ve cells/mm BM ($p<0.05$). Using the Wilcoxon-signed ranks sum test this difference was shown to be the result of a decrease in proliferation by 24 hours ($p<0.04$), increasing again by 48 hours. The difference between 24 and 48 hours was significant ($p<0.03$). Increased epidermal thickness was observed at 6 hours ($p<0.04$), the trend continued at 24 and 48 hours but was not significant. This may be due to small sample size ($n = 5$).

APOPTOSIS

Only the prickle and basal layers were assessed for the presence of apoptosis. The granular layer was not analysed as the dark keratohyalin granules present in this area present technical difficulties for scoring.

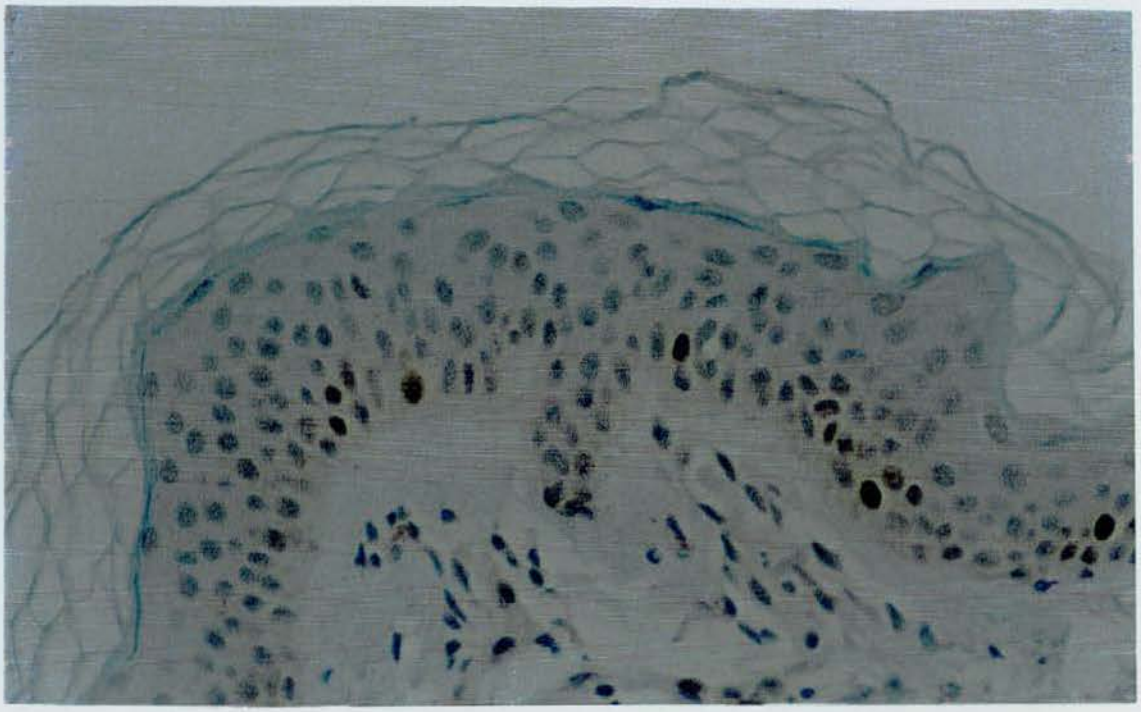
TABLE 3.8:

Median number (and range) of Ki-67+ve cells per mm basement membrane at 0, 24 and 48 hours after SLS and NA application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test).

TIME (hrs)	N	Ki-67+ve cells/mm BM	
		MEDIAN	RANGE
SLS			
0	6	28.2	12.8 - 45
24	6	28.6	8.7 - 46.7
48	6	56.2*($p<0.05$)	30.2 - 212.1
NA			
0	6	30.5	13.3 - 128.8
24	6	13.4*($p<0.04$)	6.9 - 25.1
48	6	45.9	38.6 - 78

PLATE 3.9:

a) Ki-67+ve cells in unirritated skin (mag. x20)



b) Ki-67+ve cells 48 hours after SLS irritation. (mag. x20).

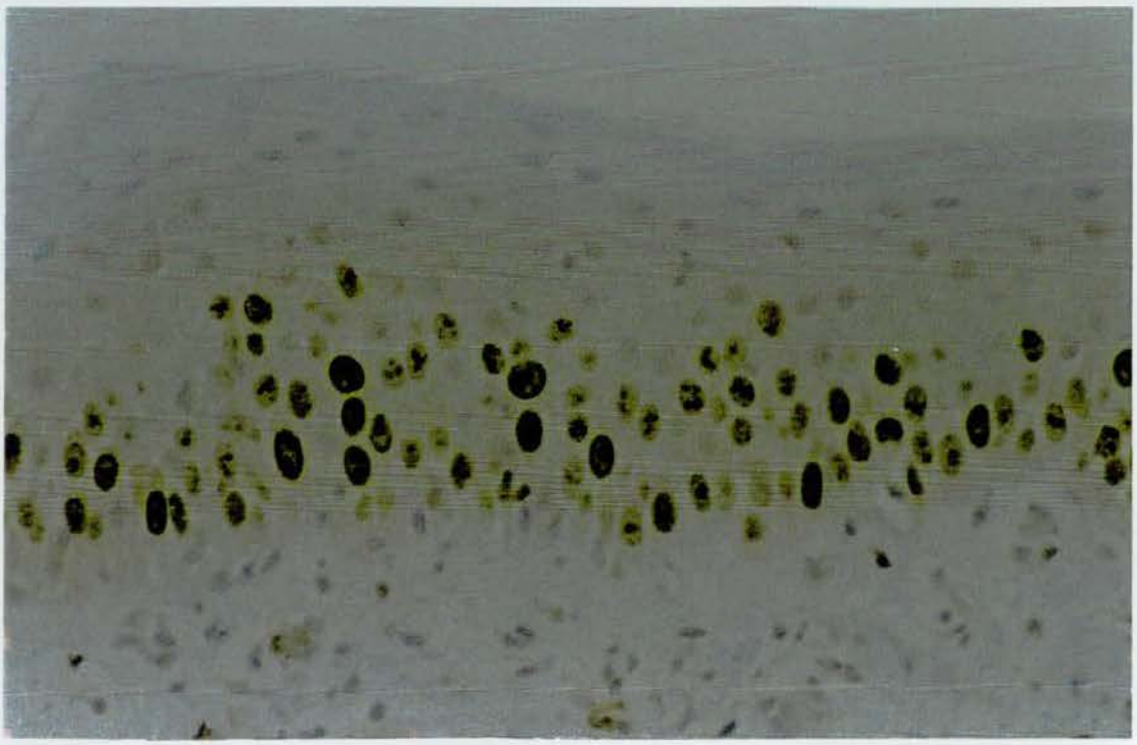


TABLE 3.9:

Median epidermal thickness in μm (and range) at 0, 1, 6 ,24 and 48 hours after a). SLS and b). NA application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test).

a) epidermal thickness after SLS application.

TIME (hours)	N	MEDIAN	RANGE
0	12	73.5	54.5 - 121
1	12	77	55.3 - 100
6	12	73.7	60 - 98.5
0	7	57	42 - 67.7
24	7	62.3	52 - 196
48	7	89.5*($p<0.02$)	73.7 - 108

b) epidermal thickness after NA application.

TIME (hours)	N	MEDIAN	RANGE
0	16	69.7	50 -102
1	16	72.5	50 - 128.3
6	16	78.2*($p<0.04$)	60 - 111.3
0	5	73.3	55.2 - 110.5
24	5	108	105 - 119
48	5	123.3	108 - 167

1). SLS

Prickle and basal layer apoptosis was observed after SLS application, yet the numbers of apoptotic cells did not rise significantly above control levels. At 24 and 48 hours 3/8 and 5/8 biopsies showed prickle layer apoptosis. Incidence of basal layer apoptosis was similar, with 5/8 and 4/8 positive biopsies at 24 and 48 hours respectively. However, the actual number of apoptotic cells/mm BM in each biopsy was low (24 hours, median 0.2, range 0 -1.4; 48 hours, median 0.2, range 0 -0.9) not rising above control levels.

Paraffin sections stained with monoclonal CD1a Ab were assessed morphologically for the presence of basal and prickle layer LC apoptosis. Apoptotic cells present after SLS application were almost exclusively of KC origin. The exception being one biopsy at 48 hours showing evidence of LC apoptosis.

2). NA (**Table 3.10**)

Prickle layer apoptosis was not evident in control or irritant treated biopsies taken at 1 and 6 hours after irritation. However, by 24 and 48 hours, 4/7 and 4/7 biopsies respectively showed prickle layer apoptosis. Findings similar to those after SLS application.. Basal layer apoptosis was present in more biopsies than prickle layer apoptosis. Basal layer apoptotic cells were observed at 1, 6, 24 and 48 hours (3/15, 10/16, 7/7 and 7/8 biopsies respectively, **Plate 3.10**). At 24 hours, the numbers of apoptotic cells were significantly larger than in control groups ($p<0.02$). Furthermore, comparison of NA with SLS treatment revealed a significantly higher incidence of basal layer apoptosis at 6 ($p<0.002$) and 24 ($p<0.04$) hours after NA application. TUNEL (terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labelling assay) was used to confirm the morphological assessment of apoptotic cells in biopsies previously shown to be positive (**Plate 3.11**).

PLATE 3.10:

Basal layer apoptotic cell 48 hours after NA application, a) mag. x20 and b) mag. x100

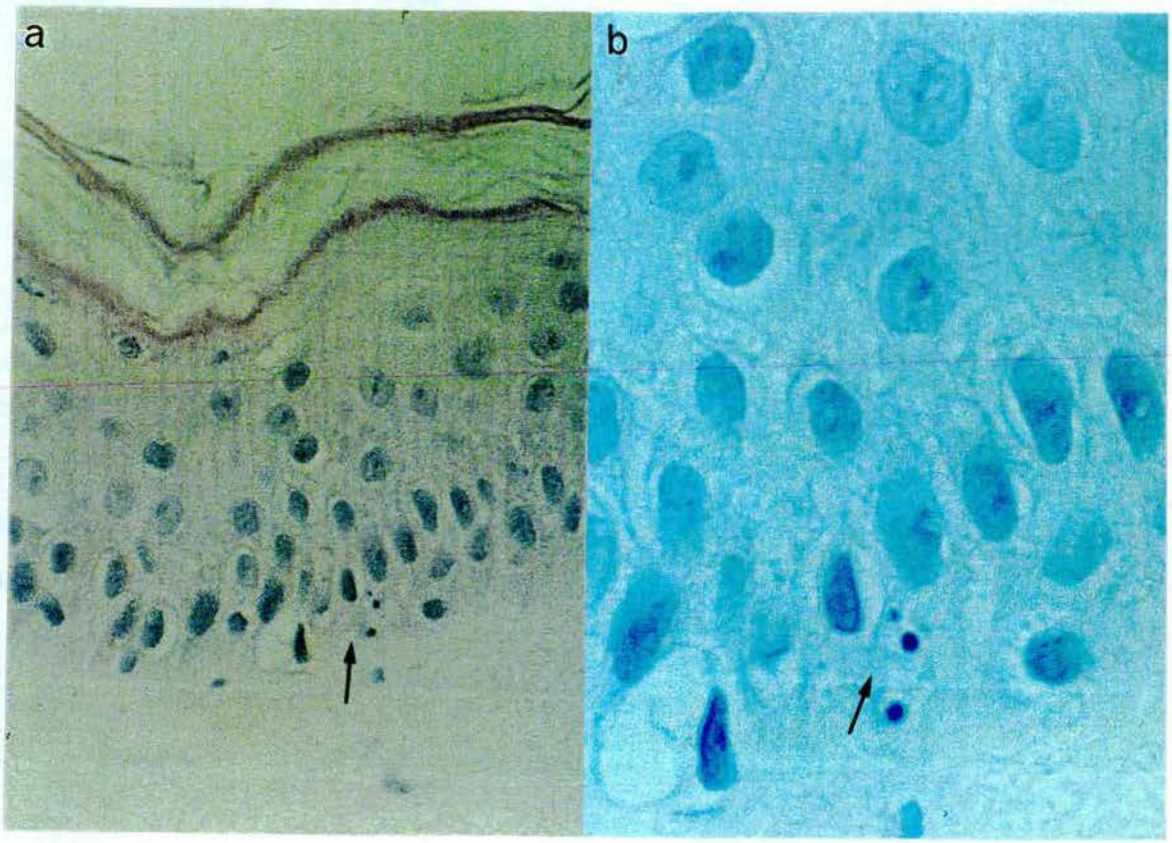


TABLE 3.10:

Number of basal layer apoptotic cells per mm basement membrane at 0, 24 and 48 hours after NA application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test).

STATISTICS	CONTROL	24 Hours	48 Hours
	0	0.2	1.4
	0	1.9	5.2
	0.3	0.5	0.2
	0	0.2	0.4
	0	6.4	0
	0	1.0	0.4
	0	2.8	1.6
n	7	7	7
MEDIAN	0	1.0*	0.4

Paraffin sections stained with monoclonal CD1a Ab were assessed morphologically for the presence of basal and prickle layer LC apoptosis. Basal layer CD1a+ve apoptotic cells were observed in biopsies taken at 1, 6 ,24 and 48 hours after NA application (2/15, 1/15, 3/7 and 1/7 biopsies respectively). Prickle layer CD1a+ve apoptosis was also observed in biopsies taken 1, 6, 24 and 48 hours after NA application (1/15, 2/15, 4/7 and 1/7 biopsies respectively). LC apoptosis was most prevalent in the prickle layer accounting for 85 - 100% of the total number of apoptotic cells, with 60% of the total LC population undergoing apoptosis by 24 and 48 hours (**Plate 3.12**).

• SUMMARY

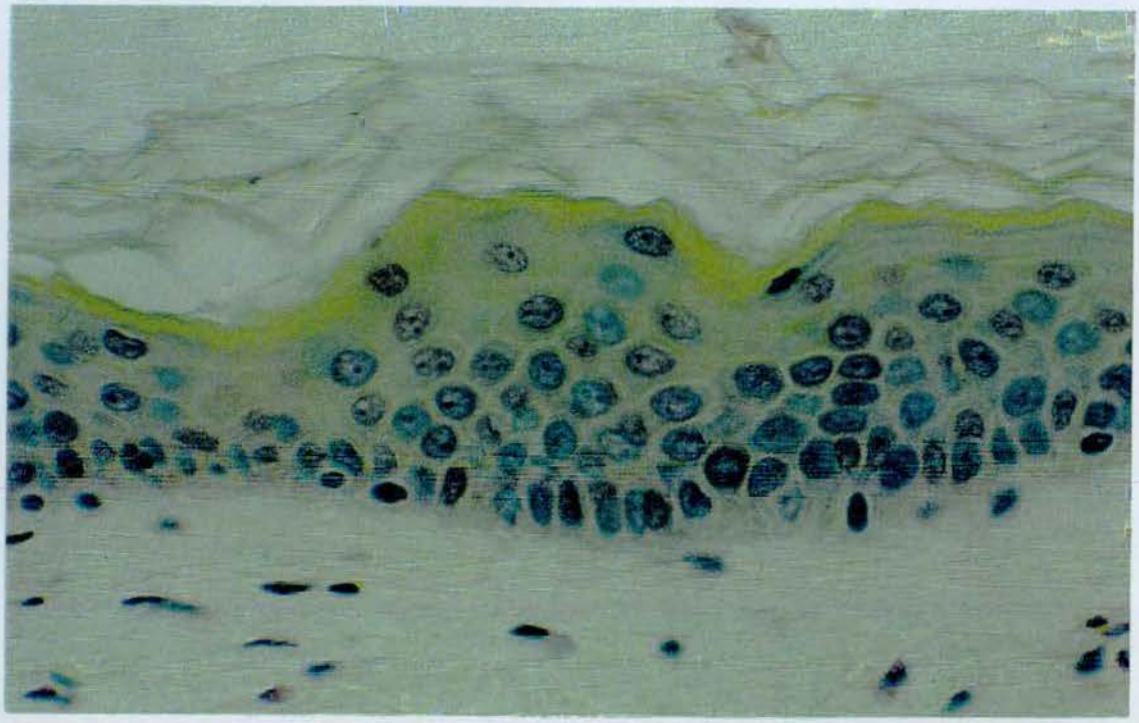
Irritation had a marked affect on basal KC proliferation and epidermal thickness. SLS application resulted in increased proliferation at 24 and 48 hours, with a corresponding increase in epidermal thickness at 48 hours. Results from NA

application were different, showing an initial decrease in proliferation at 24 hours. Basal layer KC proliferation had returned to normal control levels by 48 hours. Increased epidermal thickness was observed by 6 hours after irritation and did not appear to correlate with increased proliferation.

