CELLULAR EVENTS IN

CHRONIC IRRITANT DERMATITIS AND

EXPERIMENTAL IRRITANT DERMATITIS

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DECLARATION

I hereby declare that the work presented in this thesis is my own except where clearly stated.

The work formed part of a larger project investigating the cellular and molecular mechanisms of irritant contact dermatitis funded by the Health and Safety Executive.

All of the experiments which involved patients and volunteers had received prior ethical approval from the Lothian Ethics Research Committee.

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PUBLICATIONS

Forsey RJ, Shahidullah H, Sands C, et al. Epidermal Langerhans cell apoptosis induced *in vivo* by nonanoic acid but not by sodium lauryl sulphate. *Br J Dermatol* 1998; **139**: 453-461.

See insert at end of appendices.

ABBREVIATIONS

ACD	- allergic contact dermatitis
AD	- atopic dermatitis
APC	- antigen presenting cell
CIE	- Commission Internationale de l'Eclairage
cDNA	- copy DNA
CLSM	- confocal laser scanning microscopy
DL	- dithranol
DNCB	- dinitrochlorobenzene
EGF	- epidermal growth factor
ELAM-1	- endothelial adhesion molecule-1
ELISA	- enzyme-linked immunosorbent assay
EM	- electron microscopy
FITC	- fluorescein isothiocyanate
GM-CSF	- granulocyte colony stimulating factor
ICAM-1	- intercellular adhesion molecule 1
ICD	- irritant contact dermatitis
ICDRG	- International Contact Dermatitis Research Group
IFN-γ	- interferon gamma
IL	- interleukin
IL-1R(A)	- interleukin-1 receptor (antagonist)
KC	- keratinocyte
LC	- Langerhans cell
LDF	- laser doppler flowmetry
LFA-1	- lymphocyte associated antigen-1
LMR	- leucocyte mobilisation rate
M-CAF	- monocyte chemoattractant and activating factor
MHC	- major histocompatibility complex
MIP-1α	- macrophage inhibitory protein-1 alpha
NA	- Nonanoic acid
PAF	- platelet aggregating factor
PBS/BSA	- phosphate buffered saline/ bovine serum albumin
PGE$_2$	- prostaglandin E$_2$
PMN	- polymorphonuclear
PSGL1	- p-Selectin glycoprotein ligand-1
SLS	- sodium lauryl sulphate
TEWL	- trans epidermal water loss
TGF	- transforming growth factor
TH$_1$/TH$_2$	- T Helper type 1/ type 2 cell
TNF-α/β	- tumour necrosis factor alpha/beta
TUNEL	- TdT-mediated dUTP-biotin nick end-labelling
UVR	- ultraviolet radiation
VCAM-1	- vascular adhesion molecule-1

ABSTRACT

Irritant contact dermatitis (ICD) is a very common occupational skin disease but its pathogenesis remains poorly understood. The application of topical irritants was used to study cellular mechanisms of ICD, by sampling tissue from skin biopsies, suction blisters and skin window chambers.

The early (up to 6h) and late (up to 48h) histopathological changes were investigated in patients following the application of 80% nonanoic acid (NA) and 5% sodium lauryl sulphate (SLS). The irritants were titrated on volunteers to produce similar grades of mild erythema for the two irritants. SLS caused minimal early damage but induced parakeratosis and spongiosis at 24h. SLS also induced a prominent dermal neutrophilic infiltrate and epidermal infiltration. In contrast, NA induced basal cell layer apoptosis without significant inflammatory infiltration. These cells were later shown to be Langerhans cells (LC).

The different patterns of epidermal damage prompted the quantification of the cytokines IL-8 (a neutrophil chemoattractant) and IL-1α (a key pro-inflammatory cytokine) by ELISA in suction blister fluid in patients and volunteers after irritation with NA and SLS. It was shown that NA induced IL-1α upto 24h post irritation, but minimal IL-8. SLS, however, induced IL-8 at 6 and 24h. This result was in keeping with the histopathological findings above.

Another aspect of ICD studied was the investigation of LC after NA irritation by confocal microscopy, in both volunteers and patients with ICD. There was a marked reduction of LC in both groups, but greater in patients. The morphology of the LC was altered with a reduction in both the number and lengths of dendrites with time after irritation.

The final study looked at the effect of SLS irritation on the activation of neutrophils using skin window chambers. It was shown that this technique was suitable for collecting neutrophils from the skin but flow cytometry analysis of the neutrophil activation markers (cell surface antigens) showed no differences between control and irritated sites.

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CHAPTER 1. INTRODUCTION

1.1 Irritant contact dermatitis - definitions and concepts

Irritant contact dermatitis (ICD) has usually been defined as an inflammatory

reaction following non-immunologically mediated damage to the skin (Wilkinson,

1992). The exposure may be a solitary one such as a chemical, resulting in acute

inflammation. Some substances are only toxic under certain conditions e.g. under

occlusion or only when the stratum corneum has been breached. The duration and

nature of exposure required to elicit a transient inflammatory response varies

considerably between irritants. The clinical spectrum of acute irritant dermatitis

ranges from a mild irritant reaction with transient erythema or scaling to a more

severe picture with swelling, vesiculation and even necrosis.

The concept of chronic ICD was first proposed by Malten (1981) who

considered that it developed as a result of repeated chemical or physical insult to the

skin. Physical factors such as heat, cold and friction can contribute to its chronicity.

Once the skin barrier has been broken, milder irritants, scratching or rubbing may

perpetuate the dermatitis. Thus chronic ICD may result from a series of exposures

which individually are not sufficient to cause irritant dermatitis but when occurring

repeatedly, lead to chronicity (see below).
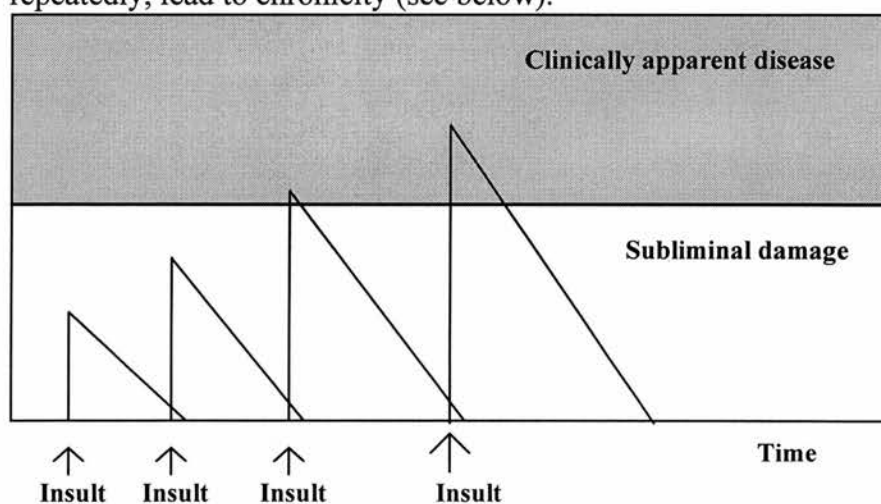
Fig1.1 Cumulative insult dermatitis adapted from Malten 1981

Diagnosis of chronic irritant contact dermatitis

The diagnosis of ICD is not straightforward, as irritant, constitutional and allergic factors may coexist. Many studies of irritant dermatitis do not mention a working definition which can lead to incorrect comparisons of data. Two notable exceptions are Malten (1981) and Rietschel (1990), both of whom listed criteria which should be fulfilled to establish a diagnosis of ICD.

Malten (1981)- all criteria have to be fulfilled

Clinical

1.   Eczematous inflammation which is relatively static

2.   Absence of vesicles and oozing

History

1.   No personal or family history of atopy

2.   No history of psoriasis

3.   Overexposure to external factors (occupational* or non-occupational)

     e.g. mechanical: gardening, wringing

       physico-chemical: shampoos

       chemical: paint removers, bleach

Investigations

1.   Patch testing reveals no relevant positive allergic reaction

* Occupations at risk: Bricklayers, Hospital nurses, Hairdressers,

                Industrial/Hospital cleaners

<u>Rietschel (1990)</u>

In this list it was stated that no arbitrary number of major or minor features need to be present for establishing the diagnosis. The greater the number of features present, the more likely the diagnosis of ICD.

Subjective

Major criteria

1. Onset of symptoms minutes to hours after exposure

2. Pain and stinging greater than itching

Minor criteria

1. Onset within two weeks of exposure

2. Other people in the environment similarly affected

Objective

Major

1. Erythema, hyperkeratosis or fissuring greater than vesiculation

2. Glazed or scalded appearance of skin

3. Healing occurs after withdrawal of exposure to substance

4. Patch test negative

Minor

1. Sharp delineation of dermatitis

2. Evidence of gravitational effect e.g. dripping effect

3. Lack of tendency to spread (on sequential examinations)

4. Vesicles related closely to patches of erythema

Wilkinson (1987) drew attention to important clinical patterns of hand dermatitis (see Fig 1.2 below) which suggest an irritant aetiology. Dermatitis affecting the dorsa and lateral palms were more commonly irritant in origin. The apron distribution is involvement of the palmar surface of the fingers extending onto the distal palm. The

Fig. 1.2 Patterns of irritant hand dermatitis
(Wilkinson, 1987)

R = ring pattern

P = pulps of fingers
(pulpitis)

APRON

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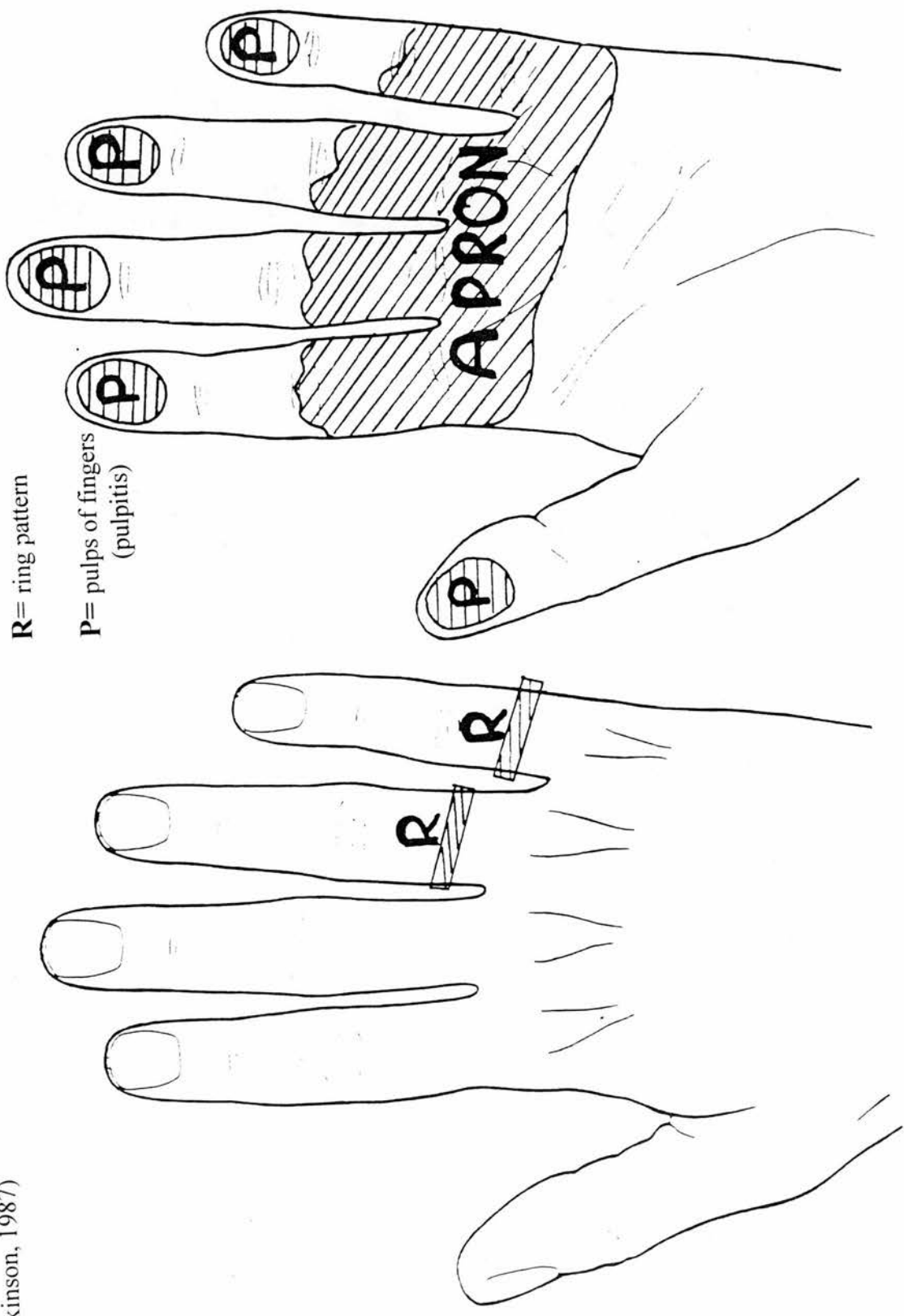
PLATE 1.1 Irritant hand dermatitis affecting dorsa of fingers

PLATE 1.2 Close up of same patient above showing web space involvement

PLATE 1.3 Irritant hand dermatitis affecting fingers and distal palm (apron pattern)

PLATE 1.4 Close up of same patient above showing palmar involvement

apron and pulp patterns were due to irritants in the majority, whereas a ring pattern or web space involvement was exclusively irritant in origin.

Plates 1.1 and 1.2 show dorsal finger and web space dermatitis. **Plates** 1.3 and 1.4 show the apron pattern.

In order to understand the magnitude of the clinical problem of ICD it is first necessary to look at the epidemiological data.

1.2 The epidemiology of irritant contact dermatitis

An occupational skin disease is defined as 'a pathological condition of the skin for which occupational exposure can be shown to be a major causal or contributory factor' (Lane, 1942). In the United Kingdom occupational skin disease statistics are recorded by the Health & Safety Commission, a body which is under the auspices of the Health and Safety Executive. There are two recent sources of statistics for occupational skin disease. The first of these, carried out in 1995, the Survey of Self-reported Work-related Ill-Health (SWI95) found a prevalence of 97,000 reported cases of skin disease caused by or made worse by work (Health and Safety Commission, 1997a). To put this figure in context, it is much less common than musculoskeletal disorders (1.4 million cases) but nearly three times more common than vibration white finger (37000 cases). The second is the EPI-DERM project which was set up in 1993 by the University of Manchester to monitor work-related skin disorders. This scheme collects data from both occupational physicians and consultant dermatologists. It has to be remembered that such specialised reporting schemes may well underestimate the true figures as many workers may not go further than the general practitioner when seeking medical help. Using estimates of the working population from the Labour Force Survey, in the period May 1993 - Dec 1996, it was calculated that 0.62 per 10,000 new cases of occupational disease per

year (0.28/10000 reported by dermatologists and 0.34/10000 reported by occupational physicians), were due to skin disease (Health and Safety Commission, 1997). Of these almost 80% occurred as a result of contact dermatitis. Irritant dermatitis accounted for between 38% (males) and 49% (females) of this sub group (Health and Safety Commission, 1996). The commonest occupations associated with contact dermatitis are hairdressers and beauticians, chemical operatives and health associates (Health and Safety Commission, 1997b). Occupational dermatoses have a significant morbidity, resulting in a loss of 132,000 working days per year in England and Wales compared with the most common cause of lost productivity- musculoskeletal disorders (4.3 million days) (Davies, 1994).

There have been relatively few population-based studies on the incidence and prevalence of ICD as allergic contact dermatitis (ACD) and ICD have usually been grouped together. In addition these studies have been hospital rather than population based. The prevalence of hand dermatitis in population based studies has varied from 2-11 % (Diepgen, 1995). The reasons for this wide variation include different definitions for the disease and different methods for ascertainment of cases (e.g. postal questionnaire or direct examination). One population based study of particular note looked at the incidence of ICD in employees in Northern Bavaria (Diepgen, 1995). Over a 3 year period all cases of occupational skin disease were recorded and the total number of employees in this time calculated from the data held by the State Institute of Labour and Occupation. The 3 year incidence rate of ICD in hairdressers was 270 per 10,000 employees, compared with an incidence rate in bricklayers of 8 per 10,000 employees. ICD was the main cause of occupational disease in bakers, cooks and confectioners.

Different groups have looked at the frequency of ICD in hospital patients suffering from hand dermatitis. In a retrospective study of 190 patients with hand dermatitis, 27% were due to ICD, 42% due to atopic dermatitis and 23% due to

allergic contact dermatitis (Frosch, 1992). Similar figures were found by Baurle et al (1985), who found that 24.2% of 683 patients with hand dermatitis had ICD. Meding (1980), using a postal questionnaire, estimated a point- prevalence of hand eczema to be 5.4% in a population sample of 20,000. Clinical examination of 1585 of this sample revealed a relatively high percentage of hand eczema due to ICD-35%. This may have resulted from the inclusion of mild cases of ICD which the author felt would be missed unless a thorough examination was performed.

'Housewife's dermatitis' is top of the list of different occupations most susceptible to ICD (Cronin, 1985). In occupations where wet work is involved e.g. cleaning, nursing and hairdressing, there is a high prevalence of ICD, mainly affecting women (Fregert, 1975; Cronin, 1985). In men, occupational contact dermatitis is commonly seen in employees of the construction and engineering industries. However, it is not clear what percentage is due to ICD and how much is due to ACD. If one strictly applies some of the criteria used to define irritant dermatitis, then almost 100% of people exposed in occupations such as food handling, hairdressing and construction have slight irritant contact hand dermatitis. In fact most employees in such occupations do not seek medical attention as they feel the symptoms are not serious and are thought to be normal for that job.

1.3 Prognosis of contact dermatitis

The prognosis of an occupational dermatitis is influenced by many variables. ICD in general terms has a better outcome than ACD. Fregert (1975) in his series of 555 patients found that, after three years, 26% of women with ICD cleared, compared with 16% of those with ACD. The corresponding figures for men were 31% and 24% respectively. Overall the dermatitis (including ACD and ICD) had healed in 25% and intermittent symptoms were still present in 50%. A postal questionnaire 5-year follow-up survey of 53 patients, 5years after being diagnosed as

having ICD indicated that 30.6% of patients had cleared completely, and that there was some improvement in 82.4% (Fitzgerald,1995).

Referring to ACD, Burrows (1981) stressed that the type of contact sensitiser was also important. Patients with nickel and chromate allergy had a poor prognosis whereas those with rubber and epoxy allergy had a more favourable outcome. A change of employment may not necessarily improve the prognosis (Fregert, 1975) as someone with, for example, nickel allergy would still be open to non-occupational contact.

Factors indicating a better prognosis include age under 40, early diagnosis and treatment (Burrows, 1981). In recent times, high levels of unemployment and lack of retraining opportunities reduce the chances of getting a different job.

The likelihood of developing an irritant dermatitis depends upon the individual and these factors will be examined in further detail.

1.4 The susceptibility to skin irritation

1. Endogenous factors

There are several host-related factors that affect cutaneous susceptibility to irritation. Age is a factor which has been studied by examining trans-epidermal water loss (TEWL) as an indicator of barrier function. The peak of TEWL occurs in childhood and then levels out between 18-50 years of age, before falling to its lowest value by 70 years (Leveque et al, 1984). The fall in TEWL is not site dependent and occurs on all parts of the body in a constant manner. However, it was shown that with age, the thighs, forearms, upper back and abdomen have the largest differences (almost 50% greater in the young age group) in irritant reactivity to sodium lauryl sulphate (SLS) (Cua et al, 1990a). The site variation of TEWL responses may be important in the understanding of clinical irritant dermatitis. Regional differences in TEWL show that higher levels occur on the palms and ankle compared with the forearm or upper

back (Cua, 1990b). Intra-site variation is also relevant as there is a significant fall in TEWL from the wrist to the elbow (Panisset, 1992).

An individual's race is another important variable in irritant responses. Black and Latin races have a higher TEWL response to SLS irritation, in contrast to whites. The same is not true for erythema (Berardesca et al, 1988).

The sex of an individual appears to be of little significance in susceptibility to SLS irritation with both basal and post irritation TEWL values showing no difference between males and females (Lammintausta et al, 1988a). Females do vary in reactivity during the menstrual cycle, with increased reactivity on day 1 compared with day 10 (Agner, 1991b). Hormonal influences on both the mediators of inflammation and hydration of the stratum corneum could explain such differences within the cycle (Berardesca et al, 1989).

The presence of a pre-existing dermatitis, whether active or quiescent, modulates irritant responses. Patients with an active dermatitis had a greater irritant response to SLS irritation compared with controls. Conditions other than dermatitis e.g. xerosis can cause similar responses to SLS (Lammintausta et al, 1988b).