SLS irritation did not result in significant apoptosis when compared with control biopsies. In contrast, NA irritation induced significant levels of basal layer KC apoptosis at 6 and 24 hours. NA also induced LC apoptosis, accounting for 85 - 100% of the prickle layer apoptosis observed. Approximately 60% of the LC population were seen to be undergoing apoptosis by 24 and 48 hours, suggesting that this may be a contributory factor in the reduction of LC number observed in the previous section.

PLATE 3.11:

a) In situ cell death detection, unirritated skin. (mag. x20)



b). In situ cell death detection 48 hours after NA application. (mag. x20 and x40).

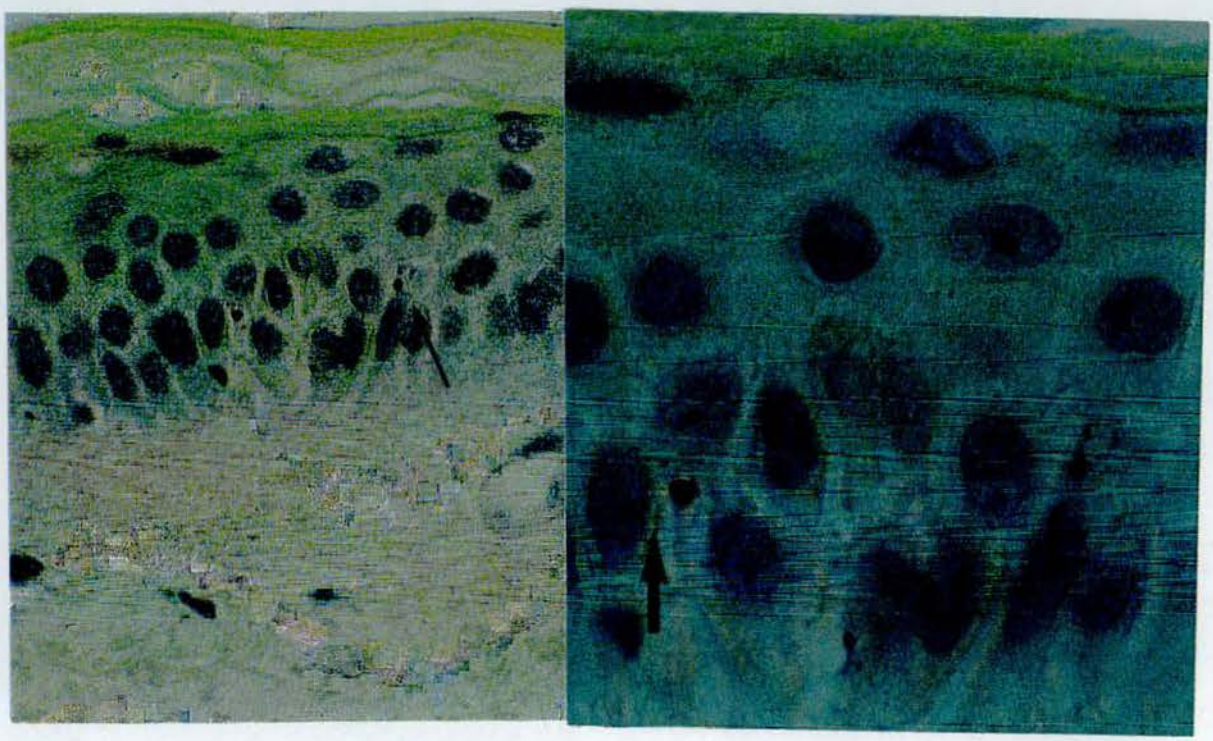
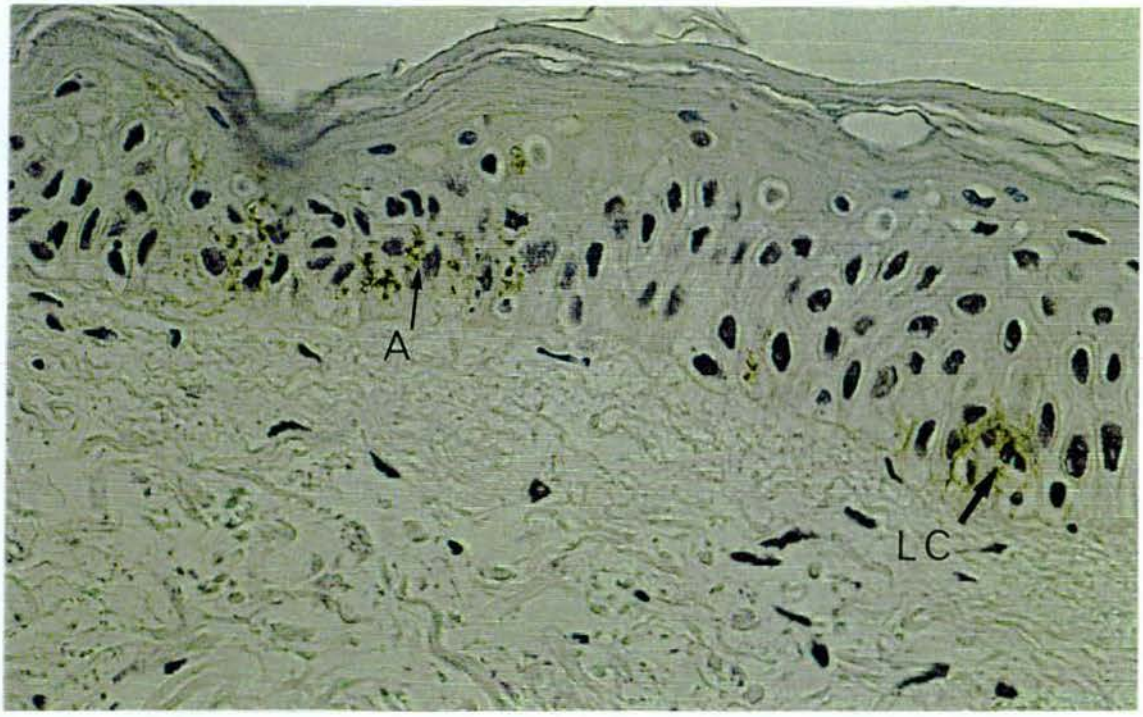


PLATE 3.12:

CD1a+ve apoptotic cells (A) 48 hours after NA application. (mag. x20).

Normal LC (LC).



• CD95, AND THE BCL-2-LIKE GENE FAMILY

This section investigates genes that might be important in induction of apoptosis in the skin.

1). CONTROL BIOPSIES (**Table 3.11**)

Mild CD95/fas expression was seen in the basal, prickle and granular layers of all control biopsies.

Mild bcl-2 expression was observed in the basal layer of all biopsies and the prickle layer of 60% of biopsies. In contrast, bcl-2 expression was not observed in the granular layer. The pattern of bcl-x expression was similar in the basal, prickle and granular layers of all biopsies. Moderate bax expression was observed in the basal, prickle and granular layers in the majority of biopsies. The stratum corneum was negative for all bcl-2-like genes investigated.

Comparison of bcl-2, bcl-x and bax expression revealed significant differences in the basal, prickle and granular layers. In all three layers bcl-x and bax expression was significantly stronger than bcl-2 expression (**Table 3.12**), with no difference found between bcl-x and bax.

2). SLS

CD95/fas expression was not significantly different from control levels. Bcl-2, bcl-x and bax levels were not significantly different from control levels.

Comparison of bcl-2, bcl-x and bax levels at 24 and 48 hours after SLS irritation revealed significant differences in the basal, prickle and granular layers. Bax and bcl-x expression were found to be significantly greater than bcl-2 expression in the basal layer at 48 hours after SLS application (Friedman test). Due to small sample size this test was not significant using the McNemar test. Results from the McNemar test are shown in **Table 3.13**. Prickle and granular layer differences were noted only

at 24 hours, showing significantly stronger bcl-x and bax expression when compared with bcl-2.

3). NA

CD95 expression observed was similar to control levels. Bcl-2, bcl-x and bax levels were not significantly different from control levels.

Comparison of bcl-2, bcl-x and bax levels at 24 and 48 hours after NA irritation revealed significant differences in the prickle layer. By 48 hours after NA application bcl-x and bax expression were significantly stronger than bcl-2 expression (McNemar test, $p<0.03$ and $p<0.004$ respectively).

• SUMMARY

In control skin bcl-2 expression was present in the basal and prickle layers and absent in the granular layer. Bcl-2 expression was not observed in the granular layer. The results show that bcl-2 expression decreases as the KC move towards the outer epidermal layers. Bcl-2 expression was not observed in the granular layer and stratum corneum where the cells eventually die as a result of terminal differentiation.

Similar levels of bcl-x and bax expression were observed in all three layers of normal skin. Expression of which was significantly stronger than bcl-2.

In irritant treated biopsies differences in the intensity of staining occurred between bcl-2, bcl-x and bax. As with control skin, bcl-x and bax staining intensity was stronger than bcl-2. After SLS irritation bcl-x and bax staining was significantly greater at 24 hours and basal layer staining at 48 hours. NA application induced differences in the prickle layer only, showing significantly stronger bcl-x and bax staining by 48 hours.

TABLE 3.11:

The patterns of bcl-2, bcl-x and bax expression in the a). basal layer, b) prickle layer and c). granular layer of unirritated control skin from patients with chronic ICD.

a). Basal layer expression

	% OF BIOPSIES (n = 13)			
MOLECULE	**NEGATIVE**	**MILD**	**MODERATE**	**SEVERE**
bcl-2	0	100	0	0
bcl-x	0	31	62	7
bax	0	23	54	23

b). Prickle layer expression

	% OF BIOPSIES (n = 13)			
MOLECULE	**NEGATIVE**	**MILD**	**MODERATE**	**SEVERE**
bcl-2	39	61	0	0
bcl-x	0	8	77	15
bax	0	23	54	23

c).Granular layer expression

	% OF BIOPSIES (n = 11)			
MOLECULE	**NEGATIVE**	**MILD**	**MODERATE**	**SEVERE**
bcl-2	100	0	0	0
bcl-x	0	0	64	36
bax	0	27	36	37

Table 3.12:

McNemar Test, comparison of the intensity of bcl-2, bcl-x and bax expression in control biopsies.

	P VALUE		
GROUPS COMPARED	BASAL	PRICKLE	GRANULAR
bcl-2 vs bcl-x	$p<0.04$	$p<0.0004$	$p<0.001$
bcl-2 vs bax	$p<0.02$	$p<0.002$	$p<0.001$
bcl-x vs bax	NS	NS	NS

Table 3.13:

McNemar Test, comparison of the intensity of bcl-2, bcl-x and bax expression after SLS irritation. Time-point stated in brackets is the time after SLS application to which the results refer.

	P VALUE		
GROUPS COMPARED	BASAL (48 hrs)	PRICKLE (24 hrs)	GRANULAR (24 hours)
bcl-2 vs bcl-x	NS	$p<0.02$	$p<0.03$
bcl-2 vs bax	NS	$p<0.02$	$P<0.03$
bcl-x vs bax	NS	NS	NS

3.4. DERMAL CHANGES - SLS and NA

• MONONUCLEAR CELL RECRUITMENT

1). SLS (**Table 3.14a**)

Moderate mononuclear papillary perivascular infiltration was observed in approximately 75% of control and SLS treated biopsies taken at 1 and 6 hours. Infiltration increased significantly by 24 and 48 hours ($p<0.05$, Friedman test) with 88% of biopsies showing heavy perivascular infiltration by 48 hours after SLS application ($p<0.02$, McNemar test).

Reticular dermis mononuclear cell infiltration was also found to be moderate in control and irritant treated biopsies taken up to 24 hours. However, by 48 hours heavy reticular infiltration was evident ($p<0.02$, McNemar test) reflecting the increased perivascular infiltration also seen at this time.

2). NA (**Table 3.14b**)

Moderate mononuclear cell perivascular infiltration was observed in approximately half of control and NA treated biopsies taken at 1 hour. By 6 hours, infiltration had significantly increased ($p<0.03$, McNemar test), with moderate infiltration in 70% of biopsies and heavy in 18%. Although not significantly greater than in control biopsies, increased infiltration continued to be evident at 24 and 48 hours after irritation.

In the reticular dermis infiltration was significantly greater ($p<0.02$) at 6 hours after NA application. As in perivascular areas the trend of increased infiltration continued at 24 and 48 hours; however, this was not significantly greater than in control biopsies.

Table 3.14a:

Analysis performed on H&E stained sections. Mononuclear cell infiltration after SLS application, + (mild), ++ (moderate) and +++ (heavy).

Data presented as % of positive biopsies.

% OF POSITIVE BIOPSIES															
	Control n=24			1 HR n=16			6 HRS n=16			24 HRS n=8			48 HRS n=8		
	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Perivascular	32	62	4	25	75	0	13	81	6	37	25	38	0	12	88
Reticular	75	25	0	69	31	0	38	56	6	62	0	38	11	25	64

Table 3.14b:

Analysis performed on H&E stained sections. Mononuclear cell infiltration after NA application, + (mild), ++ (moderate) and +++ (heavy).

Data presented as % of positive biopsies.

% OF POSITIVE BIOPSIES															
	Control n=24			1 HR n=16			6 HRS n=16			24 HRS n=8			48 HRS n=8		
	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Perivascular	42	58	0	53	47	0	11	71	18	0	71	29	0	57	43
Reticular	79	21	0	82	18	0	47	53	0	43	57	0	29	71	0

• CD3+ve LYMPHOCYTE INFILTRATION

1). SLS

As with mononuclear cell infiltration normal levels of CD3+ve lymphocytes were observed in control biopsies. However, by 1 and 6 hours after SLS application lymphocyte movement from perivascular areas into the surrounding papillary dermis had occurred. By 24 and 48 hours this had progressed to heavy CD3+ve leucocyte infiltration throughout the dermis (3/5 and 4/5 biopsies respectively, **Plate 3.13**).

2). NA

Control biopsies showed a few scattered perivascular CD3+ve lymphocytes. NA application resulted in lymphocyte movement from the perivascular areas into the papillary dermis by 6, 24 and 48 hours (6/13, 2/3 and 2/3 biopsies respectively).

• NEUTROPHIL RECRUITMENT

Neutrophils were assessed morphologically (H&E sections) and using a monoclonal CD15 Ab. Neutrophil infiltration was not observed in control biopsies. Dermal neutrophils were observed after both NA and SLS irritation (**Table 3.15**). NA induced significant levels of infiltration earlier than SLS ($p<0.04$ at 6 hours) but was ot seen at 24 hours. Higher neutrophil numbers were observed at 24 hours in SLS treated biopsies ($p<0.05$).

• EOSINOPHIL AND MAST CELL RECRUITMENT

Eosinophils were assessed morphologically using H&E stained sections. Eosinophils were absent in control biopsies. Dermal eosinophils were noted in irritant treated biopsies (**Table 3.16**), with the numbers of positive biopsies significantly greater than controls at 6 hours after SLS application (perivascular eosinophils).

Mast cells (assessed using an Ab directed against mast cell tryptase) were observed in all control and irritant treated biopsies (range 7.0 - 82.9), with no significant difference between groups.

• ADHESION MOLECULE EXPRESSION

1). SLS

Intensity of expression was difficult to assess due to the poor dermal architecture in frozen sections; therefore conclusions were only made about presence or absence of expression. CD54 and CD62E expression was observed on the endothelium of all biopsies. CD54 expression was also observed on cells in the dermal infiltrate. Dermal infiltrate expression was present in 4/10, 4/14, 4/6 and 4/5 biopsies at 1, 6, 24 and 48 hours respectively.

2). NA

As with SLS application, CD54 and CD62E expression was observed on the endothelium of all biopsies. CD54+ve infiltrating cells were also observed, with 3/15, 5/12, 2/4 and 1/3 biopsies at 1, 6, 24 and 48 hours respectively.

• SUMMARY

Dermal cellular infiltration occurred following application of both irritants. However this was more pronounced after SLS application, resulting in heavy perivascular and reticular dermis infiltration by 48 hours. The CD3+ve lymphocytes present in SLS induced dermal infiltrate moved from the perivascular areas into the surrounding papillary dermis producing a heavy CD3+ve infiltration throughout the dermis by 48 hours.

CD54+ve infiltrating cells were observed after irritation, again these were more pronounced after SLS application. These results suggest that a large proportion of the

dermal infiltrate observed 48 hours after SLS application comprises activated T lymphocytes.

Differential recruitment of neutrophils was observed. In the case of NA irritation, neutrophils were observed in the papillary dermis by 6 hours, but, this influx was not sustained at 24 and 48 hours. In contrast, SLS application resulted in the dermal accumulation of neutrophils by 24 and 48 hours.