An important group, which merit separate attention are atopic individuals. Berardesca et al found that TEWL on the uninvolved forearm skin of atopics was higher than equivalent sites in non-atopics (Berardesca et al, 1990). This would suggest that atopics may have a damaged or disrupted skin barrier which is not clinically apparent. One possible explanation is the reduction in intercellular lipids, especially ceramides (Imokawa et al, 1991) which alters the balance between inter- and intracellular lipids, leading to a decrease in the size of corneocytes (Berardesca, 1994). Other aspects of atopy will now be considered in more detail.

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Atopy and contact dermatitis

The study of atopic individuals has revealed various intrinsic vulnerabilities to external factors. The most consistent clinical feature is a diminished threshold for skin irritation. The groups of irritants, which have been tested, have varied from soaps and detergents to alkalis (Nilzen, 1962). The reduced threshold for skin irritation is worse at sites where dermatitis is common (e.g. hands, behind knees, fronts of elbows) and during exacerbations of dermatitis. In terms of occupational skin disease, this is very important, as repeated exposure to marginal irritants will provoke a clinical dermatitis initially among those individuals with a lowered threshold for irritation i.e. atopics (Shmunes, 1986).

Atopic subjects also have intrinsically dry skin. Winter weather provokes exacerbations and chapping of the skin is common in work environments that combine low humidity and low temperature. The irritability of atopic skin may also be due to mechanical provocation. Intolerance to wool was recognised during World War II when wool blankets and uniforms were issued. Davies reported 110/670 (16%) of admissions to hospital involved wool intolerance (Davies, 1944). Hanifin also lists wool intolerance as a specific minor criterion for the diagnosis of atopic dermatitis (Hanifin, 1983). In the workplace, sanding, cutting or breakage of fibre glass of critical diameters (3.5μm) affect atopic individuals in the same manner as wool (Shmunes, 1984).

It is generally felt that contact dermatitis (both allergic and irritant) occurs more frequently in patients with atopic dermatitis (AD) than in normal people. As long ago as 1937, it was shown that routine patch tests in patients with various dermatoses, revealed that the incidence of reactions in AD patients did not significantly differ from the incidence seen in patients with other dermatoses (Rostenburg, 1937). One possible explanation for their low frequency of positive reactions could be that tar, one of the most common sensitisers in atopics, was not

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included in their patch test series. In addition, neomycin sensitivity had yet to be described (Epstein, 1964). The numerous studies, which have tried to address this issue have not really clarified the picture. Table 1.1 summarises the major studies. Comparisons between the studies are hampered by the different diagnostic criteria used in the diagnosis of AD. Some studies used clinical criteria alone whilst others used a history of AD and only three used the strict Hanifin and Rajka criteria (Hanifin, 1980). Another drawback was that some groups only used one allergen. Uehara et al (1989) only used one allergen, dinitro-chlorobenzene (DNCB), but managed to sensitize 100% of patients by using high doses of DNCB. If the studies which are comparable (i.e. Magnusson, Cronin, Forsbeck and Rystedt) are combined this would imply approximately 22-35% of atopics have a positive contact sensitivity but a control population have a positive sensitivity in 29-45%. Hence there appears to be no apparent difference in the development of positive patch reaction between normal people and atopic individuals.

Despite the drawbacks of patch testing as a model for real life exposure to irritants and contact allergens, it still serves as a useful clinical method by which the diagnosis of allergic contact dermatitis can be established. AD patients with the longest history of severe symptoms had the highest frequency of positive reactions (Lammintausta et al, 1992). Some other points are worthy of note in the same study. Allergic reactions to nickel were more frequent in the younger subjects whereas both fragrance sensitivity and chromate sensitivity increased with age. Irritant reactions (macular erythema) varied from 24-40% of the atopics, being highest in the older atopic group. The commonest substances to elicit an irritant reaction were formaldehyde, fragrance mix and propylene glycol.

Table 1.1 Summary of studies comparing allergic patch test reactions in atopic and non-atopic individuals

STUDY	No.of patients		No. of allergens	Criteria for atopic dermatitis	% positive	
	Total	atopics			Atopics	non-atopics*
Epstein et al,1964	100	100	>13	past clinical diagnosis	28%	9%[a]
Magnusson et al, 1969	1027	n.s.	6	past /present clinical AD	26%	39%
Cronin et al, 1969	4000	233	20	personal/family & clinical AD	26%	41%
Jones et al, 1973	88	40	1(Rhus)	clinical AD	15%	61%[b]
Rudzki et al, 1975	93	93	41	history & past/present AD	18%	n.d.
Forsbeck et al, 1983	186	186	27	Hanifin & Rajka criteria	35%	
Rystedt, 1985b	368	145	n.s.	Hanifin & Rajka criteria	22%	45% (n=223)
Rystedt, 1985a	289	n.s.	31	mod/severe childhood AD	17-23%	
Marghescu, 1985	4609	129	60	n.s.	39.5%	
Blondeel et al, 1987	4362	n.s.	1 (DNCB)	n.s.	40%	51%
Uehara et al, 1989	150	150	>20	Hanifin & Rajka criteria	33-100%	
de Groot, 1990	499	214	>20	+ve prick tests & clinical AD	37%	52%
Lammintausta et al, 1992	1040	851	24	n.s.	33-57%	25% (n=189)

* patients with suspected contact dermatitis; [a]patients with psoriasis; [b]healthy normal volunteers; n.s. - not stated; n.d. -not done

One would surmise that contact allergens readily penetrate traumatised skin compared with intact skin in atopics. However, Rystedt's findings do not bear this out (Rystedt, 1985a). She looked at two different groups of patients with AD- one severe and the other mild. The percentage of positive allergic patch test reactions were very similar in both groups (severe, 17% and mild, 23%). It could be that, because of a defect in cell-mediated immunity in atopics (see below), individuals are not often sensitised in spite of damaged skin. The higher prevalence of hand eczema, in patients with contact sensitivity(42%) compared with those without hand eczema (23%), would suggest that in some patients, hand eczema is mainly the result of contact sensitisation. Previous work has shown that irritants play a more significant role than contact sensitivity in the development of hand eczema in patients with atopic dermatitis in childhood (Forsbeck et al, 1983; Rystedt, 1985b).

Moller (1959) showed that in patients with atopic dermatitis, abnormalities of the barrier layer occur in both the normal and clinically affected skin. In atopic skin, there is an increased transepidermal water loss (Blank, 1952). Irritation from many allergens and non-allergens placed on or over the skin is commonplace although the exact nature of this barrier abnormality is unknown. In histological sections of skin affected by atopic dermatitis, the stratum granulosum layer is thickened. Cutaneous inflammation and an increased local skin temperature may alter the normal skin barrier. Sweat glands in atopic skin have an increased sensitivity to acetylcholine (Blaylock, 1976). A novel approach to examining the irritability of atopic skin compared with non-atopic skin was performed by Gehring (1990). He incubated frozen sections of skin biopsies taken from these two groups with different irritants and recorded the swelling of the stratum corneum, optical loss of nuclei and structural damage of the epidermis. This technique should enable one to examine the pure toxic effect of the irritant on the epidermal structure. Seven different irritants caused more damage in the atopics and significantly increased (absolute) swelling of

the stratum corneum. He concluded that increased irritability in AD must be due to a reduced resistance of the whole epidermis and not just a reduced barrier function. However, a major criticism of such a conclusion is that the result was based on only 2 atopic dermatitis patients and 10 volunteers.

Even though clinical findings in AD patients point towards defective cell-mediated immunity (e.g. susceptibility to certain viral infections and dermatophyte infections) it is not clear whether the low incidence of allergic contact sensitivity in AD is primary or secondary to the disease. A DNCB challenge study by Uehara et al (1989) showed that sensitisation decreased with increasing severity of atopic dermatitis (clinically), suggesting it is secondary to the disease process. The mechanisms by which this lowered sensitisation is effected have yet to be elucidated. The responsiveness of lymphocytes to antigens (in-vitro) is reduced as is the number and activity of natural killer cells (Jensen, 1985). However, it should be remembered that lymphocyte abnormalities vary with the activity of the dermatitis (Champion et al, 1992).

From the large variation in frequency of contact sensitivity seen i.e. 15-57% (see Table 1.1), it is quite clear that finding suitable control groups is important. Thus a control group of healthy normal volunteers would be more appropriate than patients attending the dermatology department for another problem such as psoriasis. In most cases the atopic patients have the longest history of dermatitis and consequently have the greatest chance of becoming sensitised. An age-sex matched control group would be almost impossible to obtain. A few studies of contact sensitivity in normal volunteers are thus useful for comparison. Magnusson (1979) tested 274 normal volunteers with the ICDRG standard series of which 22% (60/274) showed one or more positive reactions. Only one study compared the incidence of sensitisation in AD patients (15%) with age-matched healthy normal volunteers (61%), although only one allergen- rhus oleoresin- was tested (Jones et al, 1973).

In clinical practice, however, comparison with patients with non-atopic dermatitis is more important. One could therefore conclude that most of the evidence indicates that even though atopic dermatitis patients are less frequently sensitised than non-atopic dermatitis patients - 30% and 42% respectively (Table 1.1) - 3 out of 10 patients with AD will have one or more contact allergies. This should alert clinicians to consider patch testing most patients with atopic dermatitis, especially with increasing age.

2. Exogenous factors

The exogenous factors, which influence the irritant response of human skin, include the type of irritant, its penetration and mode of exposure. Of these, the most important is the inherent toxicity of the chemical. The mode of action of the three irritants chosen for this study will be discussed.

Sodium lauryl sulphate (SLS) (CH_3-$(CH_2)_{10}CH_2OSO_3Na$)
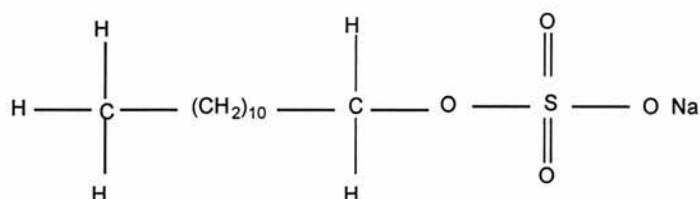
The model most widely used in the study of experimental irritant contact dermatitis is SLS irritation. Sodium lauryl sulphate is an anionic surfactant which is used as an emulsifier in different pharmaceutical vehicles, cosmetics and some foods (Nikitakis et al, 1991). It has always been believed that SLS works as a surfactant by removing stratum corneum lipids, which promote TEWL. Fulmer et al showed that SLS did not alter the total quantity of stratum corneum lipids, but changed the type of lipid, by reducing the long chain free fatty acids (C_{22-28})(Fulmer et al, 1986). More

recently it was shown that the increase in TEWL after SLS is due to increased stratum corneum hydration through spongiosis (Leveque et al, 1993; Wilhelm et al, 1993). Moreover any extraction of lipids was felt to be minimal and only occurred in the early phase of SLS irritation. Hydration is possibly increased by the alteration of the secondary and tertiary structures of keratin which allows new water-binding sites to become available (Rhein et al, 1986). Other groups have confirmed the normal lipid content in the upper stratum corneum after application of SLS but found premature keratinisation and damage to lipids in the lower layers of the stratum corneum (Fartasch et al, 1991). SLS also increases epidermal turnover (Fisher, 1975) and is pro-inflammatory being both chemokinetic and chemotactic for polymorphonuclear leucocytes (Frosch, 1987).

One should not forget that a single application of SLS does not reflect the cumulative open application of surfactants in real life. A model such as the soap chamber test (Frosch, 1979) was designed to discriminate the irritancy potential among soaps. A Duhring chamber is used to expose the irritant on the forearm for 5 days, initially for 24h and then 6h daily on the subsequent days. More recently the arm-immersion model attempts to simulate the reactivity of skin damaged by wet work and housework. This procedure uses repeated immersion of the forearm in an agitated solution of SLS at a constant temperature until a pre-determined alteration of skin appearance is reached. This compromised skin acts as the baseline on which irritants can then be applied (Allenby et al, 1993)

Another factor of great importance in SLS irritation, demonstrated by Agner et al (1989), is the quality and purity of SLS used. The inclusion of additional C_{10}, C_{14}, C_{16} and C_{18} chains confers a lower irritancy potential (as measured by clinical grading, TEWL, blood flow and oedema) when compared with SLS which is >99% pure (i.e. C_{12} chains only). This probably explains why the concentration of SLS used in studies varies so widely from 0.25% to 10% (Agner et al, 1989).

Nonanoic acid (NA)

$$H \overset{\displaystyle \overset{H}{|}}{\underset{\displaystyle \underset{H}{|}}{C}} (CH_2)_8 \overset{O}{\underset{OH}{C}}$$
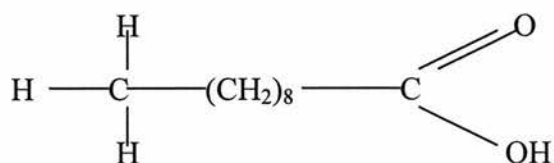
It was Bjorneberg, whilst studying irritant reactions in patients with hand dermatitis, who realised that it was impossible to predict the magnitude of the skin reaction to one primary irritant by knowing the intensity of the reaction to another irritant (Bjorneberg, 1968). During a study of the irritancy of various free fatty acids in the pathophysiology of acne, the C_8-C_{12} fatty acids were found to be the most irritating (Stillman et al, 1975). It was by chance that nonanoic acid was chosen from this group as a model irritant for study. The following qualitative criteria were deemed desirable for a chemical to be classified as a model topical irritant (Wahlberg, 1980):

- positive reactions in 90% of the population
- reproducibile
- a known chemical structure
- non-volatile or solid
- no extreme pH's
- not an allergen
- no systemic toxicity
- non-scarring
- easily read skin reactions.

Nonanoic acid (pelargonic acid) is a C_9 fatty acid and is usually tested in the vehicle propan-1-ol. When its potential for irritation was being investigated, NA was applied under patch test occlusion at concentrations of 5-40% to healthy volunteers and 5-100% to patients with a suspected allergic contact dermatitis. Results showed

a dose response curve with 98% of volunteers reacting to 39.9%NA but no response to the vehicle, propan-1-ol, alone. The reactions were monomorphic with typical well-defined erythema confined to the patch test site. Similar responses were elicited in dermatitis patients. It was concluded that NA fulfilled all the above criteria (Wahlberg, 1980). In subsequent studies the irritancy of NA was more predictable compared with the irritant Benzalkonium chloride (Wahlberg et al, 1985). It was for these reasons that NA has been widely adopted as a model irritant in experimental irritant dermatitis.

Dithranol(DL)

Dithranol (anthralin) has been used in the treatment of psoriasis for over a century, but its use has been limited by the inflammation and staining which it causes. Its mode of action is diverse and has yet to be fully elucidated. Dithranol has selective antiproliferative effects on human epidermal keratinocytes in vitro. It suppresses transcription of transforming growth factor-α mRNA (TGF-α mRNA) by keratinocytes and reduces epidermal growth factor (EGF) receptor binding by reducing the affinity of the EGF receptor for its ligands. Both TGF-α and EGF receptor expression is correlated with epidermal hyperplasia in lesional psoriatic epidermis (Gottlieb et al, 1992). Dithranol also changes the pattern of keratin expression by reducing the proliferation-associated keratins K6, K16 and K17 and increasing the differentiation keratins K1, K2 and K10. The effect of dithranol on T-lymphocytes in psoriatic epidermis is to significantly reduce the CD8+ population (Gottlieb et al, 1995). The other main effect of dithranol is to inhibit polymorphonuclear neutrophil chemotaxis and the subsequent release of reactive oxygen species from stimulated neutrophils (Mahrle et al, 1994).

In order to understand dithranol irritation its chemical structure needs to be appreciated. Dithranol is based on the anthracene nucleus (Fig 1.3a) and exists in the

anthrone form (Fig 1.3b) which is in equilibrium with the tautomeric form, 1, 8, 9-tri-hydroxyanthracene (Fig 1.3c). There are two unstable points in the anthracene nucleus at the hydroxyl groups at C_1 and C_8 (*) and the reactive hydrogen atoms at C_{10}.

Figure 1.3 Dithranol -oxidation and free radical formation
a) anthracene nucleus

b) anthrone form

c) 1,8,9-tri-hydroxyanthracene
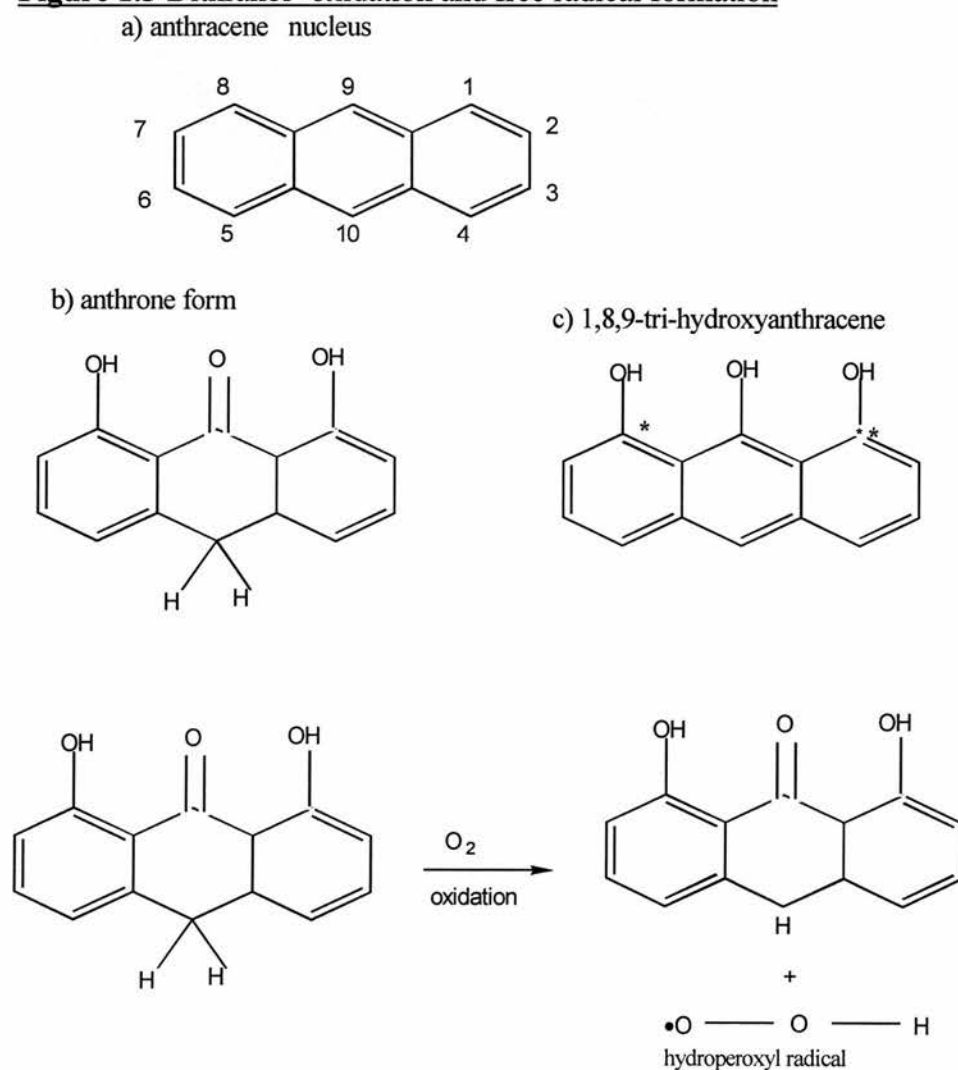
Oxidation of anthralin by light or air yields an active free radical before oxidation to anthraquinone and anthraquinone dimer. The hydroperoxyl radical reacts with different bases to form the superoxide anion (Ashton et al, 1983)- Fig 1.4

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Fig 1.4 Dithranol – mechanism of free radical formation

anthralin free radical

hydroperoxyl radical

dihydroxyanthraquinone

anthraquinone dimer
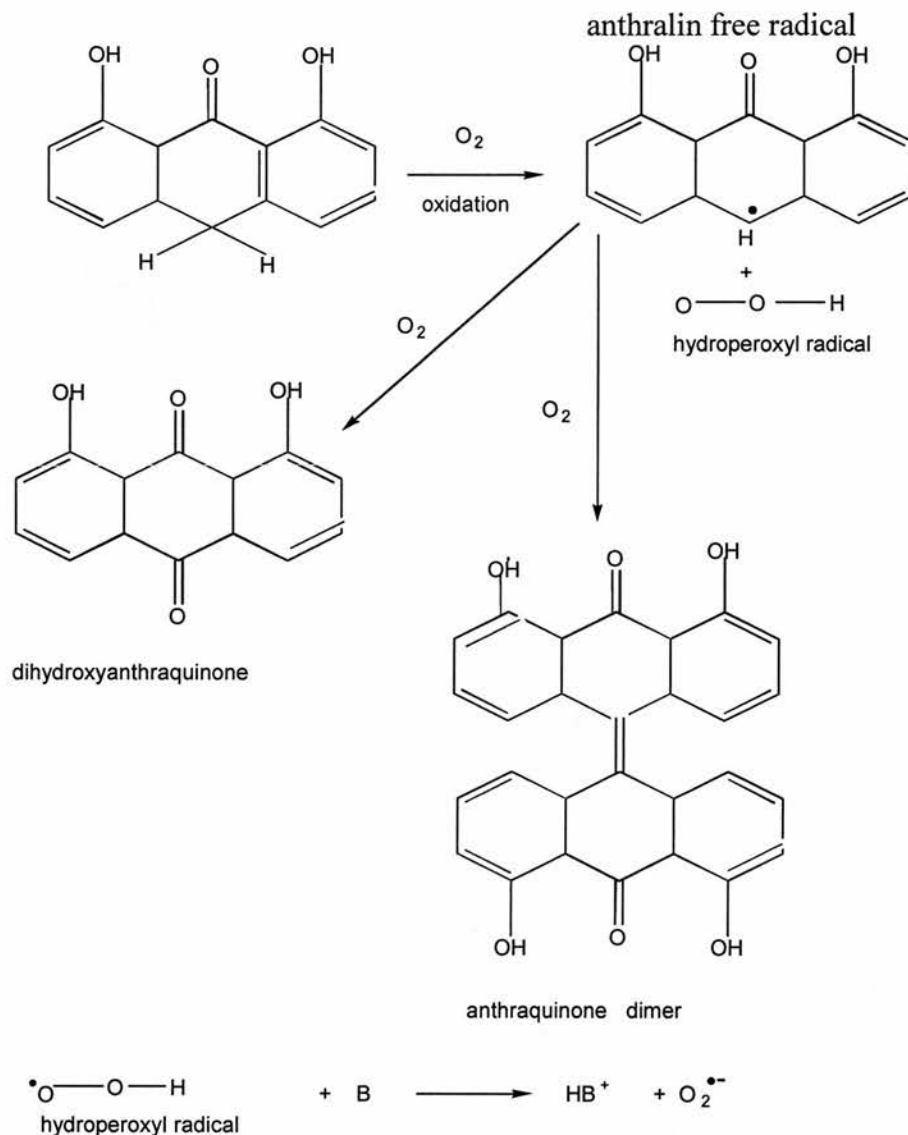
The superoxide anion ($O_2^{\bullet-}$) is responsible for dithranol irritation and is known to be toxic to tissues (Mustakallio, 1981). Various attempts at reducing the erythema induced by dithranol have produced good results with free radical scavengers such as α-tocopherol, retinol palmitate and butylated hydroxyanisole (Finnen et al, 1984). The mechanism by which free radicals induce an inflammatory response is unclear but a non-enzymatic effect on arachidonic acid leading to the formation of the pro-inflammatory prostaglandins PGE_2 and $PGF_{2\alpha}$ may be responsible.

As irritants act in different ways, the detection of irritant responses can also be measured by a variety of instruments. These tools are being used to make objective assessments of irritation.

1.5 Evaluation of irritant patch tests

Experimental irritant patch testing is frequently employed to simulate irritant contact dermatitis. Over the last ten years several different non-invasive measurement procedures have been developed to quantify irritant patch test reactions. The components of the irritant response include erythema, oedema and barrier disruption. Numerous comparative studies have tried to find the gold standard for irritant patch test measurement. The following table (adapted from Lee, 1995) shows different techniques and the functions which they measure.

Bioengineering method	Parameter measured
Evaporimetry	Transepidermal water loss
Laser Doppler flow velocimetry	Blood flow
Ultrasound	Thickness
Colorimetry	Erythema
Impedance, conductance, capacitance	Hydration

The visual scoring system for assessing an irritant reaction is open to criticism as it is subjective and liable to marked inter-observer variation. It does however, have the advantage of taking into account both epidermal changes (e.g. crusting and scaling) and inflammatory changes (e.g. erythema and oedema).

Evaporimetry records the total evaporation of water from the skin and the diffusion rate is dependent on the ambient relative humidity, ambient and skin temperature and the integrity of the stratum corneum. When the probe is applied

over the skin, a gap of approximately 10mm between the skin surface and the end of the probe is formed. It is across this gap that the water vapour pressure gradient is measured i.e. the transepidermal water loss. Any changes in the surroundings or even draughts can alter the readings obtained. For the irritant SLS, a positive dose response is consistently found when TEWL measurement is used (Serup, 1987; Agner, 1990a). As for the irritant NA, evaporimetry has not been so successful which probably reflects the fact that NA only influences the barrier to a minor degree (Agner, 1990a).

One of the methods for assessing erythema is by skin colour reflectance. The Commission Internationale de l'Eclairage (CIE) standardised a tristimulus colour system where colour is expressed as a 3-dimensional co-ordinate (see below).

White

L*   Yellow (+ve)

                  b*

Green (-ve)_____a*___ Red (+ve)

Blue (-ve)
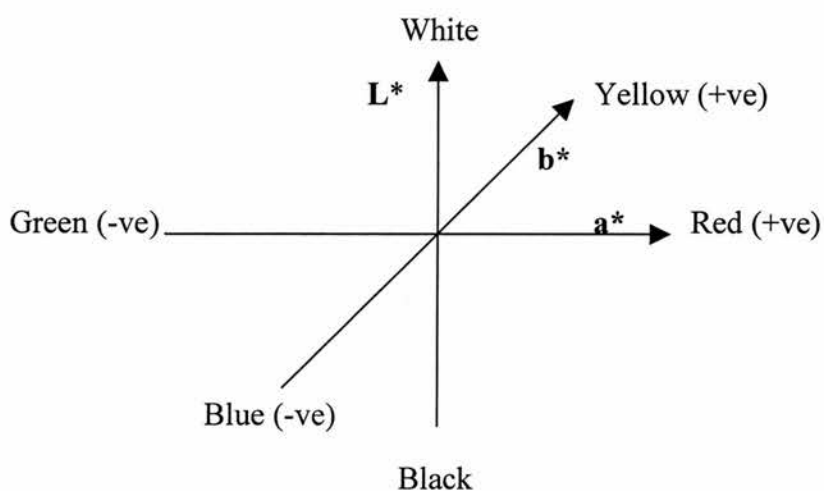
Black

The a*-axis represents the colour range from green (-) to red (+) ; the b*-axis represents the colour range from blue (-) to yellow (+). L* expresses the brightness of reflected light ranging from black (low values) to white (high values). The a* value has been shown to have a good correlation with visual scoring of irritant reactions (Agner, 1990b) and with different doses of SLS (Wilhelm et al, 1989). In comparative studies, the sensitivity of colorimetry was not as high as for evaporimetry (Agner, 1990b). The benefits of colorimetry however, include its reproducibility and ease of use in the clinical setting (Wilhelm et al, 1989).

The other major tool for the investigation of erythema is laser doppler flowmetry (LDF). The extent of erythema, as a reactive increase of cutaneous blood flow, can be quantified with a laser doppler flowmeter. Light from a laser source is transmitted to the skin through an optical fibre and reflected with a frequency shift (Doppler effect) from moving blood cells. The machine records the blood flow as the product of the number of erythrocytes encountered and their mean velocity. The output of the flowmeter is proportional to the cutaneous blood flow (de Boer et al, 1989). Good LDF positive dose responses for SLS (Nilsson et al, 1982; Staberg, 1988) and negative dose responses (Blanken et al, 1986) highlight the difficulty with LDF measurement. This may in part be due to patchy erythema (Freeman, 1984).

Ultrasound scanning has the advantage that, unlike TEWL measurement, no pre-conditioning of the subject is necessary (Agner, 1992). The principle of ultrasound is that the interval between the echo from the skin surface and the interface between the dermis and subcutaneous fat is a measure of the thickness of the skin. The inherent variation of skin thickness limits the accuracy of thickness (and therefore oedema) results. Seidenari has shown a good correlation between TEWL values and B-mode (2-dimensional) ultrasound after SLS irritation (Seidenari, 1992). The expense of the equipment and the time taken to become proficient in its use, limits wider uptake of this method routinely.

In order to understand the immunological and cellular effects of irritant induced inflammation, some basic aspects of the skin immune system and neutrophil biology will be discussed.

1.6 The skin immune system

The idea that the epidermis is more than just a barrier has been developed over the last 15 years. The concept is based on different epidermal cells being able to exert autocrine, paracrine and endocrine influences. These effects are modulated by the secretion of cytokines. Cytokines are low molecular weight regulatory glycoproteins which are produced transiently and are active at picomolar concentrations. They exert their effects via specific cell surface receptors (Luger , 1990). After a cytokine binds to the target cell receptor, this can trigger a cascade of cellular events that includes the release of further cytokines which may possibly mediate further release of the initiating cytokine (Sauder, 1990). Keratinocytes(KC) which compromise the largest cell population of the epidermis, are a rich source of cytokines. However other epidermal cells are capable of secreting cytokines, such as Langerhans cells, melanocytes and Merkel cells (Ansel et al, 1990). Some of the important KC cytokines are shown below:

Interleukins	IL-1, IL-6, IL-8
Colony stimulating factors	Granulocyte macrophage colony stimulating factor (GM-CSF)
Others	Tumour necrosis factor-alpha (TNF-α) Transforming growth factors alpha and beta (TGF-α, TGF-β)

Of these cytokines, IL-1, IL-8 and TNF-α will be discussed in more detail.

IL-1 (Ansel et al, 1990; Luger, 1990)

Interleukin-1 was one of the original cytokines studied and exists in two forms, IL-1α and IL-1β. These two sub-types are encoded by distinct genes on Chromosome

2. Although there is only 27% sequence homology between the sub-types, they share the same biological activity. KC mainly produce IL-1α, whereas macrophages produce IL-1β. Since both forms bind to the same receptor (IL-1R) with identical affinity and have the same biological activity, this differential production by cells is of no biological significance. Keratinocytes constitutively synthesise IL-1 but its synthesis can be induced by numerous different stimuli such as ultraviolet radiation (UVR), and by cytokines produced by tumour cells, fibroblasts and endothelial cells. Within normal epidermis, it is stored in keratinocytes. IL-1 is eliminated by normal desquamation and following injury to the epidermis, both newly synthesised and pre-formed IL-1 is released. Some of its effects on different cells are summarised below.

IL-1 effect on	
T cells	Stimulates mitogen responsiveness, IL-2 secretion Stimulates GM-CSF and IL-2R expression
Macrophages	Activated to synthesise PGE$_2$ Increases cytokine production Increases cytotoxicity
Fibroblasts	Induces production of collagen
Endothelium	Induces expression of ICAM-1 leading to lymphocyte, monocyte and neutrophil adherence
Acute phase of inflammation	Fever, muscle proteolysis Synthesis of acute phase proteins and hypoalbuminaemia

IL-1 Receptor antagonist (IL-1RA)(Austyn, 1993)

IL-1 receptor antagonist has structural homology with IL-1β and is synthesised by keratinocytes and macrophages. It binds to the IL-1 receptors blocking their function. IL-1RA may have physiological significance by limiting IL-1 activity. In humans there are two IL-1 receptors, types I and II, both of which bind IL-1α, IL-1β and IL-1RA. However, only type I receptors can signal and type II could function as an IL-1 antagonist. Thus IL-1RA and type II IL-1 receptor expression may be part of the normal homeostatic response to an excess of IL-1α and β.

IL-8 (Luger, 1990)

Interleukin-8 is a pro-inflammatory cytokine and is a member of a group of chemoattractant factors which includes monocyte chemoattractant and activating factor (M-CAF) and macrophage inflammatory protein. IL-8 is produced by many cell types, including monocytes, fibroblasts, KC, and endothelial cells and is also found in increased amounts in psoriatic epidermis.

IL-8 is chemotactic for polymorphonuclear cells (PMN) and activates neutrophil degranulation, enzyme release and respiratory burst. It also modulates the adhesion of neutrophils to vascular endothelial cells by inducing MAC-1 expression on the surface of neutrophils. IL-8 is also chemotactic for basophils causing a release of histamine and leukotrienes. Unlike IL-1, IL-8 does not produce fever, acute phase proteins or ICAM-1 expression on endothelial cells.

TNF-α (Luger, 1990)

During the study of tumour breakdown, the mediator which led to tumour necrosis and cachexia was called cachexin or TNF-α. A different lymphocyte derived cytotoxic mediator previously known as lymphotoxin, has been renamed as TNF-

β. These two proteins are distinct antigenically but bind to a common receptor and have similar biological activity. TNF-α is initially produced as an inactive peptide but is cleaved at several sites to produce the active moiety.

It is produced by numerous cells including KC, macrophages, T and B lymphocytes, fibroblasts and some epidermal carcinoma cell lines. The synthesis and release of significant amounts of TNF by epidermal cells, is induced by IL-1, IL-6, ultraviolet radiation (UVR) and the tumour promotor phorbol myristate acetate.

One of the most important biological effects of human TNF is the stimulation of production of PGE_2 , IL-1 and IL-6 leading to fever. TNF activates neutrophils, eosinophils, macrophages and the production of prostaglandins and collagenase by synovial cells and fibroblasts. It also induces MHC class I and class II antigens and ICAM-1 expression on endothelial cells. The effects on T-lymphocytes include expression of IL-2 receptors and IL-2 production.

Interferon- gamma (IFN-γ) (Halloran PF, 1993)

Human IFN-γ is coded by a single gene copy of 4 exons on Chromosome 12. The peptide (M.Wt-17 kDa) is glycosylated and dimerised to form the active protein. It is produced by activated T cells triggered by the T cell receptor (TCR), natural killer cells and even keratinocytes (Howie et al, 1996).

IFN-γ has particular effects on gene expression in many cells, especially on those coding MHC class I and II, adhesion molecules and other cytokines. T cell release of IFN-γ can be stimulated by oxazolone (a skin sensitiser), superantigens and other infectious agents. IL-2 and other cytokines including IFN-γ can induce natural killer (NK) and T cell release of IFN-γ. The self amplification by IFN-γ recruits non-specific T cells to augment production of IFN-γ by specific T cells. The advantage of the systemic effects of IFN-γ is that a local infection will increase MHC and adhesion molecule expression in all tissues and so enhance immune

surveillance of a potential spread of infection. The following summarises the key effects of IFN-γ:

i) regulation of membrane molecule synthesis and expression

- role in normal MHC class II expression
- induction of MHC class I and class I transcription
- induces ICAM-1

ii) induction of other cytokines

- expression of TNF-α and its receptor

iii) proinflammatory

- macrophage migration inhibition and activation
- nitric oxide production by macrophages (suppressing T cell proliferation)

iv) immune regulation

- B cell immunoglobulin secretion
- suppresses TH$_2$ cells and antagonises TH$_2$ cytokines

1.7 The role of cytokines in inflammation

As has been discussed previously, cytokines initiate their biological action by interacting with target cells bearing cell surface cytokine receptors. The final response to a particular cytokine is determined by the interactions between synergistic and antagonistic cytokines. They provide the communication between infiltrating inflammatory cells and resident cutaneous cells. Over the last decade more evidence has accumulated that KC have an active and key role in inflammation in vivo in both mice and humans. The major element central to this belief is the ability of KC to respond to diverse environmental insults by producing TNF-α and IL1-β (Nickoloff, 1993)(see Fig 1.5 below).

Fig. 1.5 Immunological function of keratinocytes in response to diverse environmental triggers (adapted from Nickoloff, 1993)

ENVIRONMENTAL CHALLENGES

UVR   Chemical irritants   Allergens   Tumour promotors   Infections

IMMUNOLOGICAL CHARACTERISTICS OF KERATINOCYTES

Function 1   Function 2   Function 3

KC   KC   KC

Sentinel role   *Accessory cell role*   *Antigen-specific Target cell role*

Non-specific response   Amplification of   T cell mediated effects
barrier disruption   immune response   on epidermal keratinocytes

                          HLA-DR   ICAM-1

Release of TNF-α   Elimination of virus
                          T cell proliferation   Removal of transfected cells
                          and cytokine release

induction of
chemotactic
factors and adhesion
molecules

influx of T cells, APCs

Previous explanations of contact hypersensitivity have suggested that low molecular weight haptens bind to a carrier protein and are processed by Langerhans (antigen presenting) cells which transport the complex to a local draining lymph node, where an antigen-specific T cell proliferative response is mounted . The latter still occurs in the new model, but it occurs long after keratinocytes release TNF-α and IL1-β. These cytokines stimulate neighbouring endothelial cells to express adhesion molecules such as E-Selectin (previously known as endothelial adhesion

molecule-1), vascular adhesion molecule-1(VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). This paracrine stimulation of endothelial cells by KC may then facilitate chemotactic factors such as IL-8 and M-CAF and the subsequent influx of memory T cells from the circulation (Nickoloff, 1993).

Within normal uninflamed skin, ICAM-1 is weakly expressed on the surface of microvascular endothelial cells. The discovery of ICAM-1 as the ligand for lymphocyte function associated antigen-1 (LFA-1) on T lymphocytes explained the intimate molecular and cellular relationship between KC, ICAM-1 expression and intraepidermal accumulation of T cells (Kupper, 1989).

Molecular models of inflammatory skin disease postulate that a breakdown in the regulation of growth factor production and signal transduction results in abnormal proliferation and cell behaviour (McKenzie, 1990). Cytokine production whether it be in abnormal amounts or a failure of cytokine regulation are being investigated in inflammatory dermatoses. For instance, very high levels of IL-1 are found in cutaneous T cell lymphoma; increased levels of IL-6 and IL-8 in psoriasis. The mechanisms of inflammation in ICD at the molecular and cellular level are poorly defined. Different groups have shown that the abrogation of epidermal barrier by a non-specific irritant stimulus (tape-stripping, Wood et al 1992; Nickoloff, 1994) and the model irritant SLS (Hunziker et al, 1992) leads to the production of TNF-α. Willis et al have shown that ICAM-1 is upregulated in the presence of LFA+ leucocytes in irritant reactions (Willis et al, 1991). When one compares ICD with ACD, ICAM-1 expression is a predominant feature of ICD whereas HLA-DR expression is usually seen in ACD. Since ICAM-1 expression is upregulated by TNF-α, the latter is believed to be a key mediator of inflammation in ICD.

1.8 The role of neutrophils in inflammation

Acute inflammation involves the combination of a variety of cellular and humoral mechanisms leading to a rapid change in vascular permeability, changes in blood flow and the accumulation of leucocytes. Monocytes and neutrophils are the main leucocytes mobilised. The injurious effects of such cells have been implicated in the pathogenesis of various inflammatory diseases. However, inflammation has a beneficial role as a defence mechanism against such agents and this host response usually results in resolution with minimal tissue damage (Haslett et al, 1989). The study of the resolution phase of acute inflammation may add to the understanding of the pathogenesis of chronic inflammation as in chronic irritant dermatitis.

The role of the neutrophil is of particular interest. It is the first cell to migrate to the site of 'attack' and subsequent inflammatory processing such as monocyte migration, may depend on neutrophil accumulation. The contents of neutrophils are toxic to cells but can also break down matrix proteins into molecules which themselves are chemotactic. The fate of the neutrophil after this initiation and amplification is interesting as it must be removed from the inflamed site before resolution occurs.

When inflamed skin is injected with radiolabelled neutrophils and probed for their detection, it has been demonstrated that neutrophil migration ceases very early in the acute phase (2-4h). Neutrophils have been observed in early severe irritant reactions (Lever, 1990) but in very much smaller numbers in biopsies from skin after mild experimental irritant reactions (Willis et al, 1986). As skin biopsies provide a snapshot of what is a dynamic process, the use of skin window chambers allows in-vivo collection of neutrophils. This technique has previously shown a significant neutrophil influx in irritant reactions induced in volunteers. Skin windows have been modified for this thesis to examine neutrophil surface markers which indicate their state of activation and fate after experimental irritation.

Previous dogma has stated that dead neutrophils eventually disintegrate in the tissue, but now there is an alternative hypothesis which is that intact neutrophils are engulfed by macrophages at the inflamed site. In-vitro studies have demonstrated that macrophages, attracted to the site or derived from monocytes were capable of neutrophil (aged in-vitro) ingestion. There is now good evidence that with time, increasing numbers of neutrophils undergo one form of programmed cell death (apoptosis) with characteristic morphological changes and chromatin fragmentation (Savill et al, 1989).