PLATE 3.13:

Dermal CD3+ve lymphocyte infiltration 48 hours after SLS application (mag. x10)

(P- perivascular, D - diffuse)

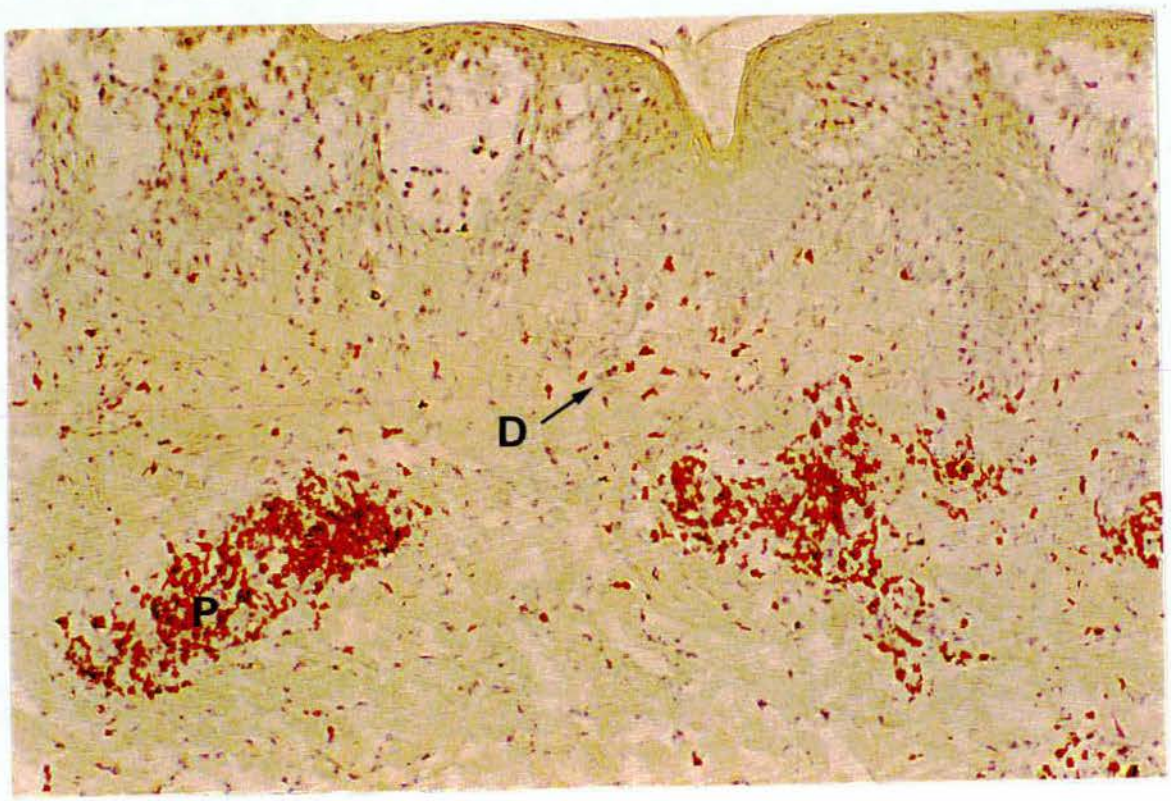


TABLE 3.15a:

Median number (and range) of dermal CD15+ve cells per mm^2 dermis at 0, 1, 6 ,24 and 48 hours after **SLS** application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test). ☆ denotes statistical differences between NA and SLS (Mann-Whitney U test).

a) median number and range of CD15+ve (neutrophils) after **SLS** application.

TIME (hours)	N	MEDIAN	RANGE
0	16	0	0 - 0
1	16	0	0 - 0.4
6	16	0	0 - 7.9
0	8	0	0 - 0
24	8	5.9☆	0 - 88.9
48	8	1.6	0 - 88.6

TABLE 3.15b:

Median number (and range) of dermal CD15+ve cells per mm^2 dermis at 0. 1. 6 .24 and 48 hours after **NA** application. *denotes results that are significantly different from control data (Wilcoxon-signed ranks sum test). ☆ denotes statistical differences between NA and SLS (Mann-Whitney U test).

b) median number and range of CD15+ve (neutrophils) after **NA** application.

TIME (hours)	N	MEDIAN	RANGE
0	17	0	0 - 0
1	17	0	0 - 5.1
6	17	10.3*☆	0 - 48.8
0	7	0	0 - 0
24	7	0	0 - 12.1
48	7	0	0 - 9.8

TABLE 3.16:

Dermal eosinophil recruitment after a). SLS and b). NA application. * denotes data that is significantly different from contol. data (McNemar test).

a). percentage of biopsies with dermal eosinophils after SLS application.

TIME (hrs)	N	PERIVASCULAR (PAPILLARY)	RETICULAR
0	16	6	0
1	16	0	0
6	16	38*	19
0	8	0	0
24	8	38	25
48	8	38	38

b). percentage of biopsies with dermal eosinophils after NA application.

TIME (hrs)	N	PERIVASCULAR (PAPILLARY)	RETICULAR
0	17	0	0
1	17	18	0
6	17	24	0
0	7	0	0
24	7	0	0
48	7	14	0

3.5. CYTOKINE PRODUCTION

• PILOT STUDY

The small amounts of blister fluid obtained from each subject (50 - 200μl) limited the number of cytokines that could be quantified using ELISA. For this reason a pilot study was carried out (using normal volunteers) to determine the cytokines of major importance in our model system.

We measured IL-8, MIP-1α, TNFα, IL-2, IFNγ and IL-1α, all suggested to play important roles in cutaneous inflammation. **Table 3.17** summarises the results from this pilot study. On the basis of these results we chose to include IL-1α, IL-8 and IFNγ in the main study.

TABLE 3.17:

Cytokine assay results from pilot study. Table shows the mean concentration of cytokine at 1, 6 or 24 hours after NA irritation.

CYTOKINE	1 HOUR	n	6 HOURS	n	24 Hours	n
IL-8	negative	3	1.6 ng/ml	3	not tested	-
MIP-1α	not tested	-	negative	3	negative	1
TNFα	not tested	-	negative	1	negative	1
IL-2	not tested	-	not tested	-	negative	2
IFNγ	not tested	-	3.2 ng/ml	1	0.6 ng/ml	3
IL-1α	not tested	-	97.8 pg/ml	1	not tested	-

- **IFNγ**

IFNγ was not detected in patient samples at 1 or 6 hours after NA irritation. Unfortunately the data from all 24 hour samples was not valid due to a faulty assay kit.

- **IL-1α (Graph 3.1)**

Untreated control samples from normal volunteers showed detectable levels of IL-1α (5/5 samples). IL-1α was detected in blister fluid from both patients and volunteers after NA application. In patients IL-1α was found at 1, 6 and 24 hours (4/19, 4/6 and 4/11 samples respectively) with no significant difference found between groups. In contrast detectable levels of IL-1α were seen at only 1 and 24 hours in volunteers (4/10 and 6/10 samples), with significantly higher cytokine levels at 24 in comparison with 6 hours ($p<0.03$, Mann Whitney-U test).

- **IL-8**

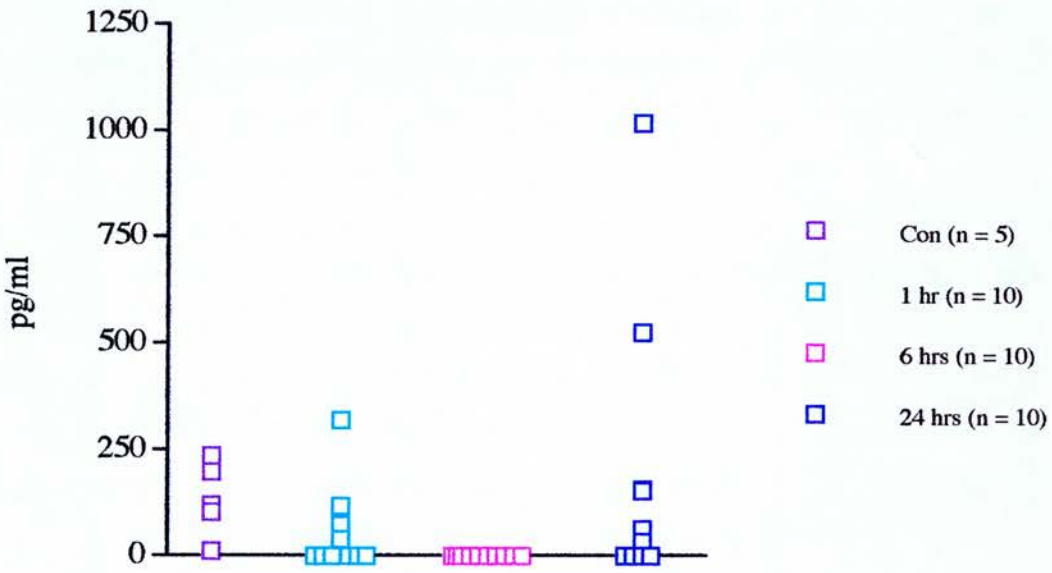
IL-8 assays were carried out after both NA and SLS irritation.

1). SLS

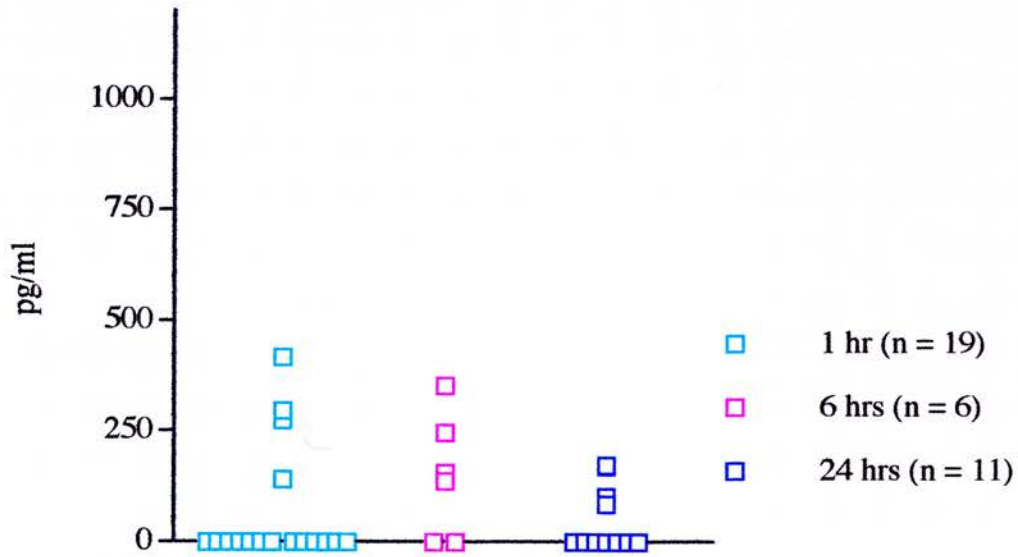
Assays confirmed IL-8 to be present in blister fluid from both patients and volunteers at all timepoints. Samples were only taken from patients at 24 hours and all 3 showed detectable IL-8. In the volunteer group IL-8 was detected in untreated control samples as well as in SLS treated samples taken at 1, 6 and 24 hours (4/16, 2/3, 7/8 and 6/8 respectively). Concentrations of IL-8 detected are outlined in the **Table 3.18.**

GRAPH 3.1: IL-1 alpha ELISA results.

a) IL-1alpha ELISA results in volunteers after NA application



b) IL-1 alpha ELISA results in patients after NAapplication



Volunteer samples taken 1 hour after irritation were not significantly different from unirritated controls. However, by 6 and 24 hours after irritation significantly increased levels of IL-8 were detected ($p<0.006$ and $p<0.003$, Mann-Whitney U test).

Comparison of 24 hour patient samples with unirritated volunteer controls revealed that SLS irritation induced a significant increase in IL-8 production ($p<0.004$, Mann-Whitney U test). IL-8 levels in irritant treated patient and volunteer groups were not significantly different, possibly due to small sample size in the patient group.

TABLE 3.18:

Shows the median (and range) concentrations (pg/ml) of IL-8 detected at 0, 1, 6 and 24 hours after SLS application. *determines data which is significantly different from control data (Mann-whitney U test).

VOLUNTEERS			
Time (hrs)	n	Median (pg/ml)	Range (pg/ml)
0	16	0	0 - 849
1	3	492	0 - 1153
6	8	340*	0 - 1398
24	8	237*	0 - 1079
PATIENTS			
Time (hrs)	n	Median (pg/ml)	Range (pg/ml)
24	3	1109*	607 - 18515

2). NA

Assays detected small amounts of IL-8 in blister fluid from both patients and volunteers although not at all timepoints investigated (**Table 3.19**). In the patient group IL-8 was detected at 1 and 6 hours after SLS application (2/19 and 1/6 samples

respectively); whereas in the volunteer group IL-8 was detected at 6 and 24 hours (3/10 and 2/10 samples respectively). Concentrations of cytokine found are outlined in **table 3.19**.

No significant differences were found between the different timepoints, including time 0 controls, in either patient or volunteer groups, suggesting that NA application has no affect on IL-8 production. There were also no differences found between patient and volunteer samples.

TABLE 3.19:

Shows the median (and range) concentrations (pg/ml) of IL-8 detected at 0, 1, 6 and 24 hours after NA application.

VOLUNTEERS			
Time (hrs)	n	Median (pg/ml)	Range (pg/ml)
0	16	0	0 - 849
1	10	0	0 - 0
6	10	0	0 - 672
24	10	0	0 - 910
PATIENTS			
Time (hrs)	n	Median (pg/ml)	Range (pg/ml)
1	19	0	0 - 565
6	6	0	0 - 1200
24	11	0	0 - 0

3). COMPARISON OF NA AND SLS

Comparison of NA and SLS irritation revealed significantly higher concentrations of IL-8 in blister fluid from SLS treated patients ($p<0.004$, Mann-Whitney U test). This

comparison could only be made at 24 hours due to the small number of patients in the SLS treatment group.

In the previous section we observed that IL-8 production after NA application was no different in patient and volunteer groups, for this reason the data was pooled allowing further analysis. The pooled group was compared with data from SLS irritated volunteers. This data revealed that SLS irritation results in significantly higher concentrations of IL-8 at all three timepoints investigated (**Table 3.20**).

TABLE 3.20:

Comparison of IL-8 levels in blister fluid from SLS treated (volunteers) and NA treated (pooled data) skin.

Time (hours)	Mann-Whitney U test
1	$p<0.02$
6	$p<0.02$
24	$p<0.002$

• SUMMARY

IL-1α production was induced by NA irritation. In patients IL-1α was detected at 1, 6 and 24 hours, whereas in volunteers detection was confined to 1 and 24 hours.

IL-8 was found in both uninvolved and irritant treated groups. NA irritation did not induce a significant IL-8 response. In contrast, SLS irritation led to significant IL-8 production at 1, 6 and 24 hours, in both patient and volunteers groups.

3.6. AUTO-ANTIBODY DETECTION IN PATIENTS WITH CHRONIC ICD.

Sera from 8 patients with chronic ICD were tested against lysates from normal skin, lesional ICD skin, normal tonsil and normal liver. Endogenous biotin and other background bands were detected in normal skin but no specific staining with sera was found. In contrast, 4/8, 3/8, 5/8 and 5/8 ICD sera tested against dermal lysates from 4 different ICD patients produced a specific band at about 75 kDA (**Table 3.22**). In the two epidermal lysates investigated a band of about 75kDa was detected in 3/8 and 4/8 sera tested (**Table 3.23**). Sera from 4 normal individuals were also tested showing no specific bands.

Graph 3.2 outlines the method used to calculate the molecular weight of the auto-antigen detected in these 4 patients.

3.6.1. SUMMARY

An antigen of approximately 75kDA was detected, using sera from patients with chronic ICD, in lesional dermal and epidermal extracts.

GRAPH 3.2:

Shows the log of molecular weights plotted against relative front. Method used to calculate the molecular weight of bands seen on the gel.

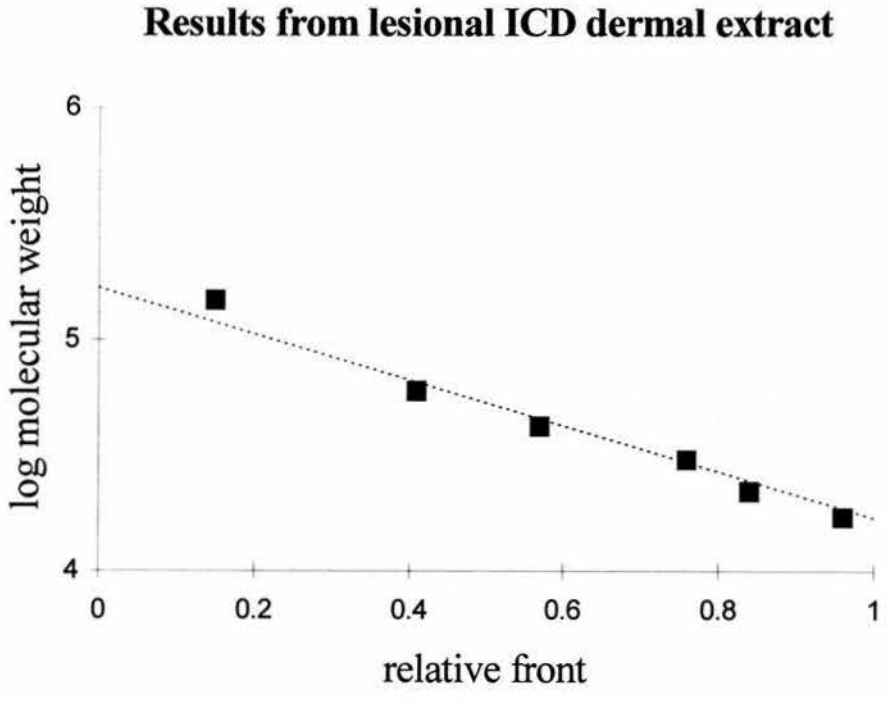


TABLE 3.22:

Relative mobility of molecular weights and specific bands, detected with sera from ICD patients, in normal dermal extracts.