Neutrophil adhesion to endothelial cells is an early event in the inflammatory response and is a pre-requisite for leucocyte accumulation. In-vitro and in-vivo studies have now established the different molecules that mediate this important interaction between neutrophils and vascular endothelium (Springer, 1995- see figs 1.6 and 1.7 below).
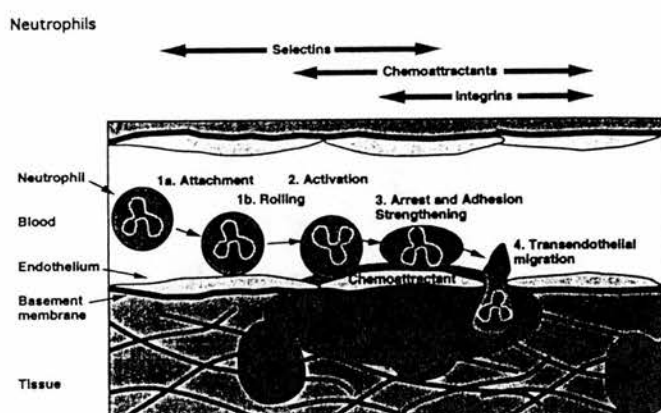
Fig. 1.6 Neutrophil adhesion to vascular endothelium (from Springer, 1995)
Three sequential steps provide the traffic signals that regulate the localization of neutrophils in the endothelium of vasculature. Selectins that bind the carbohydrate moieties of mucin-like molecules are responsible for tethering of a neutrophil to the

vessel wall and rolling adhesion (step 1). This allows the neutrophils to come into

contact with chemoattractants that are either displayed on or released from the lining

of the vessel wall. The binding of chemoattractants to neutrophil surface receptors

leads to transduction signals that activate integrin adhesiveness (step 2). The

integrins can then bind to Immunoglobulin superfamily members on the

endothelium, thereby increasing firm adhesion and neutrophil arrest (step 3). Further

directional cues from chemoattractants and use of integrins for traction enables

neutrophils to migrate out of the vascular endothelium into the surrounding tissue.

The interaction at the molecular level is shown below (Fig 1.7)

P M N
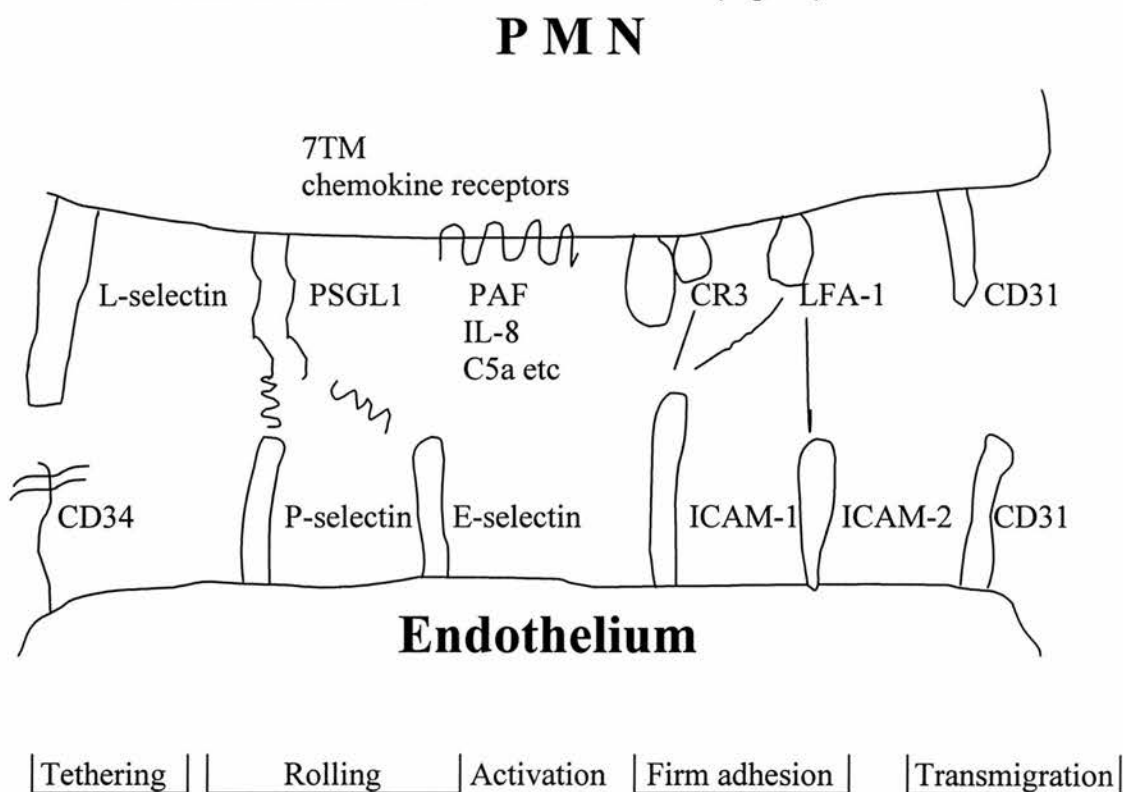
Fig. 1.7 Schematic representation of molecular interaction between neutrophil and
vascular endothelium. PAF, platelet aggregating factor; LFA-1, lymphocyte
associated antigen-1; PSGL1, P-selectin glycoprotein ligand-1; ICAM, intercellular
adhesion molecule;7TM, 7 transmembrane (personal communication from Dr. I.
Dransfield)

Thus the distinct distribution of receptors on leucocyte subsets (neutrophils) for signals found on endothelium, regulates selection of which subset emigrates at the site of inflammation.

The central purpose of this study is to define better the inflammatory and immunological responses in the pathogenesis of chronic irritant dermatitis. In order to do this the studies will determine the nature of the early inflammatory events in acute reactions in both patients with ICD and in healthy volunteers. The studies were performed on material derived from skin biopsies, suction blisters and skin window chamber fluid before and after the application of irritant patch tests. The following hypotheses will be tested:

1. The pathogenesis of irritant reactions is related to the degree of epidermal damage.

2. The initial damage caused by irritants, results in rapid secretion of cytokines known to be chemotactic for neutrophils and macrophages.

3. The damage may then be increased by accumulation, activation and secretion of enzymic contents by neutrophils.

4. Further epidermal damage results in local production of IFN-γ, which results in upregulation of keratinocyte ICAM-1 expression and recruitment of lymphocytes to the site.

1.9 AIMS

1. To define the histopathological changes in the skin during the early phase of irritant contact dermatitis.

2. To examine the changes in Langerhans cells (LC) following the induction of an experimental irritant contact dermatitis.

3. To quantify the pro-inflammatory epidermal cytokines following the induction of an experimental irritant contact dermatitis.

4. To examine the fate of neutrophils in irritant contact dermatitis.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Patient Selection

The Contact Dermatitis (Royal Infirmary, Edinburgh) clinic was the main source of patients selected for the different studies. The patch test proforma records were examined retrospectively and letters sent out to those patients in whom a diagnosis of ICD had been made. The other method of recruiting patients was by screening referrals to the contact clinic with hand dermatitis. These patients were fast-tracked with earlier appointments made for patch testing.

Table 2.1 Criteria used for the diagnosis of chronic irritant dermatitis. A person with chronic dermatitis for >6 weeks plus a minimum of one criterion from each category had to be fulfilled.

History	• occupational exposure • affected areas relatively static

Clinical	• erythema , hyperkeratosis or fissuring
	• glazed, scalded appearance of skin
	if hand dermatitis (See fig 1.2): the above plus
	   • ring pattern,
	   • pulpitis
	   • dorsa of hands affected

Patch tests	Negative (or non relevant positive reaction)

The diagnostic criteria discussed previously (see pp 2-3) were adapted to provide a simple and usable list of criteria which could be applied when screening potential subjects in the clinic (see Table 2.1) and were used for the recruitment of volunteers with ICD in all the studies presented in this thesis. The use of a combination of the history, clinical and patch test findings is more appropriate than the often used definition of ICD- contact dermatitis with negative patch testing. The UK Working Party diagnostic criteria were adopted for the determination of an atopic individual as shown below:

UK Working Party diagnostic criteria (modified from Williams et al, 1994)

    1.   History of itchy skin condition

    2.   Three of :-

            onset before age 2

            history of flexural involvement

            history of asthma or hay fever

            history of dry skin

            visible flexural eczema

The subjects had the studies explained to them and were given the subject information sheet (Appx. 1). Signed consent from the subjects was obtained prior to all studies. The subject's General Practitioner was kept informed of their intention to participate by a letter similar to the subject information sheet.

Normal healthy volunteers were recruited from the general public and from staff members in the departments of Dermatology and Pathology. Ethical approval for all studies was obtained from the local Health Board Ethical Committee.

2.2 Patch testing

2.2.1 Screening

During the screening of prospective subjects, all patients had patch testing to the European Standard battery of allergens and any extra allergens which may have been relevant to the patient's occupation. For example, perfumes and flavours for chefs and food handlers. The assessment was carried out in the standard way at 48 and 96 hours.

2.2.2 Irritant patch testing

i. Histopathology/immunohistochemistry studies

The irritants were applied under occlusion using 8mm Finn® chambers to the volar aspect of the forearm of subjects in the configuration shown below.
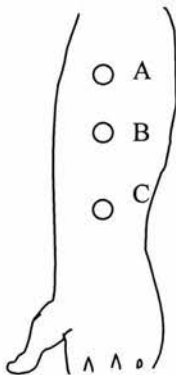
Patch A= Irritant 1 or 24h

Patch B= Irritant 6 or 48h

Patch C= Vehicle of irritant

An attempt was made to keep the distance between the patches greater than 2cm and sites A, B and C were rotated at random for each subject. In the majority of patients the non-dominant arm was used for the irritant patches.

ii. Suction blister studies (see **Plate** 2.1)
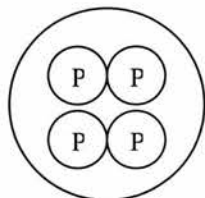
The patch tests were applied in such a way to fit the special cups. Four 8mm patches containing the irritant nonanoic acid were applied under occlusion to each forearm to maximise the yield of blister fluid. The patches were kept in place for either 1,6 or 24hours.

2.2.3 Assessment of irritant reactions

Patches were removed and left to equilibrate with the surroundings for one hour (except in suction blister studies). The reactions were assessed clinically using the clinical grading scale shown.

0 - no reaction 1- faint erythema 2- well defined erythema

3- palpable or indurated erythema

4- vesiculation and/or necrosis

2.3 Irritants

The three irritants chosen for the different studies were nonanoic acid, sodium lauryl sulphate and dithranol. Willis et al (1989) tested a panel of irritants under patch test occlusion to determine the concentrations needed to elicit mild or moderate erythema on the skin of normal healthy volunteers. This was important in the present work because less severe reactions may give clues about the pathology of the earlier stages of chronic inflammation. Pilot studies were carried out with different concentrations of the three irritants above.

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PLATE 2.1 Irritant reactions with NA prior to raising suction blisters

PLATE 2.2 Suction blisters raised at site of irritation

The sources of the irritants and their vehicles are shown below:

	Purity	Source
Sodium lauryl sulphate	99%	BDH, Poole, Dorset.
Nonanoic acid	99.5-100%	BDH, Poole, Dorset.
Dithranol	BP grade	Hillcress Pharmaceuticals, Burnley.
Yellow soft paraffin	BP grade	Thornton & Cross, Leeds.
Propan-1-ol (Analar)	99.5%	BDH, Poole, Dorset.

2.3.1 Irritant Pilot studies

Aim   To find those concentrations of irritants which will elicit a positive

reaction in over 75% of subjects tested

Subjects   All subjects were normal healthy volunteers from the department of

Dermatology or the Contact clinic.

Methods   Finn chambers of different diameters were applied under occlusion to

the volar aspect of the forearm for 24-48 hours.

Study No.1:   12mm chambers

Study No.2:   8mm   "

Study No.3:   8mm   "

Patches were removed and read visually after 30 minutes.

<u>Test irritants</u>

The following concentrations of the irritants were tested :

Sodium lauryl sulphate in distilled water (w/v):   2%, 5%

Nonanoic acid in propan-1ol (v/v):   80%

Dithranol in yellow soft paraffin (w/w):   0.01%, 0.02%, 0.04%, 0.06%

<u>Results</u>

A positive reaction was a site with a well defined erythema or palpable erythema.

Study No.1 (12mm)

Irritant	Volume/weight	no. of subjects	No. positive (%)
SLS 2%	30µl	14	7 (50)
SLS 5%	30µl	14	10 (71)
Dithranol 0.01%	35mg	11	5 (45)
Dithranol 0.02%	35mg	11	9 (82)

Study No.2 (8mm)

Irritant	Volume/weight	no. of subjects	No. positive (%)
SLS 5%	15µl	23	19 (83)
Nonanoic acid 80%	15µl	10	9 (90)
Dithranol 0.02%	25mg	27	7 (26)

Study No.3 (8mm)

Irritant	Volume/weight	no. of subjects	No. positive (%)
Dithranol 0.04%	25mg	12	8 (66)
Dithranol 0.06%	25mg	12	11 (92)

See **Plates** 2.3 and 2.4
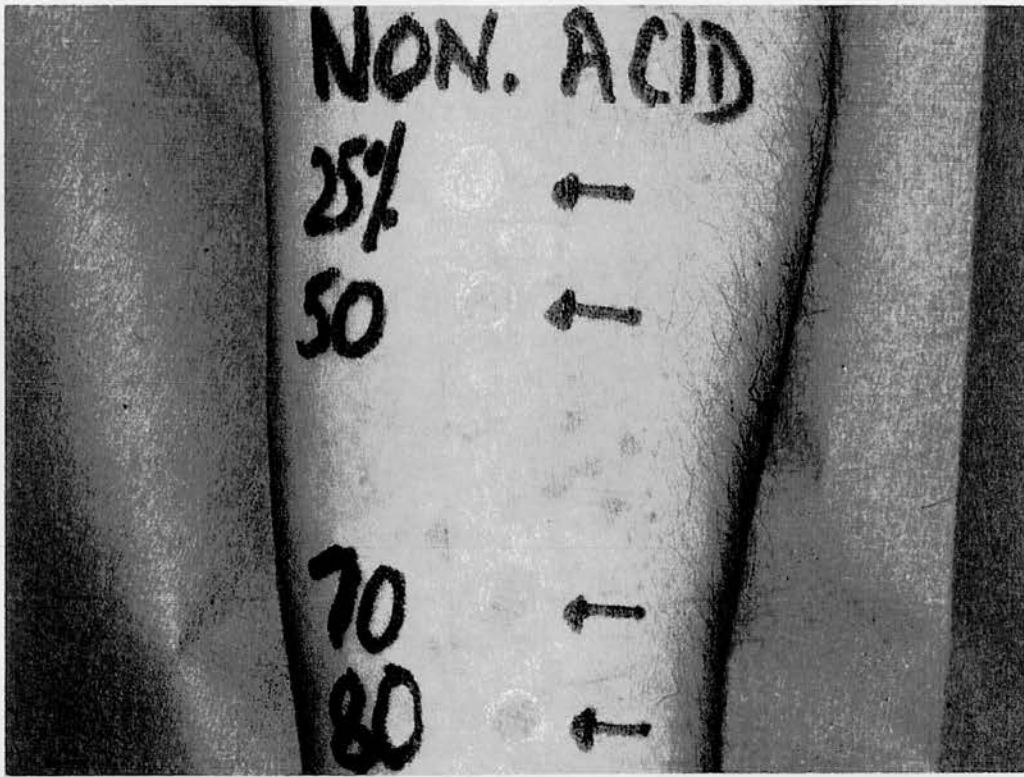
PLATE 2.3 Dose response for NA irritation, 25-80% NA in propan-1-ol

PLATE 2.4 Dose response for SLS irritation, 2-5% SLS in water.

Conclusions

Willis's group had previously shown that 2% SLS, 80% NA and 0.02% dithranol resulted in positive irritant reactions in over 75% of normal volunteers (Willis et al, 1989). The above results show the influence of area on the reaction. The volume (or weight) of irritant required for the 12mm chamber was extrapolated by calculating the volume/area of the 8mm Finn chamber. The larger Finn chambers probably required a greater volume of SLS to achieve a higher percentage of responders. I could not explain the marked variation in response to dithranol between the 12 and 8mm patches.

The final concentrations adopted for the studies were **80% nonanoic acid, 5% sodium lauryl sulphate and 0.06% dithranol.**

2.4 Bioengineering methods for assessment of irritation

The following methods were appraised by trials on volunteers and the author.

Laser Doppler Flowmetry (LDF)
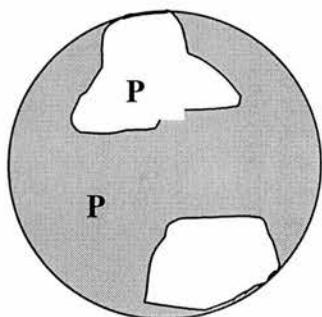
The principle has been discussed earlier. The problem with LDF is the flux variation within a test patch site. The erythema in a 8mm patch is not always uniform especially in early reactions (shaded region). The probe (P) has a diameter of 2mm and as the schematic diagram illustrates, the site of probe placement can be crucial to the reading.

Scanning laser Doppler
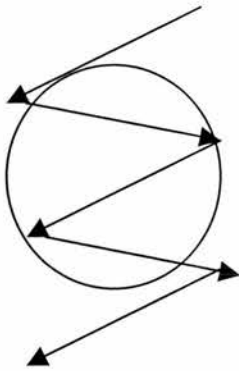
This more sophisticated device overcomes the main problem of simple LDF by scanning the whole patch and has the added advantage of measurement without the need to apply any pressure on the subject. Its major drawback apart from its great expense was the time taken to set up the apparatus. These factors led to this method being abandoned.

Digital image analysis

The Seescan photographic image analysis system (already in the department of Dermatology) was adapted to take digital images of a test colour (white silk) and its optical density compared with the optical density of irritant patch test sites. This method was technically both difficult and cumbersome for the patient, to be used on a regular basis.

Diastron colorimetry

This instrument had previously been used in the investigation of the weal and flare response to histamine injections. The probe was extremely sensitive to the pressure applied to the skin making it difficult to obtain good reproducibility of readings.

Minolta colorimetry

Following the appraisal of the above instruments it was felt the Minolta CR300 Chroma meter was the most suitable method of irritation assessment because of:

- the ease of use in the clinic setting
- the low inter-reading variation
- the speed with which repeated readings could be undertaken.

Unfortunately the machine was not available for the early subjects.

The machine was calibrated against the standard white tile supplied by the manufacturer, prior to each set of readings for a subject. An initial reading was taken from adjacent forearm skin before measurements of the irritant patch test site in triplicate. This was repeated for each of the sites A, B and C (see p40). The change in erythema (Δa^* value) was calculated by subtracting the adjacent normal skin value from the irritated site value. See **Plates** 2.5 and 2.6

2.5 Tissue sampling

2.5.1 Punch Biopsy

The irritant patch test sites were cleaned with an antiseptic solution and infiltrated with 1% lignocaine and adrenaline in a ring pattern around the edge of the site. Each site was biopsied with a 6mm disposable biopsy punch (Stiefel) including the clinically unaffected skin on the contralateral forearm. The latter served as the subjects own control.

All biopsies were coded prior to analysis in the laboratory so that laboratory research staff were unaware of the time of biopsy or whether it was an irritant or control biopsy. Tissue biopsy samples were fixed in 5% formalin in phosphate buffered saline. Three-micron sections were cut and stained with standard haematoxylin and eosin. All sections were mounted in Pertex.

PLATE 2.5 Minolta CR-300 Chroma meter with adjacent white calibration tile
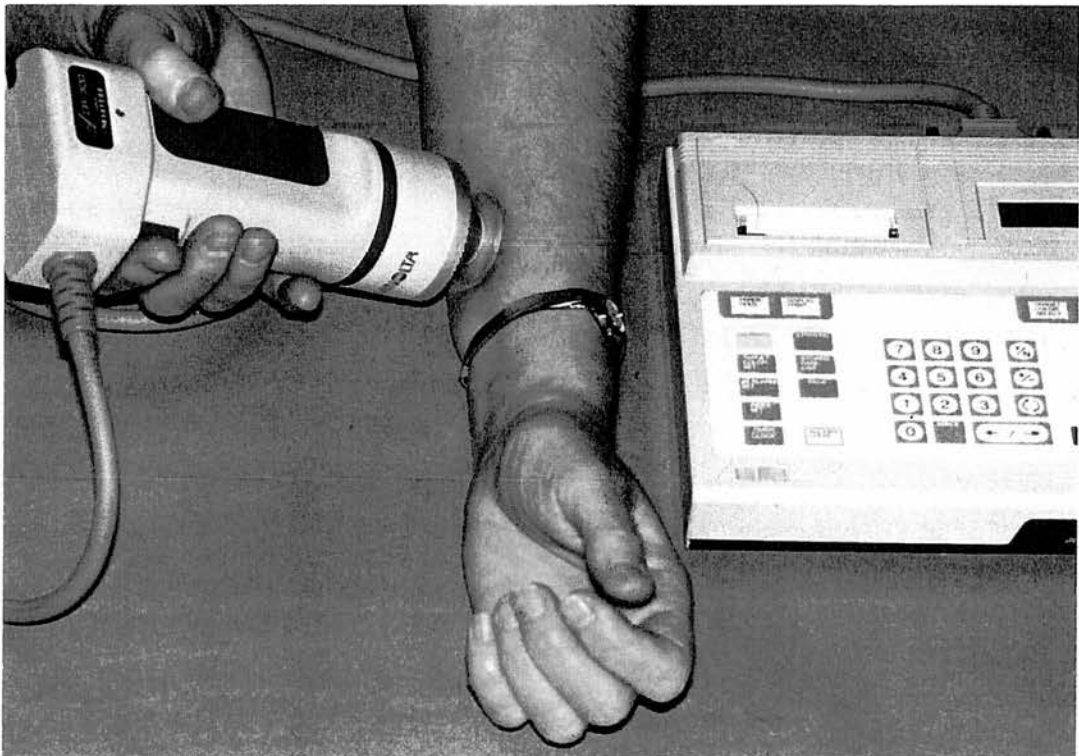
PLATE 2.6 The measurement of erythema on the forearm with the Minolta CR-300

2.5.2 Suction Blisters

Unlike the immunohistochemical study, the subjects who required suction blisters had the apparatus applied to their skin immediately after the irritant patches were removed.

The suction blister technique is based on the Dermovac® used by Kiistala (1968). Two plastic cups were attached via plastic tubing and a Y-connector to a hand operated suction pump. Previous experience with this instrument showed that a negative pressure of 30mmHg was adequate to raise suction blisters (see **Plate** 2.2). The time taken to form blisters ranged from 1-1.5 hours. The cups were removed and the blister fluid withdrawn with a syringe and stored at -40°C. The epidermal blister roofs were carefully excised and snap-frozen in liquid Nitrogen (-196°C). The roofs were later used in the confocal microscopy study.

2.6 ELISA assays

Principles

Solid phase ELISA were used, which are designed to measure IL-1α, IL-8 and IFN-γ in tissue culture media, serum, plasma and other biological fluids. The assays employ a quantitative sandwich enzyme immunoassay. A monoclonal antibody specific for the cytokine, is coated onto a microtitre plate. Standards and test samples are pipetted into the wells and any cytokine present is bound by the immobilised antibody. Any unbound proteins are washed away after which an enzyme-linked polyclonal antibody specific for the cytokine is added to 'sandwich' any cytokine immobilised at the first incubation. Any unbound antibody-enzyme reagent is washed away and a substrate solution is added to the wells. A colour develops which is proportional to the amount of cytokine bound in initial step. For IFN-γ a tertiary antibody (biotin-labelled donkey-anti-goat Ig) is used and a

streptavidin-peroxidase reaction converts a chromagen which changes the absorbance of the solution.

An optical density standard curve is calculated using the standard at different concentrations. The test sample optical densitiy is read against the standard curve, allowing the concentration of the cytokine in the unknown test sample to be calculated.

2.6.1 IL-1α(Quantikine, R&D Systems, Abingdon, UK)

Reagents

1. 20ml of the wash buffer concentrate was diluted with distilled water to prepare 500ml of wash buffer.

2. The colour reagents A and B were mixed in equal volumes to form the substrate15 minutes prior to use. 200μl of the mixture was required per well.

3. IL-1α standard was reconstituted with the appropriate calibrator diluent and diluted in the concentrations 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 7.8 pg/ml, 3.9 pg/ml and 0 pg/ml.

Assay procedure

1. 200μl of the standard or test sample was added to each well and the plate is covered with an adhesive strip and incubated for 2 hours at room temperature.

2. Each well was washed twice with the buffer via an automatic washer.

3. 200μl of IL-1α conjugate was added and left to incubate for 1 hour at room temperature.

4. The wash as in step 2 was repeated.

5. 200μl of the substrate was added to each well and incubated at room temperature for 20 minutes.

6. 50μl of the stop solution was added to each well and the plate tapped gently to ensure complete mixing.

7. The optical density of each well was read using a spectrophotometer set to 450nm with a wavelength correction set to 540nm.

The sensitivity of the assay i.e. the minimum detectable dose of IL-1α, was 0.2pg/ml.

2.6.2 IL-8 (Quantikine, R&D Systems, Abingdon, UK)

Reagents

1. Wash buffer and colour reagents were prepared as for IL-1α - see above.

2. IL-8 standards were reconstituted with appropriate calibrator diluent and diluted to 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml and 0pg/ml.

Assay procedure

1. 100μl of the assay diluent was added to each well.

2. 100μl of the standard or sample was added to the well and mixed gently for 1min. The plate was covered and left to incubate for 2 hours at room temperature.

3. Each well was washed twice with an automated washer.

4. 200μl of IL-8 conjugate was added and left to incubate for a further 2 hours at room temperature.

5. The wash was repeated as in step 3.

6. 200μl of substrate solution was added to each well and incubated for 20min.

7. 50μl of stop solution was added.

8. The optical density of each well was read using a spectrophotometer set to 450nm with a wavelength correction set to 540nm.

The sensitivity of the assay was 18.1pg/ml.

2.6.3 IFN-γ (Genzyme Corporation, Cambridge, USA)

Reagents

1. The primary antibody was coded by the manufacturer.

2. IFN-γ standards were reconstituted with the appropriate diluent to 6400pg/ml, 3200pg/ml, 1600pg/ml, 800pg/ml, 400pg/ml, 200pg/ml, 100pg/ml and 0pg/ml.

3. The secondary antibody was polyclonal goat anti-human IFN-γ.

4. The tertiary antibody was polyclonal anti-goat -biotin.

5. Streptavidin-peroxidase.

6. Substrate buffer and OPD (chromagen).

7. 2N sulphuric acid as stop solution.

Assay procedure

1. The microtitre wells were coated with the primary antibody and incubated overnight at 4°C.

2. The coating antibody was washed twice with an automated washer and blotted dry.

3. 100μl of the standard or sample was added to each well . The plate was covered and left to incubate for 2 hours at 37°C.

4. The wells were aspirated and washed as in 2.

5. 100μl of the diluted secondary antibody was added to each well and incubated at room temperature for 2 hours.

6. The wash was repeated as in step 4.

7. 100μl of the diluted tertiary antibody was added to each well and incubated at room temperature for 45min.

8. The wash was repeated as in step 4.

9. 100μl of the diluted streptavidin-peroxidase solution was added to each well and incubated at room temperature for 45min.

10. The wash was repeated as in step 4.

11. The substrate was prepared with the chromagen, OPD. 100µl of this reagent was added to each well.

12. 100µl of the sulphuric acid stop solution was added to each well.

13. The absorbance was measured at 492nm.

The sensitivity of the assay was 100pg/ml.

2.7 Langerhans cell studies

2.7.1 Immunofluorescence staining

Intact whole epidermal roofs were incubated in mouse monoclonal anti-CD1a antibody (Dako T-6, Dakopatts, Denmark) diluted 1:100 in phosphate-buffered saline/0.1% bovine serum albumin (PBS/BSA), overnight at 4°C. The sheets were washed in PBS/BSA and incubated in fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Sigma Chemical Co., St. Louis, USA) diluted 1:40 in PBS/BSA for 1 hour at room temperature. The sheets were washed again and mounted in Citifluor antifadent mountant (Citifluor Ltd., London, U.K.). The coverslip was sealed with nail varnish to prevent oxidation of the FITC. Staining was not observed in controls without the primary antibody.

2.7.2 Immunohistochemistry (performed by study co-workers R. Forsey and C. Sands; counts additionally performed by Dr. Sarah Howie)

Six micron cryostat sections were air-dried, fixed in acetone and stained using a standard procedure in a Sequenza semi-automated staining system (Shandon Laboratories). Sections were preincubated with normal rabbit serum to block non-specific binding, incubated with monoclonal anti-CD1a (DAKO UK Ltd) washed

and incubated with avidin-biotin-alkaline phosphatase complex (DAKO UK Ltd), washed and visualised with fast red (SIGMA UK Ltd) and counterstained with haematoxylin. The Zeiss HOME microscope was used to measure the total length of basement membrane for each section examined.

2.7.3 Confocal laser scanning microscopy

The Zeiss LSM 10 confocal microscope was used to examine the fluorescently labelled CD1a+ LC. This system uses an argon laser (λ=488nm) to excite the FITC, and photomultipliers to measure the signal returning from the specimen.

The sheets were optically sectioned in the z-plane and images (512x341 pixels) were collected at a scan rate of 2 seconds per scan. The whole intensity of the laser was used without a neutral density filter.

All sections were examined by one investigator (the author). Epidermal sheets were stained as above and 5 low power fields (lpf) per sheet were scanned using a Zeiss x40 water immersion lens and x20 zoom (x800 magnification). In each field 5 optical sections each of 4μm thickness were scanned and summated to produce a composite section (see **Fig 2.1** below).

For morphological assessment CD1a+ LC were examined at high power using a x40 objective lens and x80 zoom (x3200 magnification-high power field). For each LC, 40 optical sections were scanned each of 0.5μm thickness. The first section in the series was always positioned immediately above the cell and scanning continued until no further fluorescence was visible.

Fig 2.1 Optical sectioning with the confocal laser scanning microscope. Area of interest is sectioned in five segments and added together producing a composite image.
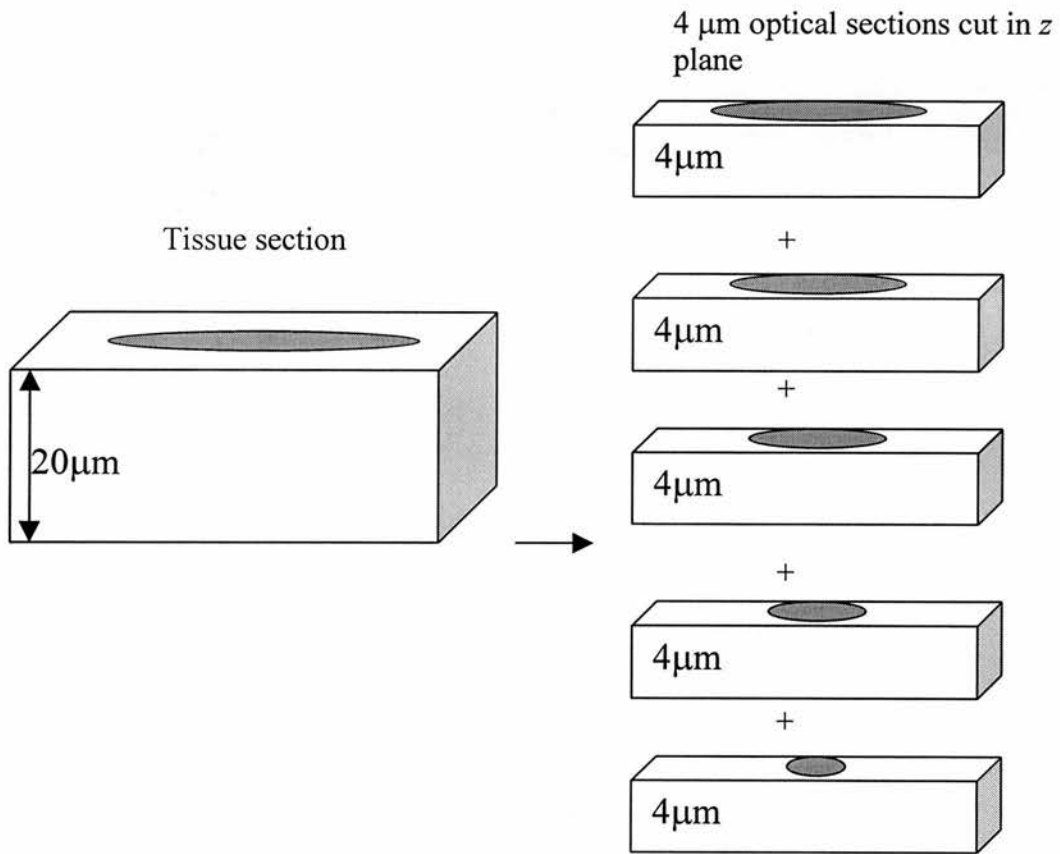
The images were analysed by a Kontron IBAS 20 image analysis system. A threshold of 32-35 was set for image data (arbitrary scale 0-255). The relative percentage of fluorescence and number of CD1a+ LC / mm^2 was measured on each composite lpf image (equal to 7.6×10^{-2} mm^2). The number of dendrites/LC and lengths of dendrites were measured on each composite high power field image, after calibrating the field using a graticule of 10μm length.

Statistical analysis

A two way unbalanced analysis of variance (ANOVA on SPSS for Windows software) was used to test the difference in the number of CD1a+ LC between patients & volunteers. This also tested for the effect of time compared with normal healthy controls (Time=0h). The factors were time and group (patients or volunteers) and the variable was the number of LC (log no. of LC used in analysis). An unbalanced test was used because of the different numbers in each group. The same method was used to look at the difference in number of dendrites/ LC and mean length of dendrites between the same groups. 2-tailed Mann-Whitney U-tests were used to test the difference in the number of functional CD1a+ LC between the control forearm site and the vehicle or irritant reaction site at the different time points.

2.8 Skin Window chambers

2.8.1 Background

It was during the search for an in-vivo technique to allow the evaluation of the role played by lymphocytes and monocytes in acute inflammation, that Rebuck, published his skin 'window' method (Rebuck, 1955). The volar part of the forearm was cleaned with alcohol and a scalpel used to abrade 3-4mm of the skin. The abrasion was continued until fine bleeding points were seen. This was felt to occur at the level of the papillary dermis. The inflammatory test agent was placed over the denuded area and a sterile glass cover slip placed over it. This was kept in situ with cardboard and surgical tape.

The inflammatory cell exudate migrates to the under surface of the coverslip. After 30-60min the coverslip is rapidly air-dried and a new coverslip is placed over

the abrasion. The coverslips were stained with May-Grunwald or Wright-Giemsa stains, thus allowing detailed examination of the nature of the cellular infiltrate. Although the Rebuck skin window yielded very useful qualitative information about the induced inflammatory cell infiltrate, little quantitative data was obtained. Moreover, it was later believed that the method itself may actually represent a foreign body reaction, rather than the early phase of the neutrophilic host response. Several groups have since modified the technique to obtain quantitative data (see Table 2.1 below). The use of scalpel abrasion was gradually phased out and dental drill abrasion substituted. Another modification of the technique was instituted to try and reduce the wide variation in the leucocyte mobilisation rate (Mass et al, 1975). In their method, the skin of both forearms was pre-hydrated with wet gauze for one hour prior to tape stripping. The skin was sterilised and a Transpore template with two holes, attached to the skin. Blenderm tape stripping was repeated 50-100 times until the skin glistened. Plastic skin chambers were securely fixed to the stripped site and ports at either end of the chamber, enabled culture medium to be withdrawn and inserted at different time points.

The variables that have been studied in the mobilisation of leucocytes include the sampling time and the nature of the chamber medium. Gowland (1964) sampled several chambers in healthy volunteers and found negligible leucocytes in the first 5hours. However all of the above groups have shown that 24hours is sufficient to pick up the maximum yield of leucocytes. The different chamber media used by Senn included autologous serum, buffered normal saline and saline with streptokinase/streptodornase. The maximum yield was from 100% autologous

serum. The predominant cell in the leucocytic infiltrate was the neutrophil (90-95%).

Table 2.2 A summary of previous leucocyte mobilisation studies

Group	No.of subjects	Chamber type	Chamber medium	Abrasion method	Leucocyte mobilisation rate/cm^2 (LMR)
Gowland, 1964	5	plastic 0.6ml	autologous serum	scalpel	2.5-6.3 x 10^6 / 7h
Senn, 1975	63	plastic 2ml	autologous serum	dental drill	73.1 x 10^6 / 24h
Mass et al, 1975	16	plastic 2ml	diluted autologous serum	tape stripping	1.43 x 10^6 / 24h
Wandall, 1980	21	plastic 2ml	diluted autologous serum	scalpel	69 x 10^6 / 24h

The skin window technique has been adapted to study many different in-vivo physiological and disease processes. Lawlor et al (1990) examined cytokine levels in chamber fluid of lesional and non-lesional skin of patients with mycosis fungoides. Brain et al investigated leukotriene activity in psoriatic skin (Brain et al, 1984). Of particular interest to the field of contact dermatitis was the investigation of the influence of nickel sulphate when added to the autologous serum just before insertion into the chamber (Lerche et al, 1981). The subjects used were both nickel sensitive (confirmed by patch testing) and normal volunteers. Their results showed that the LMR (predominantly polymorphs) in the control chamber of Ni-sensitve patients was no different from the LMR of healthy volunteers. There was however, an increase in the LMR of the nickel containing chambers of Ni-sensitive subjects

compared with their own control arm, but only after 15hours. Lerche et al concluded that for the nickel concentration used, only local LMR is altered and that the method is an effective way of investigating the cytological nature of the exudate in the evolution of a contact eczematous reaction.

2.8.2 Sellotape stripping

The method has been adapted from Mass et al (1975). Volunteers were used to refine the technique. A 12mm Finn® chamber containing 34µl of 5%SLS was applied under occlusion to the volar aspect of the forearm. This was removed after different times and then covered by a Tegaderm® template with 12mm hole, which ensured a consistent stripping site. The site was repeatedly stripped with Sellotape™ and an effort made to ensure new tape came into contact with the skin. Tape stripping was continued until the skin glistened. The variables which were changed, included the number of strippings, the volume of irritant in the patch and the duration of irritation. A similar area of non-irritated forearm skin was stripped as a control.