	RELATIVE MOBILITY			
	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4
148 kDa	0.15	0.09	0.04	0.06
60 kDA	0.41	0.33	0.30	0.31
42 kDA	0.57	0.49	0.45	0.47
30 kDA	0.76	0.69	0.65	0.64
22 kDA	0.84	0.78	0.77	0.75
17 kDA	0.96	0.91	0.90	0.89
Band	0.38	0.33	0.31	0.33
M. Wt (band)	77 kDa	77 kDa	78 kDa	71 kDA

TABLE 3.23:

Relative mobility of molecular weights and specific bands, detected with sera from ICD patients, in normal epidermal extracts.

	RELATIVE MOBILITY	
	PATIENT 1	PATIENT 4
148 kDa	0.07	0.06
60 kDA	0.3	0.3
42 kDA	0.48	0.43
30 kDA	0.61	0.61
22 kDA	0.7	0.72
17 kDA	0.86	0.87
Band	0.26	0.28
M. Wt (band)	73 kDA	75 kDA

4.0. DISCUSSION

Cells of the epidermis, including KC and LC have long been recognised as being dynamically involved in the development of contact dermatitis (Gawkrodger *et al*, 1986, Gawkrodger *et al*, 1987). The overall aim of this thesis was to investigate the epidermal and dermal changes in response to irritant application in order to clarify the role(s) of the inflammatory and immunological responses in the pathogenesis of ICD.

Previous investigations on irritant reactions have focused on events at time-points greater than 20 hours after irritant application on skin of patients allergic contact dermatitis or normal human skin. The approach adopted in this study was to investigate early events (1 and 6 hours) in comparison with events at 24 and 48 hours after experimental irritation on the skin of patients with chronic ICD. These findings were then compared with data concerning normal skin, thus providing information on differences between the two groups.

To achieve this, a detailed histopathological study was undertaken which compared clinically similar irritant reactions elicited on the clinically normal-looking skin of patients with chronic ICD. Comparisons were made between 80% NA, 5% SLS and 0.01% DL irritancy at 5 time-points ranging from 0 - 48 hours. These three chemically different irritants were chosen because they represent different modes of action on the skin.

- Anionic detergents, such as SLS, have been used widely in research and have demonstrated the greatest irritant potential of all substances tested (reviewed by Wilhelm *et al*, 1994). SLS has multiple modes of action including removal of surface lipids, denaturation of keratins and surface glycoproteins and impairment of barrier function through increased permeability to water (Kligman and Wooding, 1967).

- NA is a C9 free fatty acid which alters the skin lipid profile. It appears to have a consistent irritant effect (Stillman *et al*, 1975), hence its use as a test substance for histochemical and E.M. studies of irritancy (Wahlberg *et al*, 1985). Patch testing with 80% NA for 48 hours induced dyskeratosis suggesting a disturbance in KC metabolism and differentiation (Willis *et al*, 1989). Experimental NA irritancy has been shown to result in an increase in the density of proliferating KC (Willis *et al*, 1992, 1993).

- DL, an anthralin derivative, is commonly used in the treatment of psoriasis. It causes the release of prostaglandins which are considered to be the major factor involved in the decreased KC proliferation observed upon treatment. When applied to the skin, DL is oxidised which results in the generation of free radicals, thought to be responsible for irritation.

Gawkrodger and colleagues have shown epidermal T cell and macrophage exocytosis in response to 48 hour application of 0.1% DL (Gawkrodger *et al*, 1986). The data presented in this thesis relates to 1 and 6 hour applications of 0.01% DL, and the lack of cellular infiltration is probably due to insufficient exposure time and/or irritant concentration. Two biopsies taken at 48 hours after 0.01% DL application showed evidence of ICAM-1 expression. This would suggest that KC activation and T cell infiltration might be evident if a larger patient group was investigated.

DL has been shown to affect LC, resulting in disrupted mitochondrial function and decreased (Gawkrodger *et al*, 1986) or unchanged (Kanerva, 1984) LC density. There is some uncertainty in the literature concerning epidermal LC density, but this again may simply reflect differences in experimental design.

KC mitochondrial damage has also been observed (reviewed by Kanerva *et al*, 1990, Gawkrodger *et al*, 1986) although this was less severe than that demonstrated in LC. Mitochondrial damage has been postulated to contribute to the decreased epidermal proliferation for which DL is therapeutically utilised.

The data presented in this study clearly show that 80% NA , 5% SLS and 0.01 % DL induce different patterns of epidermal damage, cellular activation and infiltration. Despite early experiments to titrate suitable concentrations of the irritants 0.01% DL irritation evoked only mild erythema, even after 48 hours. These findings were reflected in the minimal histopathological changes observed which amounted to mild spongiosis in less than 15% of patients and basal layer intracellular oedema in the epidermis. Irritation had no apparent effect on the dermis. Evaluation of the erythema was carried out using a Minolta chroma meter which measured the degree of skin redness. The brown staining due to DL affected measurements making it difficult to grade the induced erythema. Further investigation, by eye, of the erythema produced by DL suggested that this was not comparable to that provoked by NA and SLS. For this reason dithranol was then omitted from the study. In summary it seems likely that the poor inflammatory response to DL reflects an inadequate concentration of this irritant but it is also possible that DL may be slower acting than the other irritants, requiring 48 hours or more to induce erythema equivalent to that seen with NA and SLS at 24 hours.

As I aimed to compare irritant reactions of similar intensity, further discussion will focus on the irritant reactions following application of 80% NA and 5% SLS.

4.1. VEHICLE CONTROLS, GENDER, ACTIVE DISEASE AND ATOPY.

NA was applied to the skin in the vehicle propan-1-ol whereas SLS was made up in an aqueous solution. Both vehicles induced a minimal epidermal response, but this was not significantly different from control (unirritated, normal-looking) skin taken from the same group of ICD patients. Four biopsies were taken from each subject, one from clinically normal skin at 0 hours, one vehicle control and two irritant

treated samples. This design meant that there were only half the number of vehicle control biopsies in comparison with irritant treated biopsies. Therefore, when carrying out analysis on paired data the sample groups were greatly diminished. For this reason vehicle control and time 0 control biopsies were analysed initially. No significant differences were found between these groups suggesting that the vehicle plays no significant role in these forms of experimentally induced irritation. Therefore, further analysis included irritant treated samples paired with the corresponding time 0 controls.

Comparisons were made between male and female patients and groups with and without active disease. No differences were found between these groups for any of the parameters investigated.

Previous studies have suggested that atopic skin, compared with normal skin, is more susceptible to irritation (Rystedt, 1990).. Impaired barrier function in atopic subjects is believed to contribute to this irritant susceptibility (Tupker *et al*, 1990). This does not appear to be the case in patients with chronic ICD as there was no difference in irritant susceptibility between atopic and non-atopic patients. It is possible that patients with chronic ICD have impaired cutaneous barrier function and that this factor, coupled with my observations of higher basal levels of partially activated LC and increased IL-1α, may explain the increased irritant susceptibility, regardless of atopic status. This hypothesis would explain why, in the context of this study, atopic status did not influence experimental irritation.

4.2. EPIDERMAL DAMAGE

In 1989 Willis and co-workers showed that 48 hour NA (80%) application, on normal volunteers, induced KC changes which were indicative of disordered keratinisation accompanied by mild spongiosis. In contrast, 48 hour irritation with 5% SLS was shown to result in moderate reactions, characterised by alterations in

KC morphology and parakeratosis (Willis *et al*, 1989). The results presented in this study have also shown different patterns of epidermal damage. 80% NA produced mild spongiosis, while 5% SLS caused severe spongiosis which often progressed to vesiculation at 24 and 48 hours. My results confirm Willis' findings that SLS induces parakeratosis in the later phase of irritation (24 and 48 hours) but I have shown that NA irritation may also induce parakeratosis. Parakeratosis may result from increased epidermal mitosis (Fisher and Maibach, 1975) or accelerated keratinisation (Wolff *et al*, 1987). Both of these processes may be induced through production of KC growth factors such as IL-6 and IL-8 (Grossman *et al*, 1989, Tuschil *et al*, 1992). My data show significant IL-8 production after SLS application, confirming previous reports (Wilmer *et al*, 1994, Wilmer and Luster, 1995).

4.3. EPIDERMAL PROLIFERATION

SLS application caused increased KC proliferation by 24 and 48 hours and epidermal thickening at 48 hours. These findings corroborate previous studies carried out *in vitro* and on normal volunteers (Varani *et al*, 1991, Willis *et al*, 1993, Berardesca and Distante, 1994). IL-8 may contribute to the increased proliferation observed. In contrast, NA decreased KC proliferation, at 24 hours which returned to normal by 48 hours. This may reflect the lack of IL-8 production observed after NA irritation but also suggests that other anti-proliferative factors are involved. Two such factors could be TGF-β (Choi and Fuchs, 1990) and IFNγ (Symington, 1989) which have been shown *in vivo* and *in vitro* respectively to result in decreased basal KC proliferation.

4.4. CELLULAR INFILTRATION

ICAM-1 (CD54) mediates adhesion of KCs through binding to its ligand on T cells, LFA-1 (CD11a/CD18). It has been demonstrated that a progressive loss in KC ability to upregulate ICAM-1 expression occurs with differentiation (Little *et al*, 1996). This might suggest that KCs of the basal and suprabasal layer preferentially initiate and amplify inflammatory reactions. This study has shown that, following SLS application, the resultant focal basal KC ICAM-1 expression correlates with extensive epidermal lymphocyte infiltration. These findings suggest that basal layer KC may indeed be pivotal in the initiation of inflammation. In addition, epidermal lymphocyte infiltration was observed in areas lacking ICAM-1 expression. These findings indicate that ICAM-1/LFA-1 interactions are more important in the trapping of infiltrating cells within the epidermis rather than their recruitment from the blood stream. KC activation as defined by MHC II expression, and IL-8 production was also evident by 24 hours after SLS application. IL-8 is an important T cell chemoattractant and may contribute to T cell recruitment into the epidermis.

Investigation of epidermal T-cell subsets showed recruitment of both CD4+ve and CD8+ve T cells after SLS application. Epidermal CD4+ve T cell infiltration occurs more rapidly than CD8+ve T cell recruitment. Although the phenotype of the infiltrating T cells has yet to be characterised it is possible that the initial influx of CD4+ve T cells consists of recirculating CD4+ve, CLA+ve memory T cells that migrate rapidly into the skin upon irritation. These T cells could become activated by Ag presentation in the presence of co-stimulatory molecules even though the nature of the Ag providing the activating stimulus has yet to be determined. Antigenic stimuli could be produced as a direct result of epidermal damage, for example through degradation of epidermal proteins. It is also possible that induction of an autoimmune response could be responsible for the T cell influx observed.

Memory T cells do not require EC activation to the same degree as naïve T cells and are therefore recruited at an early stage of the response. Once in the epidermis these

cells bind to activated epidermal KC via ICAM-1/LFA-1 resulting in further T cell and KC activation and consequent cytokine production. The endothelium then becomes highly activated, expressing adhesion molecules which bind to and activate naïve CD4+ve and CD8+ve T cells. These cells then migrate into the damaged area. It is possible that CD8+ve cytotoxic T cells require prior KC activation via CD4+ve T cells before they are able to exert their effects. It is possible that CD8+ve cytotoxic T cells may also contribute to the epidermal damage which leads to autoimmunity and disease chronicity (see **section 4.9**.). Such a response could become self-perpetuating, with or without an autoimmune component. CD8+ve cytotoxic T cells would cause further epidermal damage leading to release of primary cytokines and recruitment of more memory CD4+ve T cells and so the cycle would continue. This model relies on the absence or inadequate production of inhibitory factors such as IL-10 and IL-1ra, a feature speculated to lead to exaggerated inflammation.

The prominent T cell response seen following SLS irritation (**Figure 4.1**) contrasts sharply with the lack of T lymphocyte infiltration and KC activation detected after NA application. Lymphocyte exocytosis and migration may be absent as a direct result of the lack of KC activation and ICAM-1 expression and/or chemotactic factors such as IL-8 and IL-1. ELISA studies have shown that NA irritation causes minimal IL-8 production and thereby a lack of T cell chemoattraction. It is of interest that a similar level of macrophage exocytosis was observed by 48 hours after both NA and SLS irritation. This suggests that macrophage recruitment occurs independently of T cell recruitment possibly through the production of different chemotactic agents, such as monocyte activating chemotactic factor. It is possible that macrophage exocytosis occurs as a non-specific response to tissue injury. This influx would provide protection through phagocytosis of microbes and production of a wide variety of pro-inflammatory cytokines, for example IL-1 and TNFα. IL-1α has been shown to contribute to oedema and mononuclear cell chemotaxis (Miossec *et al*, 1984, Dowd *et al*, 1988), and may explain why irritation by both NA and SLS results in mild spongiosis and macrophage infiltration. IL-1 also recruits T cells to

FIGURE 4.1:

Diagramatic representation of SLS induced T lymphocyte recruitment from the blood stream into the skin.

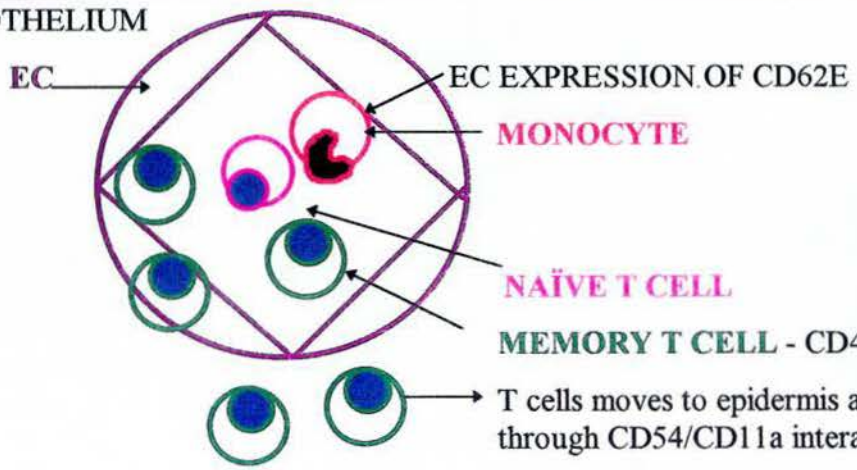
SLS IRRITATION

↓ SLS application results in release of epidermal stores of IL-1αand induces further KC cytokine production e.g. IL-8, TNFα. (see **Figure 4.2**)

EPIDERMIS

DERMIS

ENDOTHELIUM



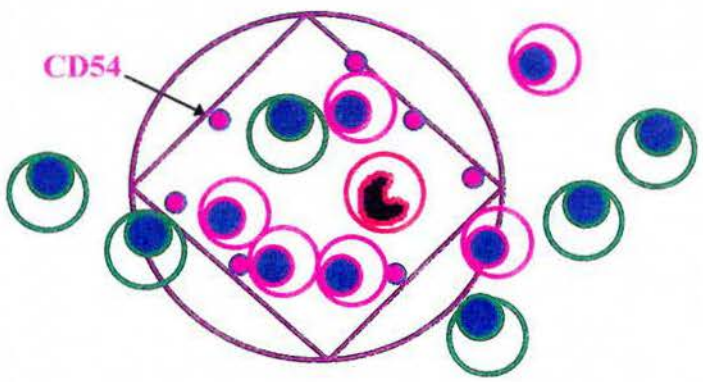
↓

Release of T cell and KC cytokines, for example IL-1, IL-8, IFNγ and TNFα

↓

ENDOTHELIAL ACTIVATION

EC activation causes adhesion molecule expression. Naive T cells bind to EC adhesion molecules and move through the endothelium. CD4+ve and CD8+ve naive T cells are present with CD8+ve numbers increasing over time. These CD8+ve cells could be involved in epidermal tissue destruction, leading to further cytokine release and T cell infiltration.



the site of tissue injury suggesting that the lack of epidermal T cell trapping after NA application may be due to the absence of KC activation and ICAM-1 expression.