2.8.3 Skin window chambers

The skin 'windows' were purchased from FAR Italia (Verona, Italy). These are plastic chambers which have an adhesive seal on the base and a detachable stopper in the roof, allowing the withdrawal and insertion of chamber medium. The diameter of the base of the chamber was 11mm which ensured that the whole of the tape-stripped site would be covered. After the chamber was stuck down, it was secured with more adhesive tape and finally bandaged with Coband. Once the chamber was in-situ the subject could move around freely (see **Plates** 2.7 and 2.8).
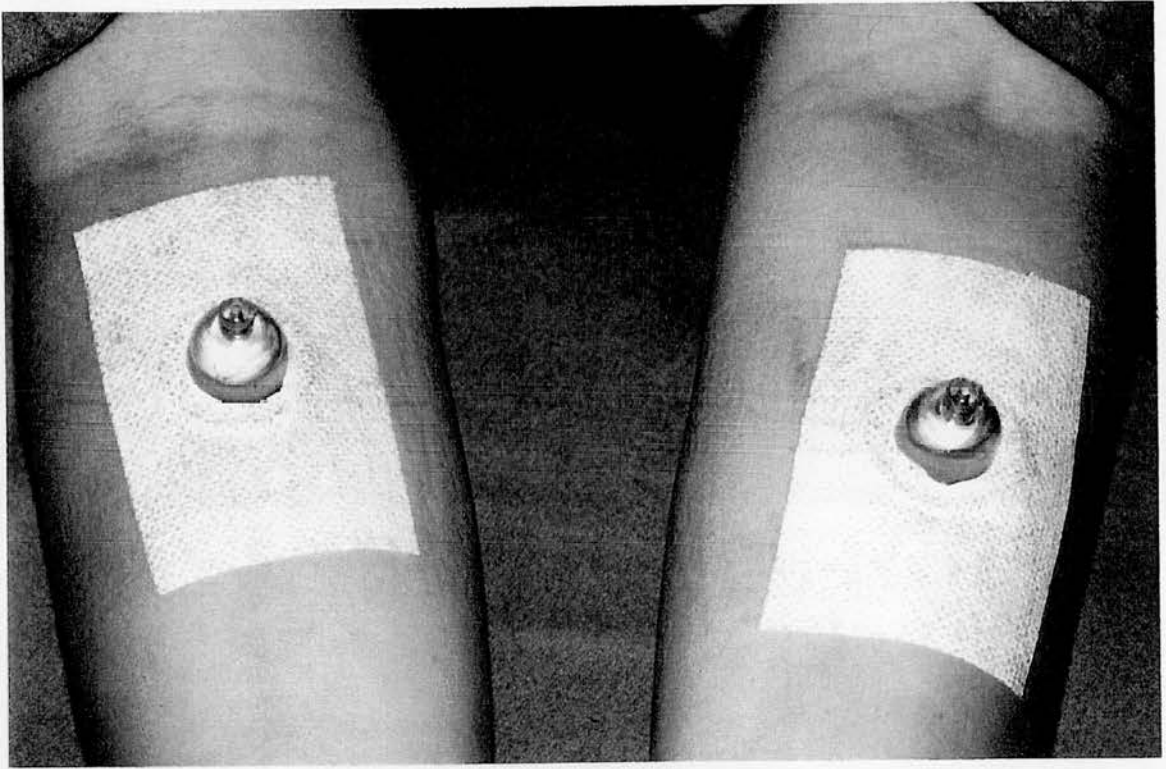
PLATE 2.7 Skin window chambers on the forearms containing autologous serum
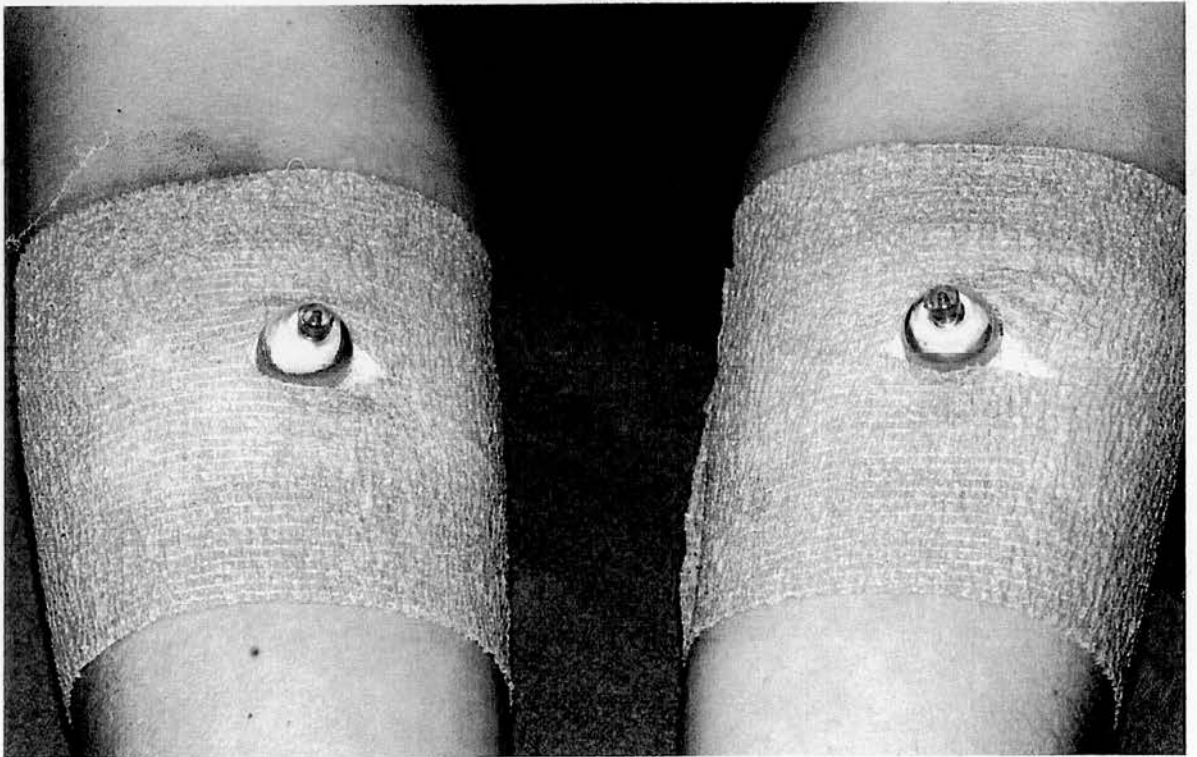
PLATE 2.8 Skin window chambers bandaged with Coband allowing free movement
of arms

The skin window chamber was filled with 1ml of autologous serum and left in-situ for upto 24h. The chamber medium was sampled at various time intervals and washed out with a 1ml aliquot of 0.9% saline to remove all possible cells. Fresh autologous serum was then added after each wash.

2.8.4 Preparation of peripheral blood neutrophils

Twenty millilitres of venous blood was removed into a citrated bottle and 5ml dextran added to it. The cells were allowed to sediment for 30minutes and the upper layer withdrawn into a separate tube. 5ml of normal saline was added and left for 5minutes before the solution was spun (1000revs/min for 5min). The cells were resuspended and 5ml hypotonic (0.18%) saline added for 30secs followed by hypertonic (1.8%) saline for 30secs. The latter steps ensured lysis of any erythrocytes that may have been present. The solution was spun again as before.

Monoclonal antibodies (kindly donated by Dr. I. Dransfield)

Antibody	Source	Concentration/ dilution
Control-mouse MOPC	European collection for Animal Cell Cultures	Neat
CD11a (WAC70)	Dr J. Ross, Royal Infirmary, Edinburgh	10μg/ml
CD11b	Dr. N Hogg, ICRF, London	1/10
CD35 (E11)	Dr. N Hogg, ICRF, London	Neat
CD16 (3G8)	Dr. JC Unkeless, Mt Sinai School of Medicine, New York	Neat
CD62L (L-selectin)	Becton-Dickinson, Oxford, UK	1/10

2.8.5 Neutrophil surface marker immunoassays

1. The cells were spun and resuspended in 1ml of phosphate buffered saline (PBS).
2. 100μl of the test sample was added to each well (>50,000 cells) in duplicate.
3. 50μl of the monoclonal antibody was added to each well and left to incubate at 4º C for 30min.
4. The plate was washed with 100μl of PBS and spun at 1000revs/min for 90secs. The excess was discarded and resuspended with a vortex mixer.
5. The wash was repeated as in step 4.
6. 50μl of fluorescein isothiocyanate (1:20) was added and incubated at room temperature for 30min.
7. Repeated wash as in 4.

The sample was placed in a test tube with sufficient buffer wash to enable it to be read in the flow cytometer.

2.8.6 Flow cytometry

Cytometry is defined as the measurement of physical and/or chemical characteristics of cells. Flow cytometry is a process in which these measurements are made while the cells or particles pass, preferably in a single file, through the measuring apparatus in a fluid stream as shown below.
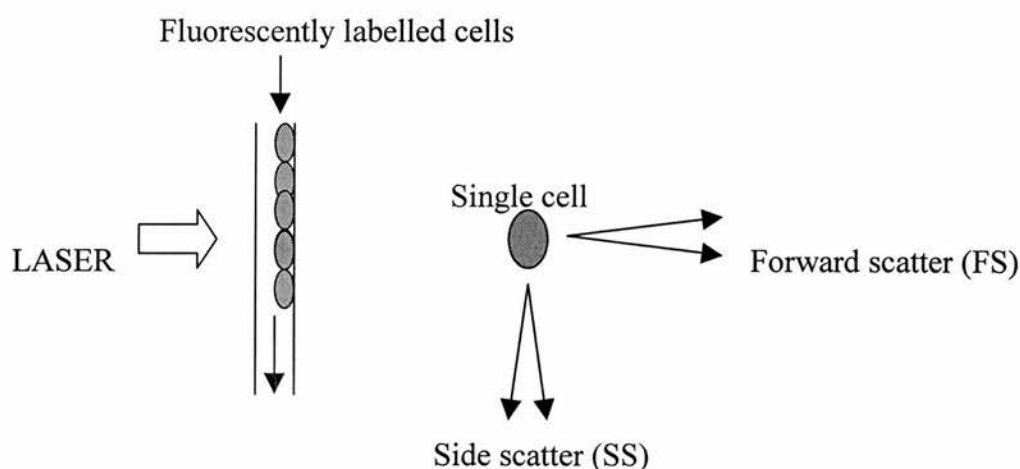
This provides quantitative data for determination of cell surface molecule expression. Cells for analysis were gated on the basis of the distinct forward and side scatter characteristics for neutrophils (see **Fig 2.2** below).

Fig 2.2a) Flow cytometry profile for peripheral blood FS= forward scatter; SS=side scatter; **b)** Histogram of the observed fluorescence for neutrophils which allows calculation of the percentages of cells with fluorescence greater than unlabelled cell populations

a)

N=neutrophils   L= lymphocytes

FS

SS
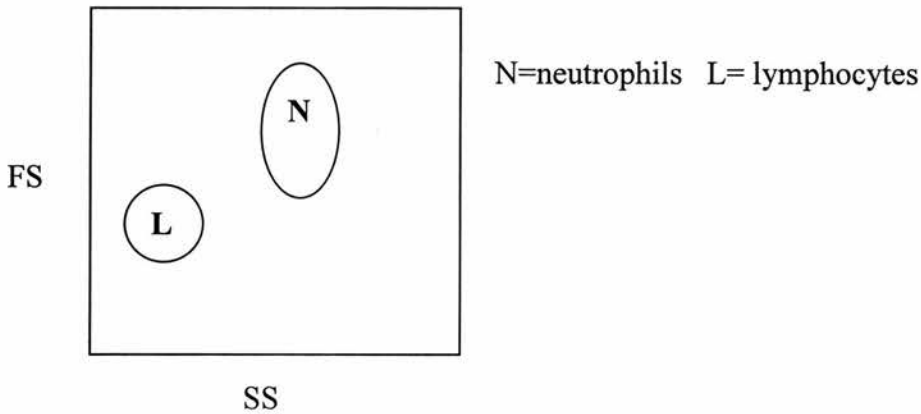
b)
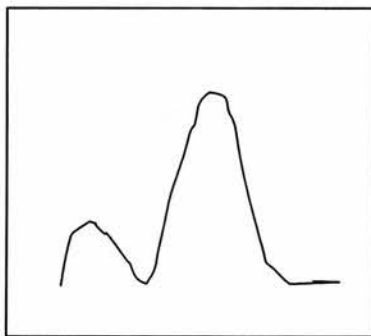
Log fluorescence

CHAPTER 3. A STUDY OF THE HISTOPATHOLOGY OF EXPERIMENTAL IRRITANT CONTACT DERMATITIS

3.1 Introduction

It is often very difficult to distinguish between allergic and irritant dermatitis on clinical grounds and this is also true histologically (Lever, 1975). Irritant reactions are more variable than allergic reactions. Early studies with guinea pigs found that high concentrations of dinitro-chlorobenzene (DNCB) induced partial necrosis and epidermal separation by 12 hours, and vesiculation with a neutrophilic infiltrate by 48 hours (Nater, 1976). A previous human study using Dettol as the irritant, found a predominantly lymphocytic infiltrate at 24 hours and 48 hours (Turk et al, 1966). It was Bjorneberg who significantly advanced the knowledge in this field. In his study of irritants and hand dermatitis he suggested that the histopathological changes vary in accordance with the mode of action of the irritant (Bjorneberg, 1968).

A summary of some of the important human studies are shown in Table 3.1 below. In most of these studies the focus has been on histopathological changes after 48 hours of irritation. We therefore decided to investigate systematically, the cutaneous histopathological changes between 1-48 hours after experimental irritation with nonanoic acid, sodium lauryl sulphate and dithranol.

Table 3.1 Summary of the relevant previous studies on the histological changes in ICD

Group	Irritant	Time of biopsy	Epidermal Changes	Dermal changes
Nater & Hoedemaeker 1976	DNCB 3-90 μg/cm^2	not stated	Minimal spongiosis Necrosis at high concentrations	perivascular mononuclear infiltrate
Reitamo et al 1981	HgCl$_2$ (0.2-0.4%) Benzalkonium chloride 0.2%		Epidermal necrosis	Sub-epidermal blistering Scanty upper dermal T lymphocyte infiltrate
Willis et al 1989	NA 80% Dithranol 0.02% SLS 5%	48h	Abnormal keratinocytes Minimal spongiosis Basal spongiosis few mononuclear cells Parakeratosis vesiculation	- - -
Willis et al 1993	SLS 5% Dithranol 0.02% NA 80%	48h	↑neutrophils, ↑lymphocytes ↑neutrophils, ↑lymphocytes ↑neutrophils only	All upper dermal perivascular infiltrate, mainly lymphocytes

3.2 Aim

To characterise the histopathological changes in the skin of patients with chronic ICD after the induction of experimental irritant reactions.

3.3 Materials and methods

Patients

Sixty-six patients who had been diagnosed as suffering from chronic ICD were recruited (see section 2.1) from the departments of Dermatology at the Royal

Infirmary of Edinburgh and the Victoria Hospital, Kirkcaldy. The breakdown of their specific diagnoses is shown in Appx. 2. The majority of patients had hand dermatitis. Atopic individuals were diagnosed by the UK Working Party's diagnostic criteria (Williams et al, 1994) as detailed in section 2.1

Irritant reactions

These were induced as described in section 2.2.2

Punch biopsies

These were taken as described in section 2.5.1 and coded so that laboratory staff were blinded to the timing of sampling and type of irritant used.

Assessment of erythema

The initial 27 patients had their reactions assessed clinically, prior to the use of the Minolta Chroma meter. Each irritant patch test site was read in triplicate.

Assessment of biopsies

As described in section 2.5.1

The appraisal of all the biopsies was carried out by 3 observers (C. Sands, R.Forsey and the author) who were unaware of the source of the biopsies, as these were coded. To reduce the inter-observer variation, 30 test sections were graded using a semi-quantitative method. In order to aid this task, a special proforma was devised, which was refined after collaboration with other members of the research team (see Appx. 3). This also facilitated the storage of results on a computerised database.

The main features of interest in the epidermis included oedema (intracellular and spongiosis/vesiculation), parakeratosis, apoptosis and any cellular infiltrate. For oedema, this ranged from no visible oedema (grade 0) to a maximum of grade 3,

which implied involvement of greater than 3/4 of the test section. A binary system was used to record the presence or absence of a cellular infiltrate. The numbers of apoptotic cells were arbitrarily ranked with the highest grade reserved for greater than 10 cells. Apoptotic cells were defined as those cells in the epidermis below the granular layer with 2 or more chromatin clumps. The three observers had received prior tuition from Dr. M. Arends, Department of Pathology, University of Edinburgh, who has a special interest in apoptosis.

The main dermal changes recorded were the nature and site of any inflammatory infiltrate. The site (if periappendageal) was felt to be relevant because it has previously been believed that irritants acted by traversing down the hair follicles on the skin.

The 3 assessors, using a triple-headed microscope, reviewed all sections with any doubtful or unusual features.

Statistics

The chi-squared test with Yates' continuity correction was applied to compare the distribution of a parameter in sections between irritants. In cases where numbers in a group was less than 5, Fisher's exact test was used. p values < 0.05 were considered to be statistically significant.

3.4 Results

Assessment of irritant reactions

All values for irritated sites are expressed as the Δa^* value for erythema of the patch test site compared with the adjacent normal forearm skin. For raw data see Appendix 4.

Fig. 3a. Colorimetric assessment for Nonanoic acid irritation with time
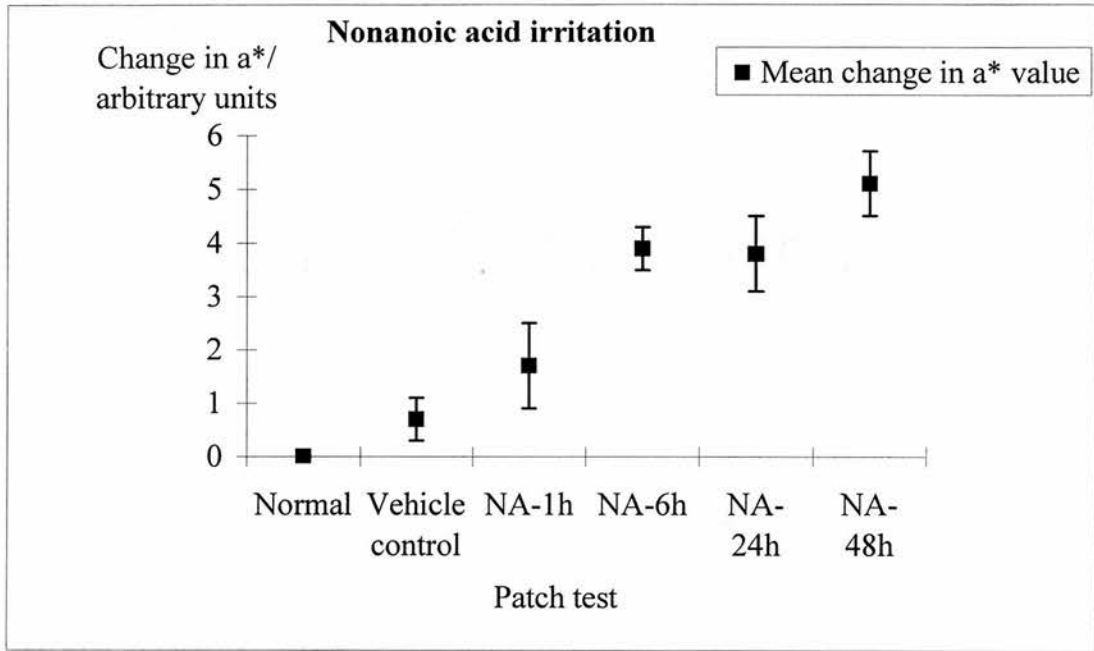
Fig. 3b. Colorimetric assessment of SLS irritation with time
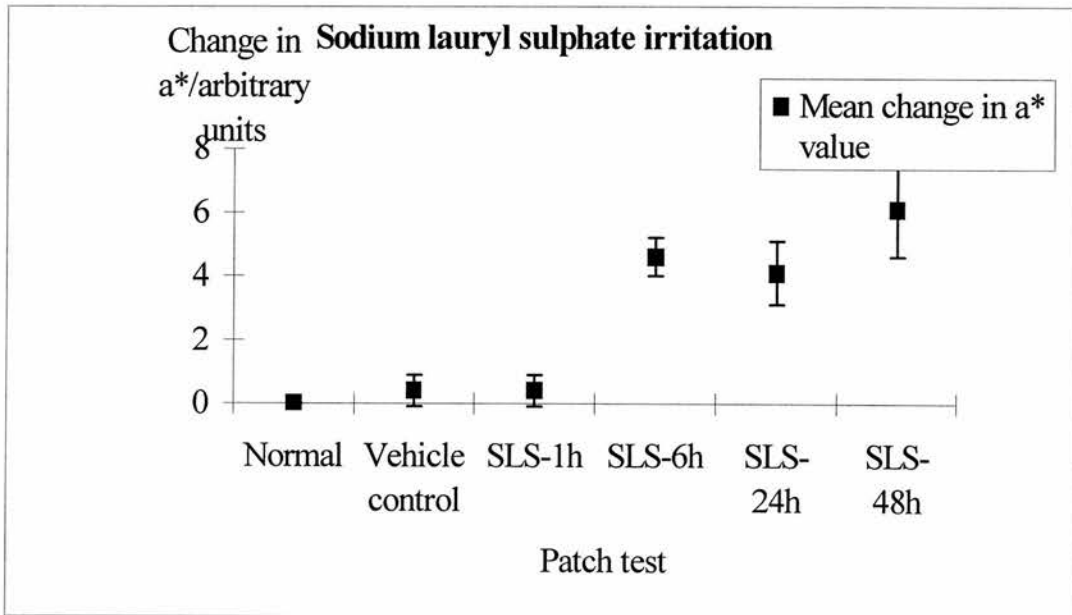
Fig 3c. Colorimetric assessment of Dithranol irritation with time
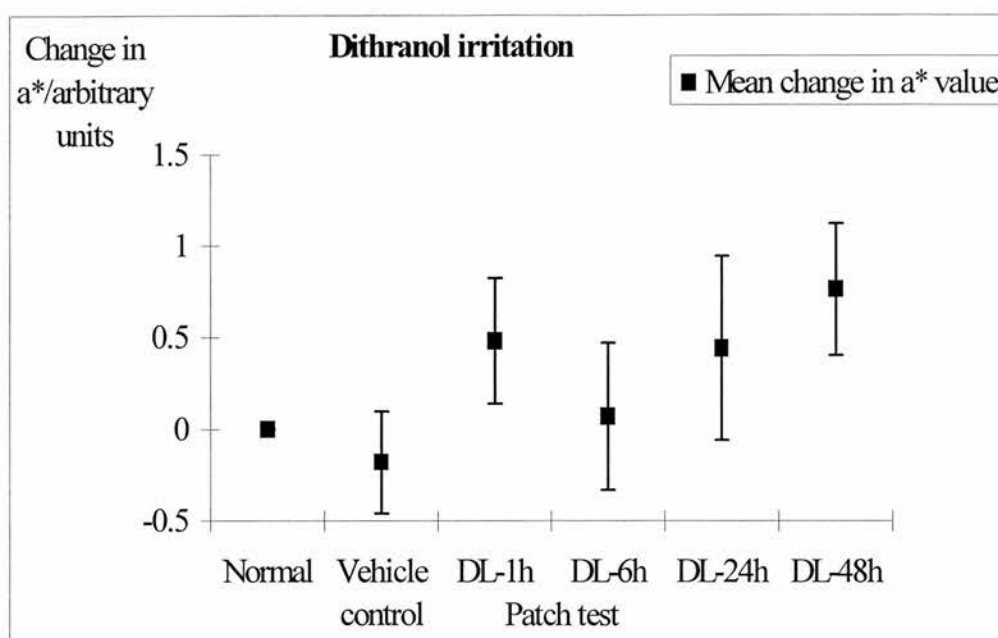
The different time-points were divided into two groups for ease of analysis: early (1 and 6h) and late (24 and 48h). To ensure irritant reactions between these groups were of a similar magnitude, the mean erythema values for each group were calculated.

Mean a* value (+/-SEM)

	NA	n	SLS	n
Early 1,6h	2.8(+/-0.5)	14	2.5(+/-0.7)	14
Late 24,48h	4.6(+/-0.5)	18	5.2(+/-1.0)	18

Assessment of biopsies

A total of 4 biopsies from each patient was taken which generated a total of 264 biopsies. One of these was lost due to the processing leaving 263 for analysis. The breakdown of the patients biopsied are as follows:

	No. of patients					
	NA		SLS		Dithranol	
	Atopic	Non-atopic	Atopic	Non-atopic	Atopic	Non-atopic
Early- 1,6h	7	10	6	10	6	10
Late- 24,48h	3	4	3	5	0	2

Vehicle controls

The main histological changes seen in 24 biopsies for the vehicle propanol (for NA) were mild intracellular oedema at all time points, a mild upper dermal perivascular mononuclear infiltrate and 1-5 apoptotic cells in 5 biopsies.

Distilled water (vehicle for SLS) caused negligible epidermal changes with mild spongiosis in 8/24 biopsies and a mild perivascular infiltrate in 14/24 biopsies.

Yellow soft paraffin (vehicle for dithranol) induced minimal change except for mild intracellular oedema in 13/18 biopsies.

Nonanoic acid – see **Plates** 3.1-3.3

The major epidermal change seen with NA was mild spongiosis at the early time-points, which became marked in all sections by 48 hours (Table 3.2). Parakeratosis was not a feature of NA irritation. There was no epidermal infiltration but there was a dermal inflammatory infiltrate. NA induced intravascular accumulation of neutrophils soon after irritation; these cells migrated into the papillary dermis (Table 3.2) at all time subsequent points. Eosinophils were seen in vessels and the papillary dermis but in smaller numbers than neutrophils.
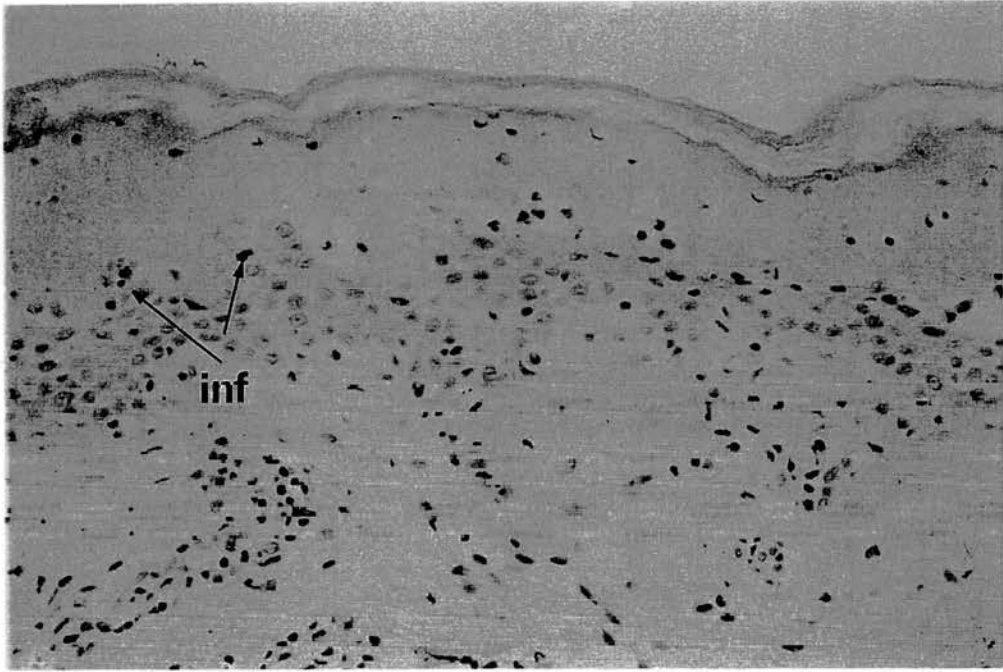
Plate 3.1 Nonanoic acid irritation at 24h showing an epidermal mononuclear infiltrate (inf)　　　x216
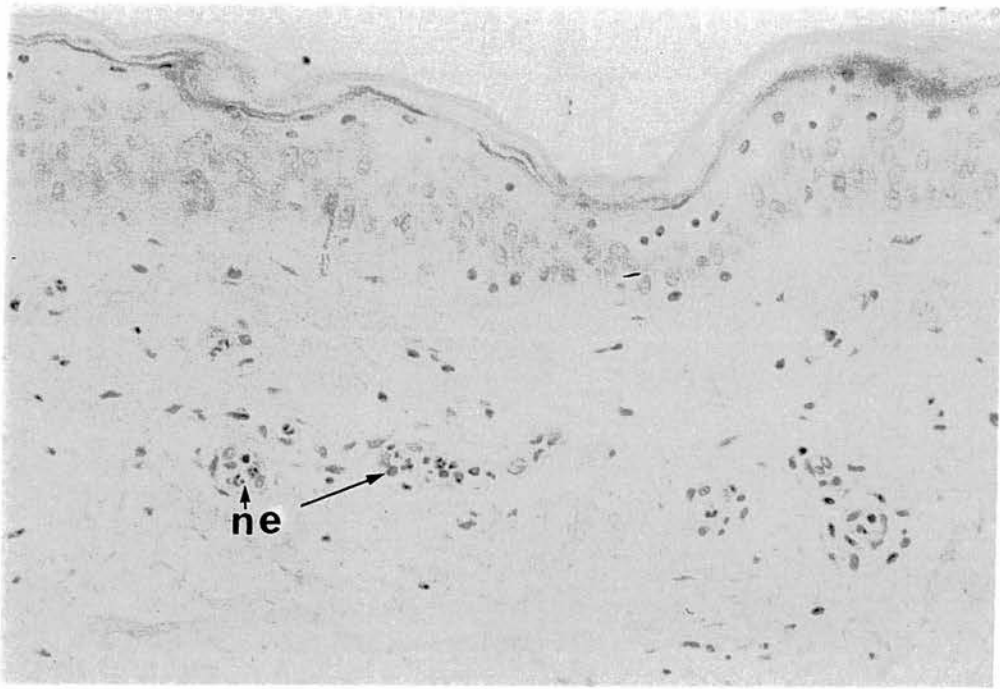
Plate 3.2 Nonanoic acid irritation at 6h showing a perivascular/intravascular neutrophilic infiltrate　　　x270
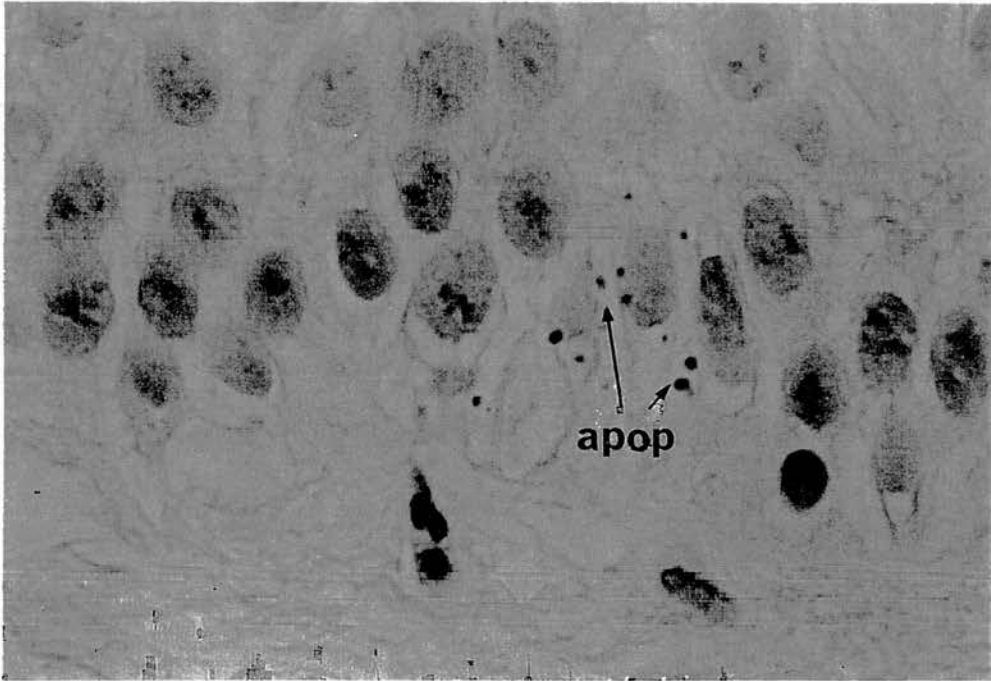
Plate 3.3 Nonanoic acid irritation at 24h showing more marked apoptosis (apop)
x1080
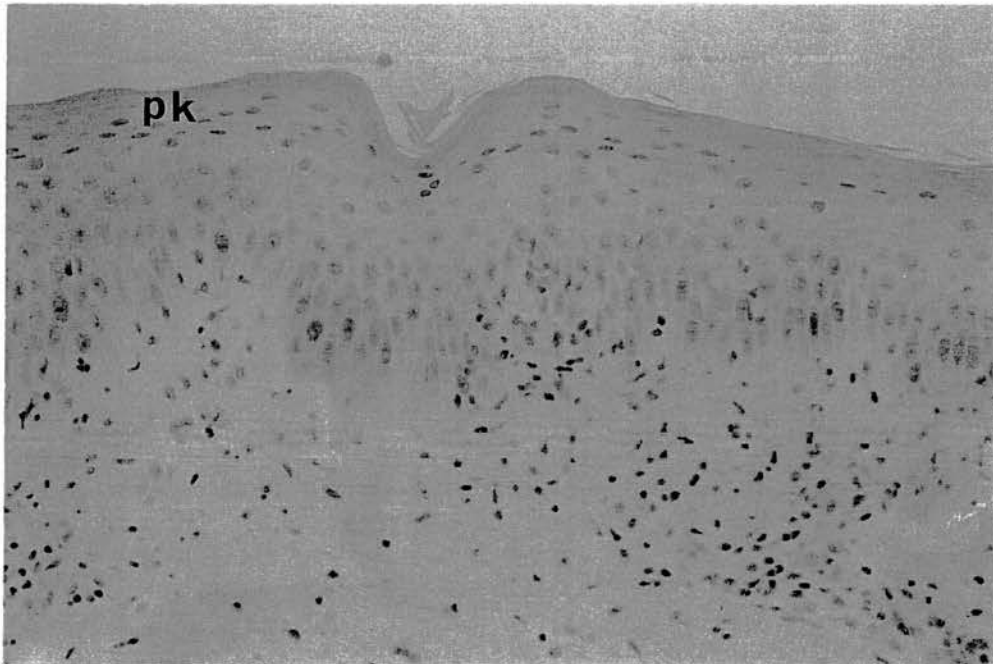
Plate 3.4 Sodium lauryl sulphate irritation at 48h showing parakeratosis (pk)
x216

<u>Sodium lauryl sulphate – see **Plates** 3.4-3.6</u>

SLS induced a basketweave hyperkeratosis and parakeratosis, the latter becoming

evident at 24 sand 48 hours (Table 3.2). The other major epidermal feature of SLS

irritation was epidermal intracellular oedema and marked spongiosis progressing to

vesicle formation at 24 and 48 hours. Basal layer apoptosis was seen in just 9% of

the early biopsies, a feature which contrasted with NA irritation at the equivalent

time point ($p<0.005$). However apoptotic cells were found in 31% of the late

biopsies. The pattern of epidermal infiltration contrasted with NA in that SLS

induced greater numbers of inflammatory cells at both early and late phases of

irritation. The dermal inflammatory cellular infiltrate was once again predominantly

mononuclear. However, the time course and pattern differed from NA. Neutrophils

were rarely seen early, unlike NA ($p<0.025$), but were observed by 48 hours

($p<0.05$). This was reflected by the neutrophil migration to the upper dermis, mainly

in the perivascular region. Upper dermal eosinophilia was found mainly in the later

stages of irritation.

<u>Dithranol - see **Plate** 3.7</u>

Dithranol evoked very few histopathological changes with the main feature being

intracellular oedema. It induced little or no inflammatory infiltrate in either the

epidermis or dermis.

From the erythema results and the lack of histological change, it was clear that the

concentration of dithranol used in this study did not induce significant irritant

reactions. The main objective of the whole study was to investigate inflammation in

the early stages of irritation. Since this was not being achieved (despite the results
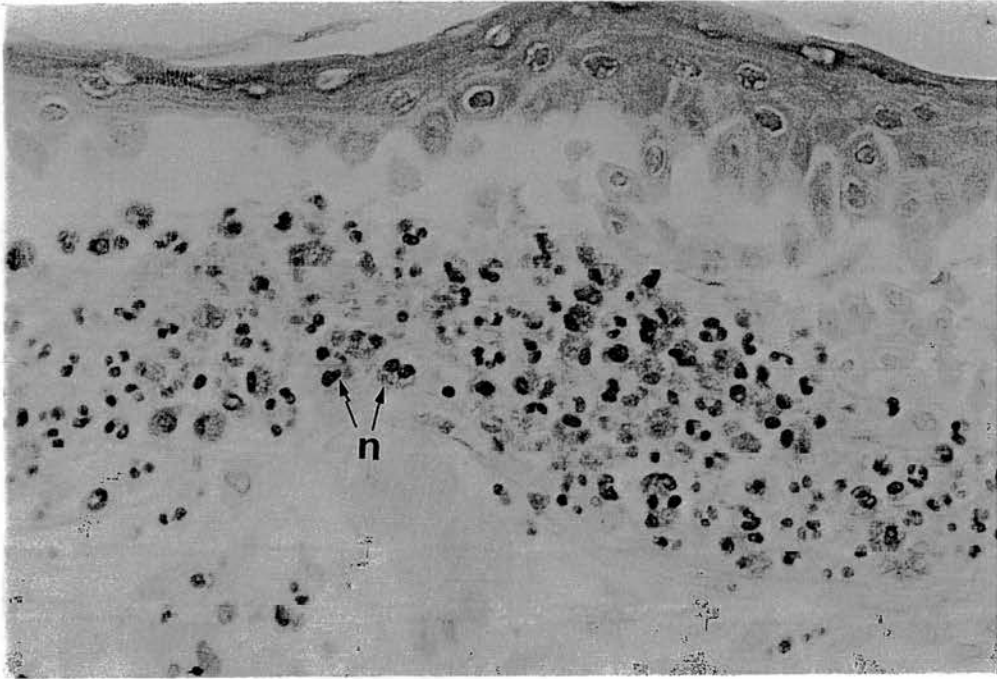
Plate 3.5 Sodium lauryl sulphate irritation at 24h showing epidermal neutrophils (n) in vesicle. x432
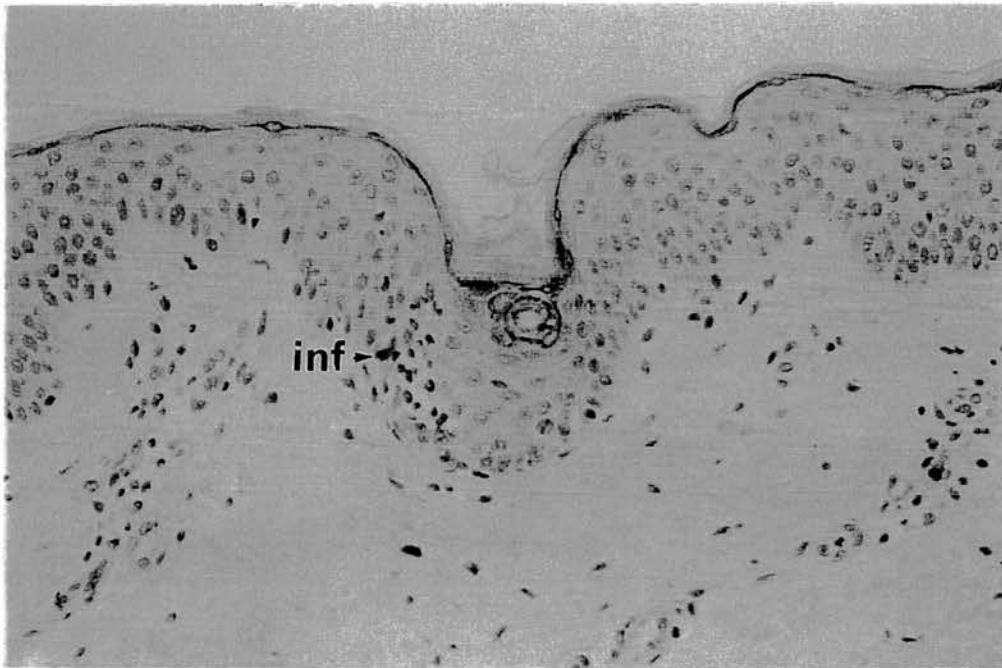
Plate 3.6 Sodium lauryl sulphate irritation at 24h showing basal mononuclear inflammatory infiltrate. x216
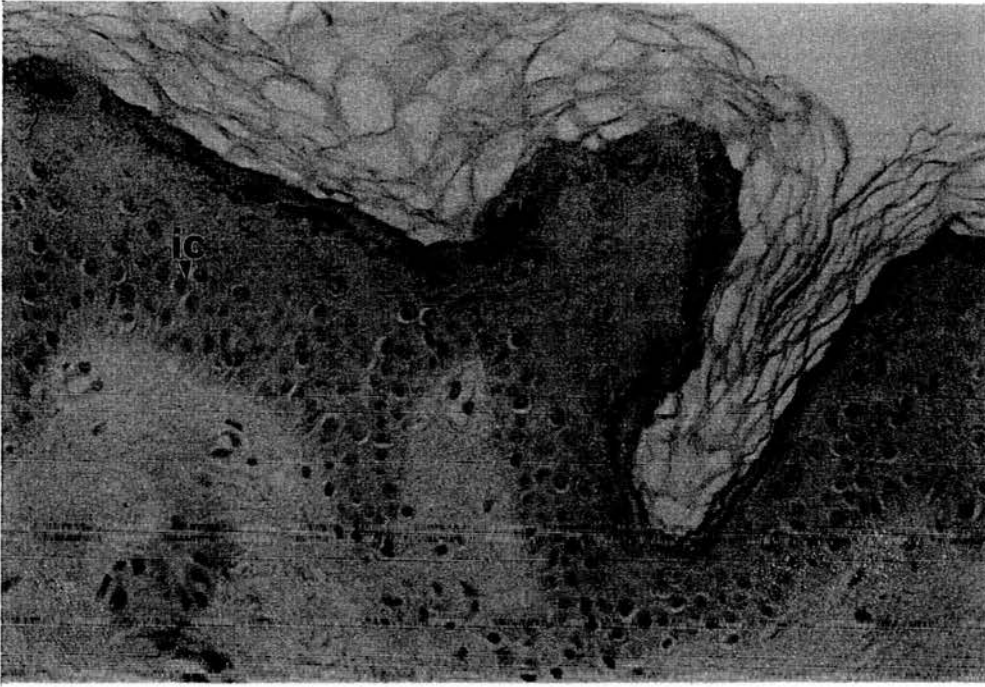
Plate 3.7 Dithranol irritation at 6h showing intracellular oedema (ic)   x216

from pilot studies which indicated 0.6% dithranol should induce irritant reactions), it was decided at this stage, that dithranol should be dropped from further study.

Table 3.2 Histological parameter assessment for nonanoic acid and sodium lauryl sulphate at 1, 6 hours (early) and 24, 48 hours (late) post irritation. Comparisons between irritants at a time point which are statistically significant are shown in bold. *p<0.05. [a] using Fishers exact test.

Histological parameter	% of positive biopsies EARLY		% of positive biopsies LATE	
	NA (n=32)	SLS (n=32)	NA (n=14)	SLS (n=16)
Parakeratosis	3	8	7	**44***[,a]
Basal cell apoptosis	**44***	**9**	64	31
Spongiosis (severe)	6	3	14	44
Vesiculation	9	9	**0**	**31***[,a]
Intravascular neutrophilia	**38***	**9**	7	**44***[,a]
Papillary dermal neutrophilia	**38***	**13**	43	50
Intravascular eosinophilia	13	6	0	19
Papillary dermal eosinophilia	16	13	7	**38***[,a]

For raw data see Appendix 5.