4.5. CYTOKINE PROFILES

Relatively low levels of cytokine is released from normal skin. In contrast, injury or disease results in KC production of several pro-inflammatory cytokines including IL-1α, TNFα and IL-6 (reviewed by Barker and MacDonald, 1992, Nickoloff and Turka, 1993). A trend towards different levels of Il-1α expression between patient and volunteer groups after NA irritation was noted. Larger numbers would be required to determine if this was a real finding. Presumably similar results would be seen after SLS irritation as IL-1α release represents a non-specific, protective response. Patients continually produced IL-1α whereas in the volunteer group IL-1α was detected at only 1 and 24 hours, with a dip at 6 hours. One explanation is that in patients with chronic ICD, the KC may be in a perpetual state of activation or partial activation and therefore continuously synthesise and release IL-1α. A generally increased state of activation is also implied by the increased levels of LC in the normal skin of patients with chronic ICD. LC can also produce IL-1α (reviewed by Sauder, 1984) which may contribute to the increased levels of this cytokine detected. In situ hybridisation and in vitro studies should be utilised to determine the contribution of these two cell types, thus giving a more complete picture of the response occurring here.

It is possible that KC in normal skin release pre-formed stores of IL-1α immediately upon NA irritation (1 hour). These stores are depleted by 6 hours, hence the dip in IL-1α detection. By 24 hours new IL-1α synthesis has increased to detectable levels. It would be of interest to investigate the IL-1α production after SLS and DL irritation. Unfortunately we could not obtain sufficient material to do this in our

investigation. Results from a similar investigation using SLS and DL would clarify the role of KC IL-1α release in this model system.

The IL-8 induction observed after SLS irritation may be important for T cell and Neutrophil recruitment as well as the increased KC proliferation and parakeratosis observed. IL-8 production was not significant after NA application and have been responsible for the lack of cellular infiltration, KC proliferation and parakeratosis observed.

Data presented in this thesis have shown that the patterns of cytokine induction upon irritation appear to be different depending upon on the irritant used. It is therefore possible that the cytokine profile induced by experimental irritation may be closely related to the ensuing cutaneous response.

4.6. LANGERHANS CELL RESPONSE TO IRRITATION

The function of LC as APC has been studied extensively in allergic contact dermatitis (Silberberg, 1973, Stingl *et al* 1977, 1978, Braathen and Thorsby, 1980, Cumberbatch *et al* 1992) but their role in ICD is as yet poorly understood. Experimentally induced ICD reactions have provided contradictory results, with groups showing increased (Scheynius *et al*, 1984), unchanged (Kanerva *et al*, 1984) and decreased (Ferguson *et al*, 1985) epidermal LC numbers. These findings may reflect differences in the concentration, application time or irritant used. This study, comparing clinically similar reactions to NA and SLS, strengthens previous suggestions in the literature (Willis *et al*, 1990) that the chemical nature of the irritant is one of the most important factors influencing the epidermal LC population.

The effect of experimental irritation on the LC population may be crucial for the outcome of an inflammatory response. NA irritation had a dramatic, damaging effect on epidermal LCs and caused minimal inflammation, whereas SLS had a minimal effect and induced a more intense inflammatory response. SLS application caused a decrease in epidermal LC numbers by 48 hours. This reduction was not due to LC apoptosis and may simply represent a migration of LCs to the draining lymph nodes as has been previously described in the literature (Brand *et al*, 1992). In contrast, NA irritation led to a redistribution of LCs, resulting in a predominantly basal position by 6 hours, with a dramatic reduction in number occurring by 24 hours as a result of LC migration or apoptosis. Eighty five to one hundred percent of the total number of apoptotic cells in the prickle layer were CD1a+ve, which may explain the epidermal redistribution pattern. LC migration may also play a role in this redistribution. By 24 and 48 hours sixty percent of LCs were undergoing apoptosis, accounting for the dramatic decrease in LC number. CSLM analysis suggests that LC redistribution is coupled with a reduction in dendrite length and possibly APC function.

I propose, that NA irritation results in a dramatic decrease in the potential for Ag-specific responses. LCs are the APC of the skin and process and present Ags present in the skin. Antigen is taken up in the epidermis and transported by the LCs to the regional lymph node where it is presented to T cells. Naïve T cells will recognise foreign peptide in association with MHC molecules on LCs and become activated. These sensitised T cells then travel to the skin and participate in the resultant antigen-specific inflammatory response. In this manner epidermal LCS enable a rapid and specific response to any epidermal damage. In the case of NA irritation LC function is decreased and cutaneous foreign or self antigen remains undetected by the immune system. In my view this implies that NA irritation induces a form of cutaneous 'immunological anergy', suggesting that irritant reactions resulting from NA application are not likely to result in chronic ICD. The lack of APC capability may however cause other problems such as increased infections. NA induced LC damage could be put to therapeutic benefit in Ag driven diseases, such as allergic contact dermatitis. This study does not indicate how long NA induced cutaneous

'immunosupression' may last. It may be that DC are rapidly recruited from the peripheral blood to replace the damaged epidermal population. Results from this study show that dermal LC numbers have not increased by 48 hours, but it seems likely that this cell population will replenish. Repopulation of epidermal LC is a factor to be taken into consideration if NA treatment is to be utilised therapeutically. It would be of interest to carry out further experiments to try and answer the question of LC replenishment. Application of NA for 48 hours should be carried out as before. The patch should then be removed but biopsies would not be taken until at least 72 hours had elapsed. Analysis of these samples would provide valuable information concerning regeneration of the LC population.

Comparison of samples from untreated normal volunteers, and clinically normal ICD patients, revealed a small difference in LC number, dendrite number and length. Clinically normal skin from ICD patients had more epidermal LC; with fewer, shorter dendrites, than those in he skin of normal volunteers. These findings suggest that LC in these patients may be in a partially activated state, with shorter dendrites, already partially rounded up and ready to migrate upon irritation.

Following NA application MHC II expression was confined to the epidermal LC. KC activation, defined by MHC II expression, was not evident. This lack of KC activation may reflect the general 'immuological anergy' as well as the increase in epidermal APC apoptosis. Antigen specific T cell activation and migration into the epidermis would not occur because of the decreased LC population and therefore lack of APC function. Cutaneous 'immunological anergy' in this context may involve a dampening down of any non-specific inflammatory response. Mechanism by which this could be achieved include induction of increased IL-10 and/or decreased IL-1 production.

NA application resulted in basal and prickle layer apoptosis at 24 and 48 hours, a pathological effect not induced by SLS. It is well known that KC produce a wide variety of cytokines, many of which may be involved in of the apoptotic process. LC

viability has been shown to be dramatically decreased by IL-10, resulting in apoptosis (Ludewig *et al*, 1995). Therefore, induction of IL-10 may be one mechanism by which NA irritation can induce LC apoptosis. Removal of growth factors or cytokines may also result in induction of apoptosis and deserves investigation. GM-CSF (Heufler *et al*, 1988, Kolenik *et al*, 1990), TNFα (Kimber and Cumberbatch, 1992) and IL-1 (Heufler *et al*, 1988) are important in LC maturation and migration. Removal of these cytokines may result in the LC remaining in the epidermis and undergoing apoptosis. TNFR ligation (reviewed by Cohen, 1992) and CD95/fas binding have also been implicated (Sayama *et al*, 1994) in induction of apoptosis. TNFα induced apoptosis has yet to be investigated in the context of irritant reactions.

Previous investigations, carried out on normal healthy volunteers show some similar findings to the results presented here in patients with chronic ICD but this was not found to be the case in the comparison of epidermal LC numbers and IL-1α production. Continuous IL-1α production was observed in patients with chronic ICD, which suggests that the KC in these individuals may also be in a partially activated state. This study has described increased numbers of LCs in normal skin from patients with chronic ICD compared with normal volunteers. This difference between the LC populations suggests that these cells may play a pivotal role in either induction or maintenance of chronic ICD.

4.7. TERMINAL DIFFERENTIATION AND CELL DEATH

UVB irradiation has been shown to induce KC apoptosis (Schwarz *et al*, 1993) in conjunction with increased TNFα production (Trefzer *et al*, 1993). In this situation TNFR ligation has been suggested as a possible apoptotic initiation signal, though other factors would also be required (Schwarz *et al*, 1993). These apoptotic or 'sunburn' cells are assumed to be KC. Recent data, presented at the 1996 September

meeting of the European Society of Dermatological Research has shown that UVB irradiation can induce apoptosis in LC (Rattis *et al*, 1996). This finding in conjunction with my investigations suggests that LC may be more sensitive to 'environmentally' induced apoptosis than KC. It would therefore be of interest to further investigate the cellular nature of 'sunburn' cells as they may be LC as well as KC in origin.

For lymphocytes the time span from initial cell shrinkage through to removal of apoptotic bodies is relatively short, in the region of 1 to 3 hours, whereas in epidermal KC, TD requires 48 to 72 hours (reviewed by Haake and Polakowska, 1993). In ICD, signs of the classical form of apoptosis as well as TD have been observed, suggesting that different initiating/control mechanisms are involved.

CD95/fas is a member of the TNF receptor family, which can initiate the apoptotic process upon ligation by Fas-ligand (Sauda *et al*, 1993). CD95 is expressed constitutively on normal KC but is not functionally active, i.e. it does not induce apoptosis upon ligation (Matsue *et al*, 1994). KC activation via IFNγ results in functional activation (Matsue *et al*, 1995) and up-regulation of CD95 expression (Sayama *et al*, 1994). Antibody binding studies have shown that CD95 ligation on T cells can result in activation and proliferation rather than apoptosis (Alderson *et al*, 1993). It is therefore possible that the levels of CD95 expressed, the activation state of the cell (T cell and KC), may determine the outcome of the response upon ligation.

In the context of this study I propose that, following experimentally induced SLS irritation, in contrast with normal KC, KC CD95/fas ligation would be functiona. SLS irritation causes KC to become activated, expressing both MHC II and ICAM-1, therefore CD95 ligation could induce both T cell and KC proliferation and/or apoptosis. SLS application results in increased dermal and epidermal cellular infiltration. Many of the T lymphocytes present in this influx will be fas-L positive and could bind to functional CD95 expressed on the epidermal KC inducing KC activation and proliferation or apoptosis. In contrast, NA irritation does not result in

KC activation, which suggests that CD95 is not functional in this situation. The fact that significant levels of apoptosis were not observed after SLS application indicates that CD95 ligation does not play an important role in this form of irritant induced apoptosis. In the context of SLS irritation CD95/fas ligation may be important in KC activation and proliferation. It would be of interest to carry out in vitro experimentation to determine the functional significance of CD95 expression in irritant treated KC.

As cells arise in the basal layer they express bcl-2 (Hockenbury *et al*, 1991) and are therefore protected from PCD. Cells that are about to undergo TD, move outwards and down-regulate bcl-2 expression. It has been suggested that this loss of bcl-2 expression may in fact be one of the triggers for KC movement. TGF-β has also been suggested to play a role as an apoptotic trigger. This cytokine inhibits KC proliferation and is synthesised within the prickle layer just prior to the onset of TD (Choi and Fuchs, 1990). Investigation into the levels of TGF-β produced after NA irritation, may provide some explanation for the increased prickle layer LC apoptosis and decreased KC proliferation observed. The production of TGF-β at 24 hours after NA application could induce the decreased proliferation observed at this timepoint, proliferation would then return to normal by 48 hours as the TGF-β activity decreases. In this model TGF-β could also provide a trigger for the induction of apoptosis which occurs by 48 hours. An apoptotic trigger is just one signal in a complex pathway. The resultant cell death may therefore not occur immediately upon TGF-β binding as further stimuli are required. Not only are there different initiating and control mechanisms in the apoptotic process but I have also demonstrated apoptosis in different cell populations upon irritation.

Understanding the complex mechanisms responsible for epidermal apoptosis will undoubtedly have an impact on our understanding of skin disease and aid development of future strategies for therapeutic intervention in cutaneous disease.

4.8. DERMAL RESPONSES

Previous studies on irritant reactions in normal volunteers have shown a predominantly mononuclear cell dermal infiltrate, of mainly T cells and macrophages, occurring by 24 hours. This study confirms that dermal infiltration, the majority of which is mononuclear, occurs after irritation, although the density and pattern of the response differed considerably between NA and SLS. SLS induced a larger infiltration, resulting in heavy perivascular and reticular dermis infiltration by 48 hours after application. The infiltrate was composed mainly of CD3+ve lymphocytes, which moved from the perivascular areas into the surrounding papillary dermis to produce the heavy infiltration observed throughout the dermis at 48 hours. The large dermal influx observed after SLS application reflects the epidermal T cell infiltration and may represent an increase in targeted cellular trafficking. Increased cellular migration occurs via IL-8 and IL-1 chemotaxisis and endothelial activation, all of which have been observed after SLS irritation.

EC expression of E-Selectin (CD62E), which binds to the CLA expressed on skin homing T lymphocytes and sialyl Lewis X on neutrophils accounts for residual T cell and neutrophil exocytosis important in immune surveillance. Irritation results in the release of stored IL-1 from the SC (Nickoloff and Naidu, 1994). This IL-1 acts together with TNFα and IL-1α secreted by subsequently activated KC, leading to endothelial cell activation and expression of E-Selectin, VCAM-1 (CD106) and ICAM-1 (Dustin, 1986, reviewed by McKenzie and Sauder, 1990). These adhesion molecules, through interaction with leucocyte ligands, play a key role in leucocyte migration (reviewed by Barker and MacDonald, 1992). The intensity of endothelial cell ICAM-1 expression has been shown to correlate with the degree of dermal infiltration and is therefore a molecule of central importance in inflammation (Griffiths *et al*, 1989).

Although, in the case of NA irritation, neutrophils were seen in the papillary dermis by 6 hours this influx was not sustained. In contrast, SLS irritation caused the dermal

accumulation of higher numbers of neutrophils at 24 and 48 hours. High numbers of neutrophils have been noted previously in SLS reactions (Willis *et al*, 1989), as this data has confirmed. It is possible that the intrinsic chemical nature of SLS itself may in part be responsible for neutrophil infiltration. Ingression may also be brought about by the release of KC cytokines, including IL-8, which is a potent neutrophil chemoattractant (Barker *et al*, 1992, Leonard and Yoshimura, 1990), the production of which occurs after SLS application.

4.9. AUTOIMMUNITY

In this thesis I have speculated that chronic ICD may be exacerbated by, or even represent an immune response to damaged epidermal components. If the early inflammatory response to epidermal irritation does not succeed in clearing the damage caused to the skin, 'damaged self' -Ags may be processed and presented by epidermal LC. This may have no consequence unless the damage is widespread and/or prolonged, in which case self-tolerance may be broken down giving rise to autoimmunity. In some individuals response against damaged self tissue may become self maintaining, resulting in chronic dermatitis even after exposure to the original irritant has ceased. This phenomenon could explain the chronicity of ICD seen in a proportion of patients, despite all attempts to avoid irritants.

In more than half of the chronic ICD lesions investigated in this study patients showed evidence of circulating auto-Abs directed against cutaneous components. Auto-Abs from the sera of one patient could detect an Ag of similar molecular weight in biopsies taken from chronic lesions in the majority of patients tested. This suggests this is not an isolated finding and that irritation results in specific self tissue damage or alteration resulting in auto-Ag formation. The antibodies present in the patient sera recognise an Ag of about 75kDa which is present in both epidermal and dermal lysates. Such an Ag could be LC derived as these cells migrate from the

epidermis into the dermis. However there are numerous other possibilities including example the Ag may represent an altered basement membrane component.

Immunological cytotoxicity is an endpoint of the immune response, which in the skin, may involve destruction of tumours and exogenous micro-organisms. It is also a mechanism of disease induction, especially in autoimmunity. Adhesion molecules, such as ICAM-1, are essential for CD8+ve T lymphocyte/KC binding, activation and finally target lysis. Macrophages are also a effector of cytotoxicity producing a less specific response which is not dependant on KC activation and ICAM-1 expression. Norris has proposed that disease specific ICAM-1 induction is a determinant in disease initiation and for the pattern of the resultant damage (Norris, 1990). The state of KC differentiation influences ICAM-1 expression (Little *et al*, 1996); therefore MHC II restricted cytotoxicity and the consequent epidermal damage is likely to occur in the basal or suprabasal layers where ICAM-1 expression is prominent. I have shown that experimentally induced SLS irritation results in T lymphocyte exocytosis from the blood stream and migration into the epidermis. This epidermal influx correlates with KC ICAM-1 expression. A proportion of these infiltrating cells are CD8+ve, the contribution of which increases with time of irritant exposure. I therefore propose a model of SLS irritation in which CD8+ve T lymphocyte mediated cytotoxity may play a central role in the epidermal damage seen after SLS application and could lead to induction of autoimmunity.

This thesis has shown that, due to lack of detection of auto-Ag in normal skin, the release of an epidermally or dermally sequestered Ag is unlikely to contribute to autoimmunity. It is however possible that production of a neo-Ag may result in the induction of an autoimmune response. Irritant mediated alteration of self KCs, LCs, a constituent of the basement membrane or other cutaneous molecules could result in an Ab or cell mediated autoimmune response to this neo-Ag and may contribute to the chronicity of the disease.

4.10. GENERAL DISCUSSION

The two irritants chosen for this study appear to interact with the skin in fundamentally different ways. The type of tissue damage and resultant cellular activation and infiltration induced is central to the outcome of the resultant irritant response.

SLS

SLS irritation induced a classical inflammatory response. Epidermal damage was evident and could be severe, with spongiosis, vesiculation and parakeratosis. I propose that this initial epidermal damage results in the release of primary cytokines such as IL-1 and TNFα. These cytokines induce EC activation which leads to leucocyte extravasation and migration into the dermis and eventually the epidermis where they are involved in KC activation. KC activation, defined by MHC II and ICAM-1 expression, may lead to further cytokine production, for example of the potent T cell and neutrophil chemoattractant IL-8. Increased IL-8 production could be one mechanism by which increased KC proliferation and parakeratosis occur in this irritant reaction.

In this model I have suggested that CD4+ve CLA+ve memory T cells are recruited initially, resulting in increased KC activation, cytokine production and eventually naïve T cell (CD4+ve and CD8+ve) epidermal infiltration. I have speculated that the CD8+ve T cells may be cytotoxic in nature and result in further tissue damage. This would initiate a second release of primary cytokines and resultant memory T cell influx into the dermis, and finally, the epidermis. If the balance of inhibitory cytokines is insufficient then this irritant response may become self-perpetuating and result in chronic inflammation. It will be important to study the changing cytokine profiles of the irritant response to SLS to determine if this switch to CD8+ve T cell infiltration alters the cytokine environment. For instance, if an increase in pro-inflammatory cytokines or down-regulation of inhibitory cytokines were described this would shed some light on the reasons for continued inflammation. The results

outlined in this study also suggest that an autoimmune response directed against CD8+ve T cell tissue damage or SLS altered self-Ag could contribute to chronicity. Previous reports in the literature suggest that LC migration is crucial only in response to allergen, however recent reports corroborated in this thesis strongly indicate that LC migration out of the epidermis may also occur during ICD reactions, particularly in the case of SLS (Mitulowska, 1994, Brand *et al*, 1993, Brasch *et al*, 1992,). The purpose of this LC migration is unclear, since ICD is reputed to be a T-cell independent reaction. I propose that the T cell response does have a role to play in ICD, and that LC migration may reflect a role in autoimmunity. The initial LC migration is probably a non-specific response to KC cytokine release but may eventually be part of an auto-Ag specific response.

In conclusion, these studies indicate that chronic inflammation is complex involving the interactions of multiple factors, many of which are not accounted for in this model It seems likely that in the case of SLS irritation, unchecked inflammation resulting from aberrant cytokine profiles and extensive T cell infiltration contribute to the severity of the response.

NA

The epidermal response to NA irritation was much less dramatic resulting in minimal epidermal damage and inflammation. As with SLS irritation the initial skin insult would result in the release of primary cytokines such as IL-1 and TNFα. These primary cytokines are responsible for the oedema observed. In the model I have proposed for NA irritation this initial insult has very different consequences from SLS. KC activation and the resultant T cell infiltration were not observed. One explanation would be the overall 'immunosuppressive' affect that NA exerts on the skin. NA results in LC apoptosis either directly or through induction of IL-10 and/or TGF-β. IL-10 induction would also account for the lack of KC activation as it down-regulates IFNγ induced MHC II expression. Other inhibitory cytokines, such as TGF-β, contribute to the dampening down of epidermal response through decreased KC proliferation and a consequently reduce the induction of chronic inflammation.

Production of pro-inflammatory cytokines such as IL-8 are decreased in this environment and may also contribute to the lack of KC proliferation and activation. NA irritation resulted in increased IL-1α production, which is a potent inducer of IL-8 (Matsushima and Oppenheim, 1989), but IL-8 production was not detected. The action of any one cytokine is therefore clearly dependent upon interactions with other factors. The whole epicutaneous environment should be taken into consideration before conclusions can be made concerning the influence of any particular factor. I feel that my findings agree with the theory put forward by Willis and colleagues (Willis et al, 1989) that a given pattern of cytokine production is specific for certain chemicals and/or irritants and may affect the way in which KC and LC contribute to inflammation. I would also suggest that NA and SLS appear to differentially affect cytokine production and induction of apoptosis. Further *in situ* hybridisation studies continuing on from the work presented in this thesis aim to define more clearly the distinct cytokine profiles induced by NA and SLS irritation. I propose that in depth investigation of the cytokine profiles initiated upon irritation and the changes undergone as the response progresses is the key to understanding chronic ICD.

NA irritation dramatically influenced the more delicate LC population. The numbers of epidermal LC decreased substantially upon irritation. This reduction was due to extensive LC apoptosis and not as a result of migration. Thus in comparison with SLS, NA has a profoundly different effect on the LC population. NA destroys the epidermal LC population, and therefore decreases the capability for Ag specific immune responses to occur. Thus in the case of NA irritation autoimmunity is unlikely to play a major role.

The data obtained from the LC study have revealed important differences in the LC population between normal volunteers and patients with chronic ICD. Patients have increased basal levels of LC, with fewer, shorter dendrites. These cells appear to be partially rounded up as if they are in a perpetually activated state ready to migrate from the epidermis immediately upon irritation. The KC in normal skin of these patients appear to be constitutively producing higher levels of IL-1α. I conclude that

patients with chronic ICD have irritant 'sensitive' skin. This may result from the increased IL-1α production observed coupled with the fact that the LC population is activated and can therefore respond more rapidly and possibly to lower irritant concentrations than normal volunteers. In vitro functional studies require to be carried out on these two populations. Results from this type of investigation may give some insight into why one person should be more susceptible to irritation than another.

4.11. IS THIS A GOOD MODEL FOR CHRONIC ICD?

The observations made in this study indicate that irritants are likely to produce a spectrum of reactions and therefore what is true for one may not be the case for another. This makes understanding of chronic ICD and potential therapeutic interventions complicated.

The model of experimental irritation used here provides useful information concerning the irritant response at certain time-points. For instance, investigation of early time-points can provide details of the induction process. With this model it is also possible to study resolving irritant responses as the experimentally induced erythema disappears with time. This is as yet an unexplored area which may reveal anomalies, with chronic ICD reflecting poor resolution of the response. I speculate that altered cytokine profiles are pivotal with a lack of inhibitory factors such as IL-10 and increased pro-inflammatory cytokines leading to the chronicity of inflammation observed in some patients.

Of course this model only investigates a single exposure to one irritant and a repeat exposure model may be closer to the situation in the real disease. Other factors must also be taken into consideration such as environmental factors and the influence of other irritants. It has also been suggested that the size of Finn chamber used can dramatically influence any resultant irritation (Mikulowska and Andersson, 1996). It

was shown that 48 hour application of 1% SLS in 8mm and 12 mm Finn chambers differentially affected the LC population. Application using the 8mm chambers could result in increased, unchanged or decreased LC numbers; whereas with the 12 mm chambers a decrease in the LC population occurred. These data indicate that this model of experimental irritation must be used with caution. Comparisons are only of value if made between groups who have used the same irritant concentration and equivalent Finn chamber size. They also suggest that larger areas of skin may be required for adequate penetration of an irritant substance. Pilot tests should be carried out to determine if a particular experimental condition gives consistent results over time. In conclusion, I feel that the complex, multifactorial nature of chronic ICD requires creation of an animal model, using defined conditions, allowing for a more detailed study.

4.12. CONCLUSIONS

The data presented here indicate that the pathological processes induced by NA and SLS are distinct despite equivalent clinical responses.

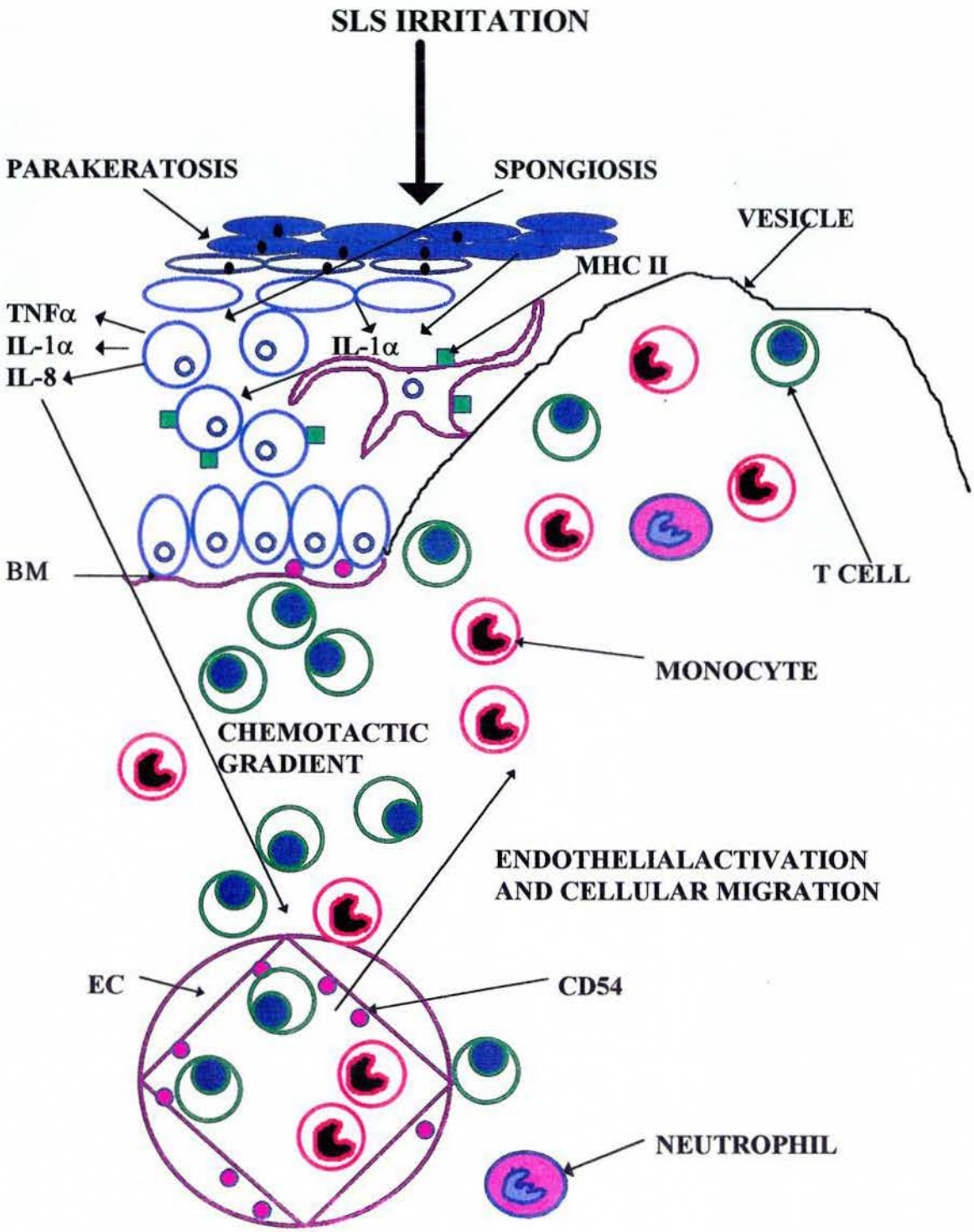
SLS (Figure 4.2)

SLS damaged the epidermis causing epidermal thickening, parakeratosis and severe spongiosis which often led to vesiculation. Increased basal layer proliferation was also induced, a factor contributing to the hyperplasia and parakeratosis observed.

SLS irritation resulted in epidermal KC activation, probably through induction of IL-1, IL-8 and probably TNFα. KC expression of MHC II and CD54 was evident, enabling infiltrating T lymphocytes to interact with KC, thus leading to further cellular activation and cytokine production. ICAM-1 expression correlated with the epidermal T cell influx, which comprised CD4+ve and CD8+ve T cells. The presence of CD8+ve cytotoxic T lymphocytes may account for some of the epidermal damage observed. ICAM-1/LFA-1 binding is probably most important for trapping

FIGURE 4.2:

Proposed model of events which occur after SLS application.



of T cells in the epidermis, while chemotactic factors such as IL-1 and IL-8 promote T cell recruitment. Heavy dermal infiltration was also observed, reflecting epidermal events.

IL-8 production was detected, accounting in part for the increased epidermal and dermal cellular infiltration and increased KC proliferation. I propose that IL-1α (not measured) will also be induced, acting in synergy with IL-8 to produce endothelial activation and increased T cell recruitment.

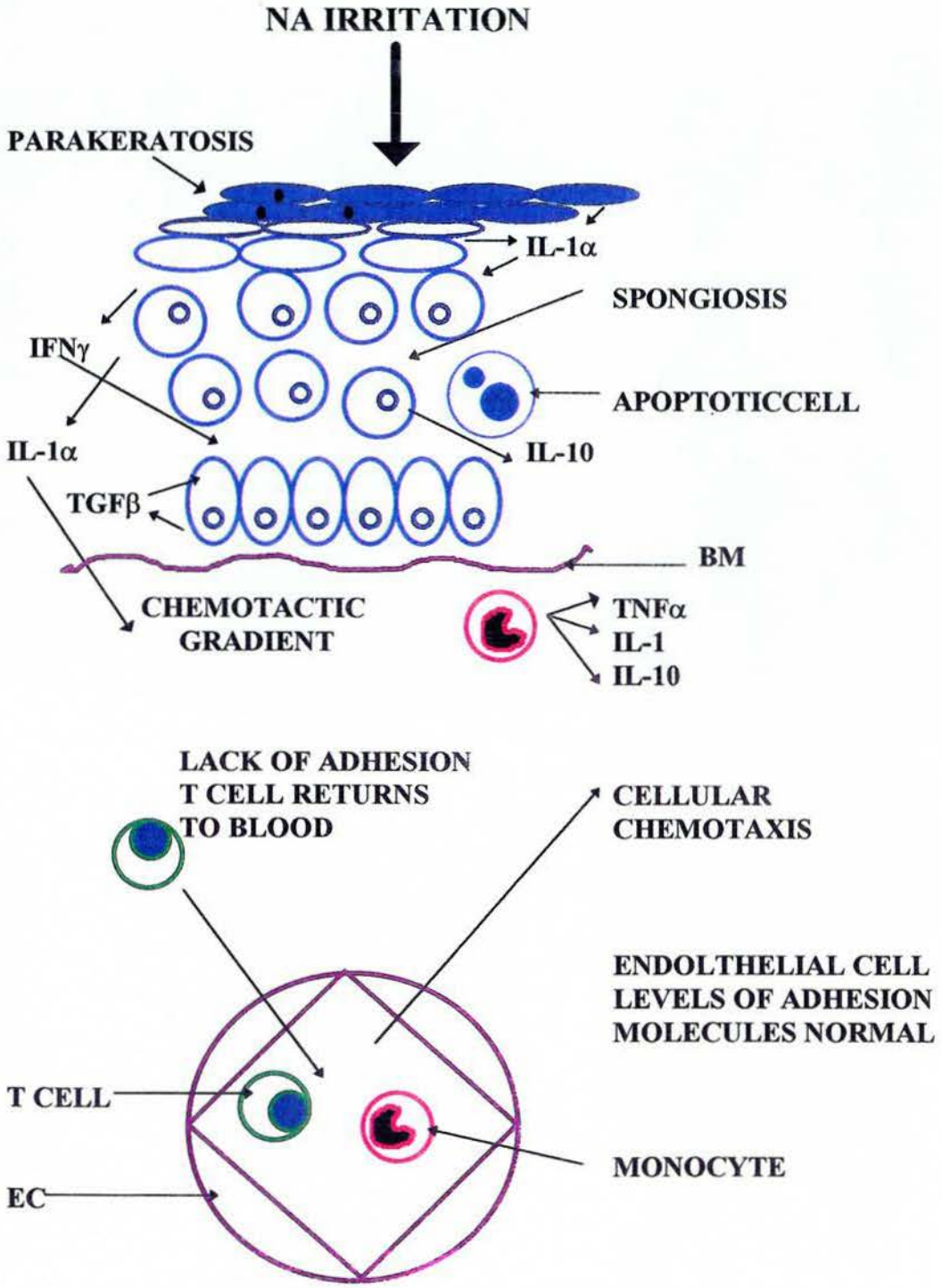
The LC population was slightly reduced by 48 hours after irritation. The reduction in number is thought to be due to migration to the draining lymph nodes under the control of cytokines produced by activated KC, such as GM-CSF and IL-1. The potential for Ag specific mediated responses remains intact after SLS irritation, unlike NA. This may affect chronicity by continued T cell activation and LC-mediated autoimmunity towards a neo-Ag.

NA (Figure 4.3)

NA irritation had a minimal effect on epidermal architecture, resulting in mild spongiosis and a degree of parakeratosis. The oedema observed was probably induced as a result of IL-1α release from the granular layer. However, the epidermal cellular population was affected by irritation, with basal KC proliferation decreasing at 24 hours. This was a transient phase with proliferation levels returning to normal by 48 hours. Decreased proliferation may be mediated by increased levels of TGF-β and IFNγ (neither measured) and/or decreased levels of KC growth factors such as IL-8 and IL-6. IL-8 induction was not observed after NA application, therefore decreased levels of this cytokine may be an important factor.

NA failed to induce KC activation or T cell infiltration, although epidermal macrophage infiltration was evident. Nevertheless, IL-1α was detectable, and should therefore have induced KC activation and T cell exocytosis from the blood into the dermis and epidermis. Lack of activation may result from increased levels of

FIGURE 4.3:
Proposed model of events which occur after NA irritation.



regulatory molecules such as IL-1RA or IL-1R2 and/or decreased IL-1R1 expression. Lack of KC activation, and therefore MHC II and ICAM-1 expression, mean that any T cells recruited through IL-1 chemotaxis had no method of binding to KC in order to remain in the epidermis to become activated. The existence of epidermal macrophages suggests that T cell recruitment did occur and that it was failure of epidermal trapping that resulted in the lack of epidermal T cell infiltration observed.

NA had a dramatic influence on the epidermal LC population by causing a redistribution and eventual decrease in LC by apoptosis. Although apoptosis induction may be a direct effect of irritation its more likely to be cytokine mediated.

The lack of epidermal LCs means that the ability to initiate Ag-specific T cell mediated responses is greatly diminished. In ICD this could be beneficial as it would reduce the possibility of autoimmunity a feature which could be utilised therapeutically. But in normal individuals it may be detrimental by dampening down an important aspect of the skin's protective function.

4.13. FUTURE STUDIES

I would extend the projects investigation of the presence of auto-Abs in patients with chronic ICD. An increase in the numbers of patients in study would be required to confirm the presence of an auto-Ag. Purification of the auto-Ag from the gel would allow characterisation of the physical and chemical properties of this molecule.

An animal model is required for in depth studies into the cellular and molecular mechanisms involved in the onset and maintenance of chronic ICD. It is possible that this purified auto-Ag could be used in the creation of an animal model for chronic ICD. Creation of an animal model, through repeat irritant exposure, would be of great value in this field.

The purified Ag could be injected into a rabbit in order to produce specific polyclonal Abs. These specific Abs could then be used in immunohistochemical and Western blotting studies to determine the nature of the Ag. For instance does it bind LC or KC on tissue sections or LC lysates on Western blots ?

In vitro studies in conjunction with *in vivo* investigations in an animal model could provide valuable information not easily obtainable from human volunteers and patients with chronic ICD. Functional studies should be carried out on LCs isolated from clinically normal skin of patients with chronic ICD. These LC populations could then be compared with normal human LCs in order to ascertain any fundamental differences in these populations. Investigation into the functional significance of CD95/fas expression on KC and LC could also be important. Is there any difference in CD95 function between cells derived from patients with chronic ICD and normal healthy volunteers? How do these different cell types respond to irritant exposure? What is the influence of cytokines, e.g. IL-10, TGFβ, on the function of CD95/fas and other adhesion/accessory molecules ?

It is of course important to carry out basic functional studies using both the *in vitro* and animal model systems. Studies could include investigation of KC and LC responses to various irritant stimuli, cytokine production and expression of activation markers. Examination of the influence of various cytokines, growth factors and anti-cytokine Abs could provide information about the control of irritant reactions.

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APPENDIX I

TABLE 1 : Patient details.

Patient Code	Gender	Age in Years	Atopic Status A-atopic NA-non-atopic	Active Disease
1	M	49	NA	y
2	M	45	NA	y
3	F	20	NA	n
4	F	27	A	y
5	M	21	A	y
6	F	29	A	y
7	F	21	A	n
8	F	32	A	n
9	F	26	NA	n
10	M	62	NA	y
11	M	24	A	n
12	M	18	A	y
13	M	58	NA	y
14	F	47	NA	n
15	M	39	NA	n
16	F	44	A	n
17	M	30	NA	y
18	F	47	NA	n
19	F	40	NA	y
20	M	40	A	n
21	F	29	A	n
22	M	54	A	n
23	F	36	NA	n
24	M	57	NA	y
25	M	43	NA	n
26	F	48	NA	n
27	M	22	NA	y
28	F	56	NA	y
29	F	18	NA	y
30	M	66	NA	y
31	M	59	NA	n
32	M	46	NA	y
33	F	40	NA	y
34	F	21	A	y
35	F	20	NA	n
36	M	24	NA	y

TABLE 1 : Patient details contd.

Patient Code	Gender	Age in Years	Atopic Status A-atopic NA-non-atopic	Active Disease
37	M	48	A	y
38	F	24	A	y
39	F	28	NA	y
40	M	52	NA	y
41	F	32	NA	n
42	M	24	A	y
43	F	43	NA	n
44	F	22	NA	n
45	M	60	NA	y
46	F	24	A	y
47	M	47	NA	n
48	F	34	NA	y
49	M	59	NA	y
50	M	32	A	y
51	F	58	NA	y
52	M	52	NA	y
53	M	31	A	y
54	F	55	A	y
55	M	35	NA	y
56	M	29	NA	n
57	F	44	A	y
58	F	58	A	y
59	F	40	A	y
60	F	46	NA	y
61	M	55	NA	y
62	M	19	A	y
63	M	28	A	y
64	M	22	A	y
65	M	61	NA	y
66	M	51	NA	y

APPENDIX II

1). Solutions required for immunohistochemistry.

All chemicals purchased from Sigma unless otherwise stated.

Fast Red

Napthol AS-MX Phosphate	2mg
N,N-dimethylformamide	0.2ml
0.1M Tris Buffer pH8.2	9.8ml
1.0M levamisole	10µl
Fast red TR	10mg

Filtered before use

Details of antibodies used on paraffin tissue sections

ANTIBODY	SOURCE	CODE	ISOTYPE
Mast cell tryptase	DAKO Ltd	M7052	IgG1
MIB-1	Immunotech	0505	IgG1
CD68	DAKO Ltd.	M0876	IgG3
CD15	DAKP Ltd.	M0733	IgG1
CD1a	Immunotech	1590	IgG1
Bcl-2	DAKO Ltd.	M0887	IgG1
Bax	Satacruz Biotechnology, Inc	SC-930	polyclonal
Bcl-x	Satacruz Biotechnology, Inc	SC-1690	polyclonal
CD95	Satacruz Biotechnology, Inc	SC-715	polyclonal

Details of antibodies used on frozen tissue sections

ANTIBODY	SOURCE	CODE	ISOTYPE
CD3	DAKO Ltd.	M0835	IgG1
CD4	DAKO Ltd.	M0716	IgG1
CD8	DAKO Ltd.	M0707	IgG1
CD54	Genzyme	2136-01	IgG1
IFNγ	Chemicon	22-19	IgG2a
L243	Keith Guy	-	-
CD1a	DAKO Ltd.	M0721	IgG2a
CD62 E	R & D Systems	BB16-E6	IgG1

2). Solutions required for gel electrophoresis and Western Blotting.

All chemicals purchased from Sigma unless otherwise stated.

10% Separating Gel (10ml)

Distilled Water	4.05ml
1.5M Tris-HCL pH8.8	2.50ml
10% W/V SDS	100µl
Acrylamide/Bis 30% (Flowgen)	3.30ml
10% ammonium persulphate	50 µl
TEMED	5 µl

4% Stacking Gel (10ml)

Distilled Water	6.1ml
0.5M Tris-HCL pH66.8	2.5ml
10% W/V SDS	100µl
Acrylamide/Bis 30% (Flowgen)	1.30ml
10% ammonium persulphate	50 µl
TEMED	10 µl

Electrode/Running Buffer (x5)

Tris	3.03g
Glycine	14.4g
Methanol	200ml
Distilled Water	300ml

Transfer Buffer

Tris	7.5g
Glycine	14.4g
SDS	2.5g
Distilled Water	500ml

Sample Buffer

50mM Tris-HCL (pH 6.8)
100mM dithiothreitol
2% Sodium Lauryl sulfate
0.1% bromophenol blue
10% glycerol

Alkaline phosphatase buffer (pH9.0)

Tris	6.05g
NaCl	2.92g
4M MgCl	1.25ml
Distilled Water	500ml

Alkaline phosphatase buffer (pH9.0)

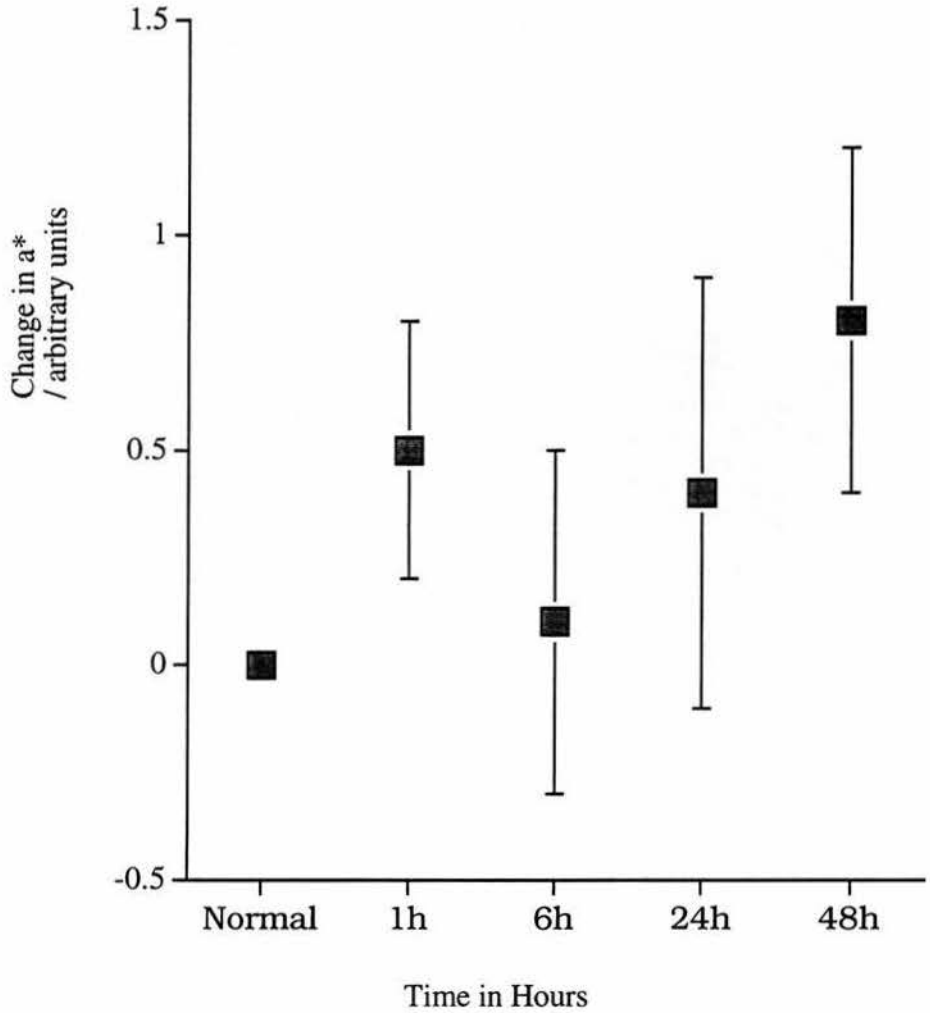
Tris	6.05g
NaCl	2.92g
4M MgCl	1.25ml
Distilled Water	500ml

NBT/BCIP Substrate solution

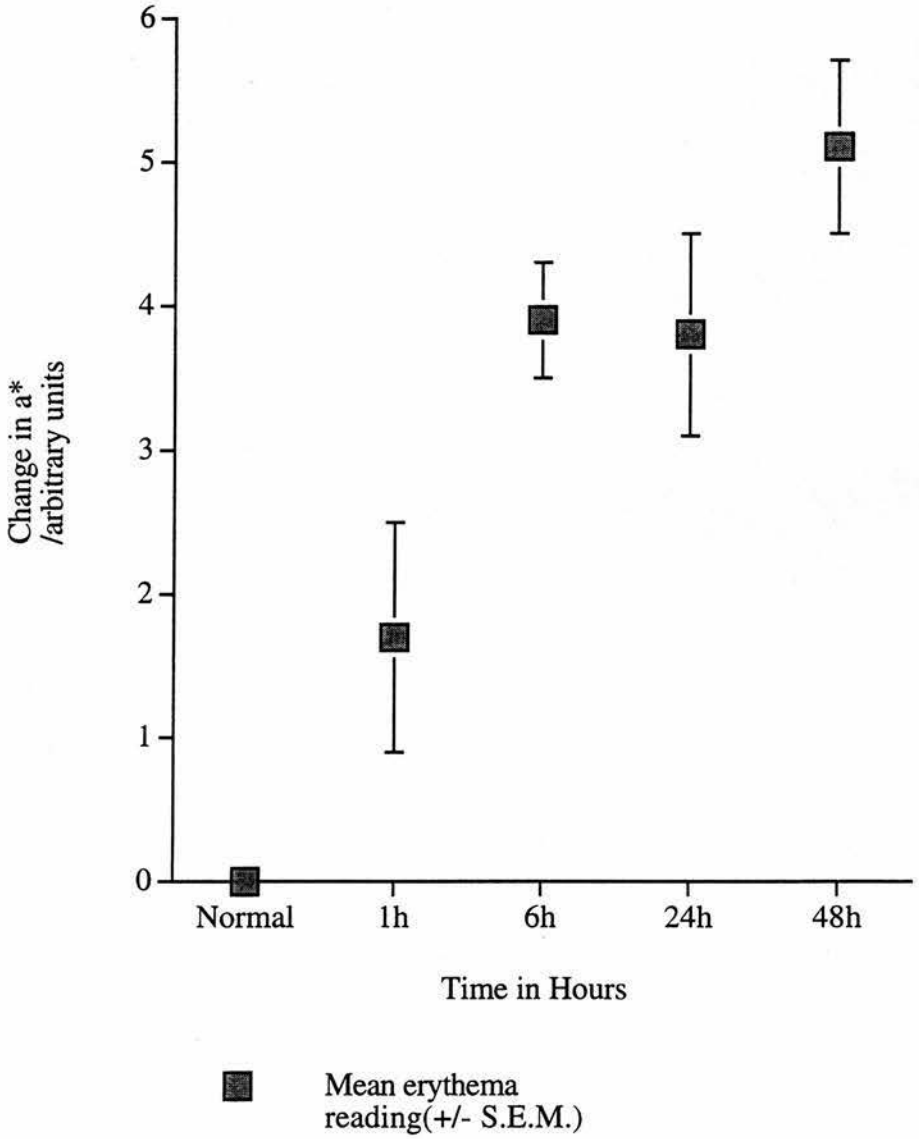
NBT	66µl
BCIP	33 µl
Alkaline phosphate buffer	10 ml

NBT - stock 75mg/ml in 70% dimethyl formamide
BCIP - stock 50mg/ml in dimethyl formamide

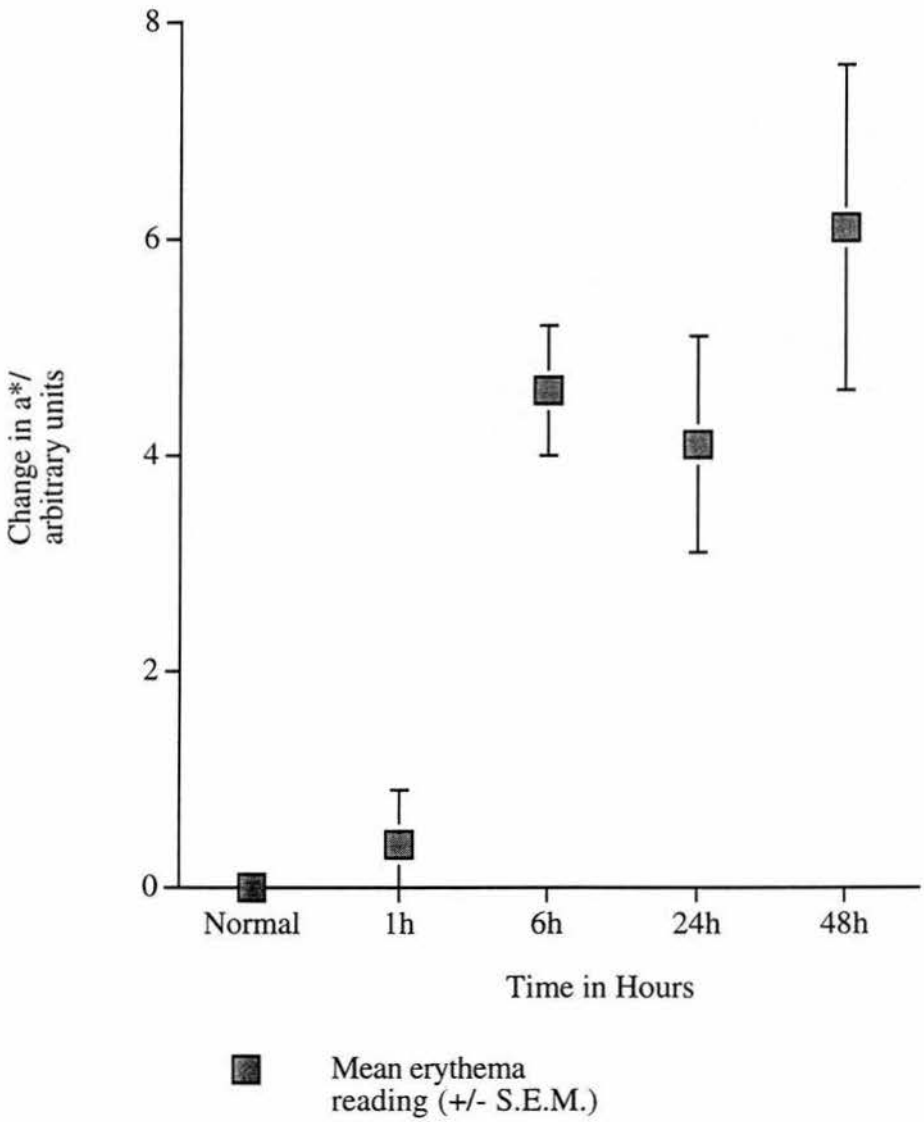
Graph shows mean erythema readings at 0, 1, 6, 24 and 48 hours after DL application. (n=9)



Graph shows mean erythema readings at 0, 1, 6, 24 and 48 hours after NA application (n=14)



Graph shows mean erythemareadings at 0, 1, 6, 24 and 48 hours after SLS application (n=15).



APPENDIX IV

1. H & E DATA

Intracellular Oedema

1. Friedman Test

DL (basal layer IO) comparison of control, 1 and 6hr biopsies $p<0.05$

Spongiosis

1. Friedman Test

SLS (prickle layer SP) comparison of control, 1 and 6hr biopsies $p<0.01$

(prickle layer SP) comparison of control, 24 and 48hr biopsies $p<0.01$

2. McNemar Test

SLS con vs 48 hrs $p<0.02$

Vesiculation

SLS 24hrs 2/8 (basal) and 2/8 (prickle)

48hrs 3/8 (basal and prickle)

NOT SIGNIFICANT probably due to small sample size

Parakeratosis

SLS 24hrs 3/7 positive biopsies

48hrs 4/7 positive biopsies

NOT SIGNIFICANT probably due to small sample size

2. Eosinophil Recruitment

1) SLS

EPIDERMAL

24 hrs 2/8 biopsies

48 hrs 1/8 biopsies

PAPILLARY DERMIS

24 hrs 2/8 biopsies

PERIVASCULAR

6 hrs 6/16 biopsies

24 hrs 3/8 biopsies

48 hrs 3/8 biopsies

RETICULAR DERMIS

6 hrs 3/16 biopsies

24 hrs 2/8 biopsies

48 hrs 3/8 biopsies

2). NA

EPIDERMAL

24 hrs 1/7 biopsies

PAPILLARY DERMIS

1 hr 2/17 biopsies

6 hrs 3/17 biopsies

PERIVASCULAR

1 hr 3/17 biopsies

6 hrs 4/17 biopsies

48 hrs 1/7 biopsies

RETICULAR

6 hrs 4/17 biopsies

3. Average Epidermal Thickness (μm)

a) NA

STATISTICS	CONTROL	1 Hour	6 Hours
	65.5	128.3	77.7
	84	109	91
	69	75	89.5
	87	63	70
	50	50	87.3
	70.3	71.6	78.7
	80.3	94.5	96
	60.6	62.8	64.5
	67.3	64.3	66.5
	50	62	60
	83.5	82.3	85.3
	74.3	73.4	60
	43.8	62.3	71.3
	102	81	95
	92.7	83.8	111.3
	54.7	70	60.8
N	16	16	16
MEAN	70.9	77.1	79.1
MEDIAN	69.7	72.5	78.2
SE	4.2	5.0	3.8

b) SLS

STATISTICS	CONTROL	24 Hours	48 Hours
	57	78.5	171
	65.7	52	76.7
	57	58.7	106.5
	54	56.7	73.7
	42	196	82
	67.7	84.3	108
	52	62.3	89.5
N	7	7	7
MEAN	56.5	84.1	101.1
MEDIAN	57.0	62.3	89.5
SE	3.3	19.2	12.7

Average Epidermal Thickness contd.

1. Friedman Test

NA	comparison of control, 1 and 6 hour biopsies	$p<0.05$
SLS	comparison of control, 24 and 48 hour biopsies	$p<0.01$

2. McNemar Test

SLS	control vs 48 hours	$p<0.02$

3. Wilcoxon-Signed Rank SumTest

NA	control vs 6 hours	$p<0.04$
SLS	control vs 48 hours	$p<0.02$

4. Mann Whitney-U Test

No difference between NA and SLS at any timepoint

4. APOPTOSIS

Basal Layer Apoptosis

NA

No. apoptotic cells/mm basement membrane.

STATISTICS	CONTROL	24 Hours	48 Hours
	0	0.2	1.4
	0	1.9	5.2
	0.3	0.5	0.2
	0	0.2	0.4
	0	6.4	0
	0	1.0	0.4
	0	2.8	1.6
N	7	7	7
MEAN	0.04	1.9	1.3
MEDIAN	0	1.0	0.4
SE	0.04	0.8	0.7

1. Friedman Test

Na comparison of control, 24 and 48hr biopsies $p<0.1$ (not significant)

2. McNemar Test

NA control vs 24hrs $p<0.03$

3. Wilcoxon-signed ranks sum test

NA control vs 24 hrs $p<0.02$

4. Mann Whitney-U Test

NA vs SLS

6hrs $p<0.002$

24hrs $p<0.04$

Prickle Layer Apoptosis

NA

No. apoptotic cells/mm basement membrane

STATISTICS	CONTROL	1 Hour	6 Hours
	0	0	0
	0	0	0
	0	0	0
	0	0	0.2
	0	0	0
	0	0	0.33
	0	0	0
	0	0.23	0
	0	0.92	0.2
	0	0	0
	0.24	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
N	15	15	15
MEAN	0.02	0.08	0.05
MEDIAN	0	0	0
SE	0.02	0.06	0.03

1. Friedman Test

NA comparison of control, 1 and 6hr biopsies not significant

5. Epidermal proliferation

No. proliferating cells/mm basement membrane, after application with a) NA or b)SLS.

a) Results after NA application, No. Ki-67+ve cells/mm basement membrane

STATISTICS	CONTROL	24 Hours	48 Hours
	17.8	17.1	44.5
	128.8	10.7	78
	38.6	13.5	38.6
	31.9	6.9	82.7
	29	25.1	39.8
	13.3	13.3	47.3
N	6	6	6
MEAN	43.2	14.4	55.2
MEDIAN	30.5	13.4	45.9
SE	17.5	2.5	8.1

b) Results after SLS application, No. Ki-67+ve cells/mm basement membrane

STATISTICS	CONTROL	24 Hours	48 Hours
	24.9	8.7	49.3
	29	32.1	30.2
	45	46.7	212.1
	12.8	16.4	117.1
	27.4	37.6	39.3
	38.2	25.2	63
N	6	6	6
MEAN	29.6	27.8	85.2
MEDIAN	28.2	28.6	56.2
SE	4.5	5.7	28.3

1. Friedman Test

NA - Comparison of control, 24 and 48 hours showed significant change $p<0.05$.

SLS - Comparison of control, 24 and 48 hours showed significant change $p<0.05$.

2. McNemar Test

SLS - Control vs 48 hours $p<0.03$

NA - control vs 24 hrs $p<0.04$

24 hrs vs 48 hrs $p<0.03$

3. Wicoxon-Signed Rank Sum Test

SLS control vs 48 hours $p<0.04$

6. CD3/CD4/CD8 - Epidermal Results

No CD3+ve cells/mm basement membrane after SLS application.

STATISTICS	CONTROL	24 Hours	48 Hours
	9	50.5	37.2
	0	5.7	12.9
	0	17.5	11.7
	0	0	8.1
	0	14.4	13.9
	0	14.1	17.3
	0	15	3.2
N	7	7	7
MEAN	1.3	16.7	14.9
MEDIAN	0	14.4	12.9
SE	1.3	6.1	4.1

1. Friedman Test

SLS - Comparison of control, 24 and 48 hr biopsies $p<0.01$

2. McNemar Test

SLS control vs 24 hrs $p<0.03$

control vs 48 hrs $p<0.02$

CD4/CD8 Ratio

24hrs 3:1

48hrs 2:1

3. Wilcoxon-Signed Rank Sum Test

SLS CD3 control vs 24 hours $p<0.04$

control vs 48 hours $p<0.02$

7. CD68 - Epidermal Results

a) Results after NA application, No. CD68+ve cells/mm basement membrane

STATISTICS	CONTROL	24 Hours	48 Hours
	0	0	1.1
	0	13	6.3
	0	1.7	1.5
	0	1.9	1.2
	0	0.1	7.1
	0	18.8	10.6
	0	3.7	2.3
N	7	7	7
MEAN	0	5.6	4.3
MEDIAN	0	1.9	2.3
SE	0	2.7	1.4

b) Results after SLS application, No.CD68 +ve cells/mm basement membrane

STATISTICS	CONTROL	24 Hours	48 Hours
	0	0	0
	0	2.9	2.1
	0	2	8.2
	0.4	0	12.4
	0	6.8	26.4
	0.2	1.5	13.4
	0	16.7	34.3
	0	0	0
N	8	8	8
MEAN	0.1	3.7	12.1
MEDIAN	0	1.8	10.3
SE	0.1	2.0	4.5

1. Friedman Test

NA comparison of control, 24 and 48 hr biopsies $p<0.05$

SLS comparision of control, 24 and 48 hr biopsies $p<0.05$

2. McNemar Test

NA control vs 24 hrs $p<0.03$

control vs 48 hrs $p<0.02$

SLS control vs 48 hrs $p<0.03$

3. Wilcoxon-Signed Ranks Test

NA control vs 24 hrs $p<0.04$

control vs 48 hrs $p<0.02$

SLS control vs 48 hrs $p<0.04$

8. CD54 - Epidermal Results

SLS controls - no focal expression

1 hr - no focal expression

6 hrs - 5/14 biopsies showed focal expression

24 hrs - 4/6 biopsies showed focal expression

48 hrs -4/5 biopsies showed focal expression

(not significant by McNemar test, due to small numbers)

Intense focal CD54 expression in the epidermis correlated (100%) with sites of CD3+ve lymphocyte exocytosis, although CD3+ve cells were found in areas lacking CD54 expression. Only 15% (5/34) biopsies without CD54 expression showed CD3+ve lymphocyte excocytosis.

9. Langerhans Cells - Epidermal Results

a) Results after NA application, No.CD1a +ve cells/mm basement membrane

STATISTICS	CONTROL	24 Hours	48 Hours
	21.3	9	ND
	10.6	2.9	0.8
	9.2	5.8	0.5
	19	5.6	5.4
	13.5	9	3.5
	11.7	10.2	5
N	6	6	5
MEAN	14.2	7.1	3.0
MEDIAN	12.5	7.4	3.5
SE	2.0	1.1	1.0

b) Results after SLS application, No.CD1a +ve cells/mm basement membrane

STATISTICS	CONTROL	1 Hour	6 Hours
	6.6	9.4	5.3
	5.6	11	9.1
	15.7	8.9	7.6
	7.8	8.6	4.11
	8.6	8.1	8
	7.6	8.6	7.2
	16.3	25.6	18.2
	13	14.9	18.8
	12.8	14.3	16.7
	12.9	12.9	7.9
	11.6	8	9.1
	15.4	5	2.5
N	12	12	12
MEAN	11.2	11.3	9.5
MEDIAN	12.2	9.2	8.0
SE	1.1	1.5	1.6

b) Results after SLS application, No.CD1a +ve cells/mm basement membrane

STATISTICS	CONTROL	24 Hours	48 Hours
	8.6	10	5.5
	24.6	18.6	19.2
	17.6	11.5	10.7
	20.2	11.1	5.7
	10.5	9.6	8.9
	30	31	11.2
N	6	6	6
MEAN	18.6	15.3	10.22
MEDIAN	19.0	11.3	9.8
SE	3.3	3.4	2.1

1. Friedman Test

NA comparison of control, 24 and 48hr biopsies $p<0.01$

SLS comparison of control, 1 nad 6hr biopsies $p<0.05$

comparision of contro, 24 and 48hr biopsies $p<0.05$

2. McNemar Test

NA control vs 24hrs $p<0.03$

SLS control vs 48hrs $p<0.03$

3. Mann Whitney-U Test

Comparision of NA and SLS

24hrs $p<0.01$

48hrs $p<0.008$

4. Wilcoxon-Signed Ranks Test

NA control vs 24 hrs $p<0.04$

SLS control vs 48 hrs $p<0.04$

Basal Layer CD1a Apoptosis

NA 1 hr 2/15 biopsies

6 hrs 1/15 biopsies

24 hrs 3/7 biopsies

48 hrs 1/7 biopsies

SLS 48 hrs 1/7 biopsies

Prickle Layer CD1a Apoptosis

NA 1 hr 1/15 biopsies

6 hrs 1/15 biopsies

24 hrs 4/7 biopsies

48 hrs 1/7 biopsies

Table 1 : Shows the percentage (and number of positive biopsies) of mean number of CD1a+ve apoptotic cells in the total apoptotic cell population after application of NA for 1, 6, 24 and 48 hours.

TIMEPOINT	BASAL LAYER % (N)	PRICKLE LAYER % (N)
1 Hour	31 (2)	100 (1)
6 Hours	14 (3)	100 (3)
24 Hours	50 (4)	86 (4)
48 Hours	50 (2)	85 (2)

Table 2 : Shows the percentage (and number of biopsies) of mean number and apoptotic CD1a+ve cells in the total CD1a population.

TIMEPOINT	BASAL LAYER % (N)	PRICKLE LAYER % (N)
1 Hour	5 (2)	2 (2)
6 Hours	2 (1)	0 (1)
24 Hours	7 (4)	60 (4)
48 Hours	14 (1)	57 (1)

10. CD15 - Dermal Results

a) NA - number of CD15+ve cells/mm^2 dermis

STATISTICS	CONTROL	1 Hour	6 Hours
	0	0	17.3
	0	5.1	48.8
	0	1.4	14.1
	0	2.1	22.8
	0	0	10.3
	0	0	33.4
	0	0	10.7
	0	0	12.5
	0	0	36.3
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
N	17	17	17
MEAN	0	0.5	12.1
MEDIAN	0	0	10.3
SE	0	0.3	3.7

1. Friedman Test

NA comparison of control, 1 and 6 hour biopsies $p<0.05$

2. McNemar Test

NA control vs 6hrs $p<0.04$

3. Wilcoxon-Signed Ranks Test

NA control vs 6 hrs $p<0.009$

4. Mann-Whitney U Test

NA vs SLS 6 hrs $p<0.04$

11. MAST CELLS

No epidermal mast cell infiltrate was observed afte NA or SLS irritation.

Dermal mast cells were observed in all biopsies but no significant difference was foun between the different timepoints or irritants.

12. Western blottting raw data

1). Dermal Extracts

	DISTANCE TRAVELLED			
	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4
Dye front	56 mm	54 mm	50 mm	53 mm
M. Wt. Marker				
148 kDa	8.5	5	2	3
60 kDA	23	18	15	16.5
42 kDA	32	26.5	22.5	25
30 kDA	42.5	37	32.5	34
22 kDA	47	42	38.5	40
17 kDA	54	49	45	47
BAND	21	18	15.5	17.5

Relative mobility of molecular weights and specific bands.

	RELATIVE MOBILITY			
	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4
148 kDa	0.15	0.09	0.04	0.06
60 kDA	0.41	0.33	0.30	0.31
42 kDA	0.57	0.49	0.45	0.47
30 kDA	0.76	0.69	0.65	0.64
22 kDA	0.84	0.78	0.77	0.75
17 kDA	0.96	0.91	0.90	0.89
Band	0.38	0.33	0.31	0.33
M. Wt (band)	77 kDa	77 kDa	78 kDa	71 kDA

2) Epidermal Extracts

	DISTANCE TRAVELLED	
	PATIENT 1	PATIENT 4
Dye front	57	54
M. Wt. Marker		
148 kDa	4	3
60 kDA	17	16
42 kDA	25	23
30 kDA	35	33
22 kDA	40	39
17 kDA	49	4715
BAND	15	15

Relative mobility of molecular weights and specific bands.

	RELATIVE MOBILITY	
	PATIENT 1	PATIENT 4
148 kDa	0.07	0.06
60 kDA	0.3	0.3
42 kDA	0.48	0.43
30 kDA	0.61	0.61
22 kDA	0.7	0.72
17 kDA	0.86	0.87
Band	0.26	0.28
M. Wt (band)	73 kDA	75 kDA