<u>Atopics and non- atopics</u>

NA-induced basal apoptosis was more common in atopic subjects (p<0.02) at the early phase of irritation. All other parameters tested were not statistically significant between the two groups.

When analysing atopic and non-atopic subjects after SLS irritation, there was no significant difference at 1&6 hours for all the parameters examined, but vesiculation occurred more frequently in atopics at 24&48 hours (not significant).

3.5 Discussion

This study has investigated early histopathological changes in the development of irritant contact dermatitis. The three irritants were chosen because of their different modes of action. Nonanoic acid alters the skin lipid profile and appears to have a consistent irritant effect (Stillman et al, 1975). Sodium lauryl sulphate, an anionic detergent, has multiple modes of action including denaturation of keratins and surface glycoproteins, removal of surface lipids and the impairment of barrier function by increasing the permeability to water (Kligman 1967, Middleton 1969). Dithranol, if applied to the skin, is oxidised by air, which results in the generation of free radicals such as superoxide anions. The latter are believed to be responsible for its irritation (Ashton et al, 1983).

A prominent feature of NA irritation in our study was marked spongiosis between 24 and 48 hours, contrary to previous reports (Willis et al, 1989). We did not observe the 'tongues of dyskeratotic cells' following NA irritation as described by Willis and her co-workers (Willis et al,1989). A finding in experimental ICD on which there have been no previous comments is apoptosis. Apoptosis is a form of

programmed cell death which does not provoke an inflammatory response. Basal cell and prickle cell apoptosis was a common finding in NA irritation (**Plate** 3.4).

Polymorphonuclear leucocytes are seen commonly in ICD induced in animals (e.g. guinea pigs), but have only been found, to any significant extent, in irritant reactions in humans induced by SLS and croton oil (Willis et al, 1993). The lack of neutrophil recruitment noted by Willis et al, who studied benzalkonium chloride, propylene glycol and the irritants used in our study, may reflect the timing of their biopsies as neutrophil migration takes place soon after irritation. This would be missed if biopsies are taken at 48 hours- the time Willis' group chose to study. In our study, NA induced the accumulation of both neutrophils (perivascular and papillary dermis) and to a lesser extent, eosinophils by 6 hours. The latter cell type is said to be found only in allergic patch test reactions and not in irritant reactions (Lachapelle, 1992).

We can confirm that SLS induces parakeratosis in the latter phase of irritation. This may in part be due to stimulation of epidermal mitosis (Fisher, 1975) or accelerated keratinisation. Sodium lauryl sulphate also produced epidermal vesiculation after 6 hours, which would be in keeping with the findings of Willis et al (1989). The degree of apoptosis, while not as marked as with NA, was observed at all phases of irritation due to SLS. The pattern of cellular infiltration after SLS contrasts sharply with that after NA. The former induces recruitment of neutrophils (and to a lesser extent, eosinophils) both into the vessels and into the papillary dermis, but not until 24 hours. Frosch showed that SLS can act as a chemoattractant *in-vitro* at concentrations of 10^{-3} to 10^{-8} % (Frosch, 1987). This would suggest that

despite 5%SLS being applied topically, the resulting *in-vivo* concentration of SLS was significantly lower than 5%.

The influence of atopy on irritation has been noted in many previous studies (Agner, 1991a;Van der Valk et al, 1985; Tupker et al, 1990) but surprisingly had little effect on the histopathological changes between the three irritants. It is possible that the numbers in the subgroups were too small to demonstrate any true differences.

Our study has thus revealed some important differences in the histopathological changes elicited when irritants are applied to the skin under patch tests. These differences are probably due to the varying chemical nature of the irritants. The different patterns of epidermal damage and inflammatory cell infiltrate induced by irritants imply that the inflammation may be mediated in different ways. In Chapters 4 and 5 the findings from the analysis of some key cytokines and antigen-presenting Langerhans cells in biopsies from this study and suction blisters at irritated sites, will be discussed.

CHAPTER 4. A STUDY OF THE CYTOKINES IN SUCTION BLISTER FLUID FOLLOWING INDUCTION OF AN IRRITANT REACTION

4.1 Introduction

As there were no previous known studies which had investigated the cytokines relevant to the mediation of inflammation in ICD, pilot studies were performed. The cytokines chosen were the key primary cytokines IL1-α, IL-2, IFN-γ and TNF-α. The results from the histological study had shown a mononuclear infiltrate in the majority of irritant biopsies and a neutrophilic infiltrate in the later SLS and NA biopsies. This prompted the inclusion of the macrophage cytokine MIP-1α and the chemotactic cytokine IL-8 in the screening panel.

The irritant reactions (erythema scores) elicited in the histopathology study showed that the most consistent dose-time response occurred with the irritant NA. This irritant was used for most of the suction blister studies.

4.2 Pilot study

Aim

To determine the level of cytokines in the suction blister fluid of volunteers after the induction of irritant reactions with 80% NA.

Materials and methods

Subjects: normal healthy volunteers from the departments of Dermatology and Pathology.

Irritant reactions: Reactions were elicited in the manner described in Chapter 2.2.2

Suction blisters: Suction blisters were raised and fluid withdrawn as outlined previously (see Chapter 2.5.2)

Cytokine assays

Solid phase cytokine ELISA (see Chapter 2.6) assays were performed for the
following: Source
IL-1α, IL-2, TNF-α, MIP-1α, IL-8   R&D Systems, Abingdon, U.K.
IFN-γ Genzyme, Cambridge, USA.

Results (see summary below)

The following shows the volumes of fluid obtained:

Cytokine	Subject (male or female)	Volume of blister fluid
IL-1α	J.C. (F)	155μl
	K.S .(F)	155μl
	J.B. (F)	150μl
IFN-γ	S.H. (F)	100μl
	C.S. (F)	60μl
	A.W. (M)	100μl
	S.R. (M)	100μl

Table 4.1 Cytokine protein levels detected after NA irritation ;
n= no of volunteers ; N.D. not detected; - assay not done

	Time after NA irritation		
	0	6h	24h
IL-1α pg/ml	N.D.	97.8	-
n	2	1	-
sensitivity	<0.2		
IFN-γ pg/ml	-	3.2ng/ml	1.8, N.D., N.D.
n	-	1	3
sensitivity	<100		
TNF-α pg/ml	-	N.D.	N.D.
n	-	1	1
sensitivity	<15.6		
IL-2 pg/ml	-	-	N.D.,N.D.,N.D
n	-	-	3
sensitivity	<31.3		
MIP-1α pg/ml	N.D.	N.D.,N.D.,N.D	N.D.
n	1	3	1
sensitivity	<46.9		
IL-8 pg/ml	-	0.6,1.6,2.5	-
n	-	3	-
sensitivity	<18.1		

Conclusions

There appeared to be no appreciable levels of IL-2, TNF-α or MIP-1α detectable between 1 and 24 hours of irritation with 80% NA. Even though the numbers of volunteers were small, significant amounts of IL-1α, IFN-γ and IL-8 were detected in blister fluid. Therefore these three cytokines were analysed in the main suction blister study as described below.

4.3 Suction blister cytokine experiments

Aim:

To determine any differences in cytokine protein levels between patients and controls following experimental irritation with nonanoic acid.

Subjects

Patients with chronic ICD were recruited as stated previously and informed consent obtained prior to the study. Normal healthy volunteers were recruited from the general public and from within the department of Dermatology.

Irritant reactions

These were induced on the forearm as outlined above. NA was applied under patch test occlusion for 1, 6 or 24h.

Suction blisters

These were induced on the forearm as outlined above. The blister fluid was stored at -40 °C for the cytokine assays.

Cytokine assays

The cytokine protein levels of IL-1α, IL-8 and IFN-γ, in suction blister fluid, was assayed by ELISA. The differences in cytokine levels at the different time points

were compared with the control (Time 0h) level by the non-parametric Mann-Whitney U-test.

Results

Thirty-six patients with ICD and thirty volunteers were studied. The breakdown of the irritant reactions and assays on these two groups is shown in Appendix 6. The majority of patients were tested after 1 hour of NA irritation. The clinical grading of the irritant reactions in patients, varied from a negative reaction (grade 0) to a well-defined erythema (grade 2) by 6 and 24 hours. The irritant reactions in volunteers were more even, with grade1 in all subjects after 1 hour, and grade 2 after 6 and 24 hours.

As with the pilot studies, the volume of suction blister fluid obtained from all subjects was limited, ranging from no blisters to 240μl. The two patients in whom blisters could not be raised (after 2 hours suction) were both atopic with lichenified forearm skin. The small volumes restricted the number of test samples for each cytokine assay.

IFN-γ

There was no detectable IFN-γ in patients or volunteers at 1 or 6 hours. Unfortunately no comment can be made for the 24 hour time point because the ELISA kit was found to be faulty at the end of the assay.

IL-1α

Following irritation at 1 hour both patients and volunteers had detectable levels of IL-1α. This fell slightly by 6h more in volunteers (not detectable in 3/4 subjects)

than in patients. However by 24 hours there was consistent detection of IL-1α in both patients and volunteers.

<u>IL-8</u>

There was no detectable IL-8 in the normal skin of both patients and volunteers. By 1 hour, IL-8 protein was found in only 2/8 patients and not detected in any volunteers. At 6 hours there was more IL-8 in volunteers, but by 24 hours there was no detectable IL-8 in patients and in only 2/8 volunteers.

Tables 4.2, 4.3 and 4.4 Cytokine levels in suction blister fluid after NA irritation

IL-1α pg/ml							
0h		1h		6h		24h	
Patient	Volunteer	Patient	Volunteer	Patient	Volunteer	Patient	Volunteer
	n.d.	275	319	246	324	155	63
	n.d.	296	38	352	n.d.	136	1017
		141	73	n.d.	n.d.	170	33
		417	116		n.d.	84	156
		n.d.				102	524
		n.d.				172	152
		n.d.		·		n.d.	n.d.
		n.d.					
		n.d.					

n.d. = not detected

IL-8 pg/ml							
0h		1h		6h		24h	
Patient	Volunteer	Patient	Volunteer	Patient	Volunteer	Patient	Volunteer
n.d.	n.d.	565	n.d.	1200	454	n.d.	650
n.d.	n.d.	454	n.d.	n.d.	624	n.d.	910
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.			n.d.	n.d.	n.d.
	n.d	n.d.			n.d.	n.d.	n.d.
	n.d.	n.d.				n.d.	n.d.
	n.d.	n.d.				n.d.	n.d.
		n.d.				n.d.	n.d.
						n.d.	

IFN-γ pg/ml					
1h		6h		24h**	
Patient	Volunteer	Patient	Volunteer	Patient	Volunteer
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.			n.d.	n.d.	n.d.
n.d.			n.d.	n.d.	n.d.
n.d.				n.d.	n.d.
n.d.				n.d.	n.d.
n.d.				n.d.	
n.d.				n.d.	

** assay kit faulty

Statistical analysis

The results for patients and volunteers appeared to be very similar and were thus

combined for both groups.

IL-1α	Time 0h v. Time1h	p=0.35	not significant (n.s.)
	Time 0h v. Time 6h	p=0.50	n.s.
	Time 0h v. Time 24h	p=0.07	n.s.

Fig 4.1 IL-1α secretion in suction blister fluid with NA irritation

87
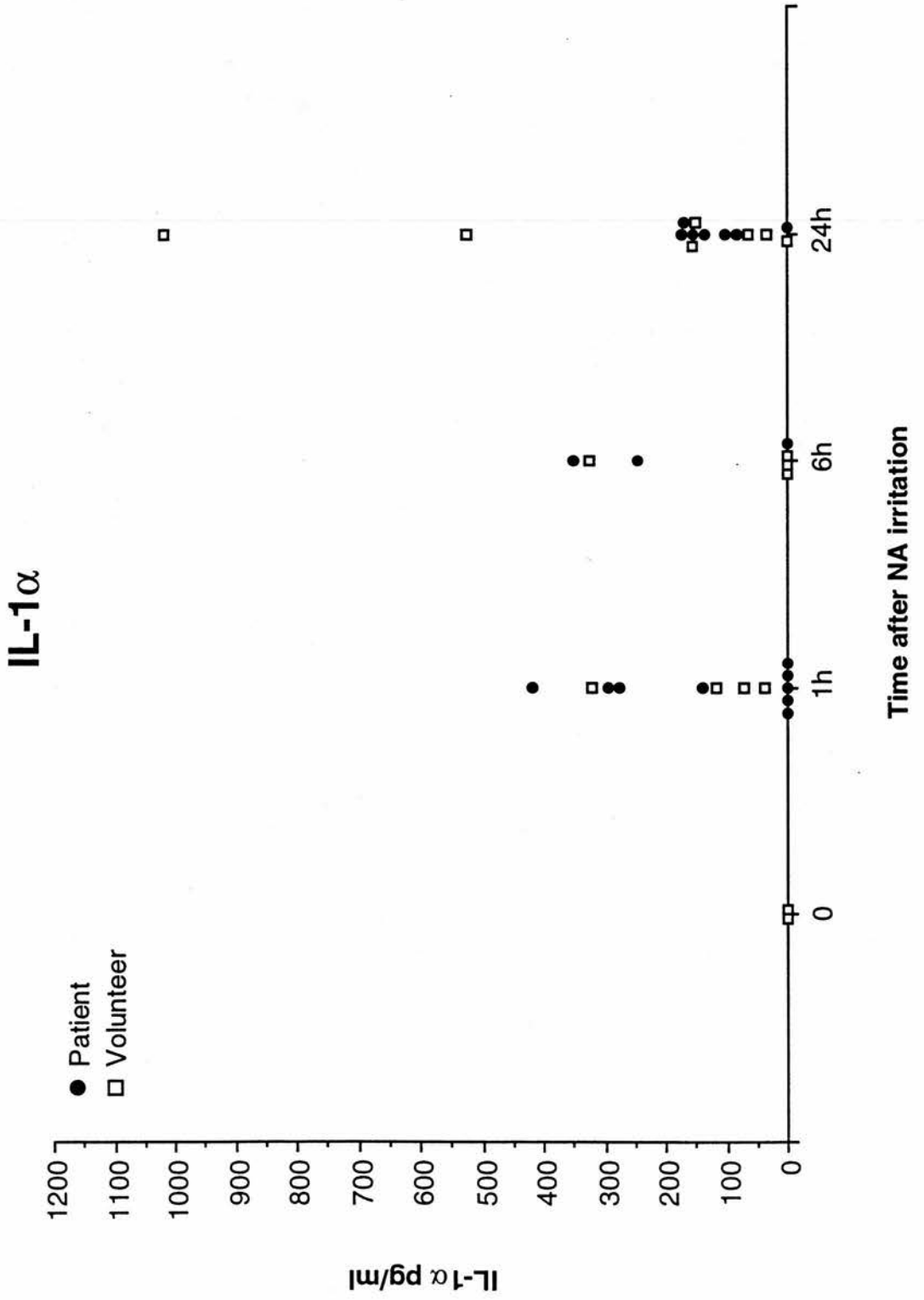
Fig 4.2 IL-8 secretion in suction blister fluid with NA irritation

88
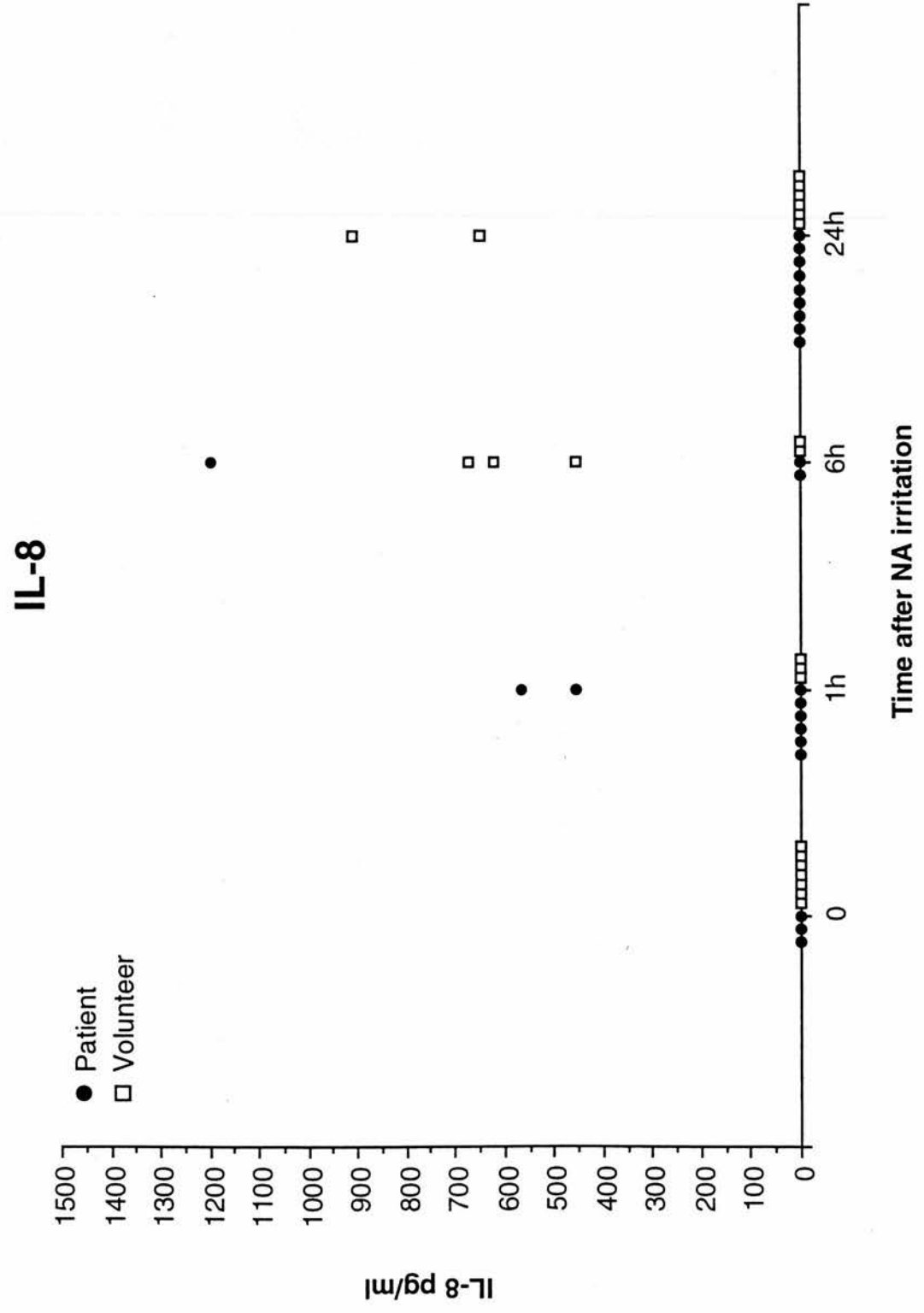
IL-8   Time 0h v. Time 1h   p=0.50   n.s.
                  Time 0h v. Time 6h   p=0.20   n.s.
                  Time 0h v. Time 24h   p=0.62   n.s.

Conclusions

There appeared to be no observable difference between atopic and non-atopic

subjects and hence the groups have been combined for analysis of data. Similarly the

patients and volunteer results were combined for statistical analysis.

The IFN-γ data was not totally surprising as previous groups have not been

able to show the presence of IFN-γ in keratinocytes. However, our research group

have demonstrated the presence of IFN-γ mRNA and protein in keratinocytes from

patients with allergic contact dermatitis who have been challenged, in vivo, with a

relevant antigen (Howie et al, 1996). One should note this only applied to biopsies

taken at 6h and that these results cannot be extrapolated to irritant reactions.

The IL-1α results could be explained by the release of pre-formed IL-

1α from keratinocytes at 1h, the stores being depleted by 6h. Furthermore at 24

hours the consistent production of IL-1α would suggest the induction of 'new' IL-

1α secretion by the irritant.

The IL-8 data are rather more difficult to explain. There was minimal, if any

IL-8 secreted in blister fluid from patients at all time points. In 3/5 volunteers there

was detectable IL-8 by 6h but this was not maintained by 24h. The histopathology

study results showed a different effect on granulocyte induction between NA and

SLS. The latter induced an epidermal neutrophilia, whereas NA induced a

perivascular and intravascular dermal infiltrate. Since the major effect of IL-8 is on

neutrophil chemotaxis, this might be reflected in the cytokine expression in irritated skin. The following experiment was performed to test this hypothesis.

4.4 IL-8 secretion with SLS

Aim:

To determine the level of IL-8 protein in patients with ICD and healthy normal volunteers.

Subjects

6 patients with ICD; 26 healthy normal volunteers

Methods

As for above suction blister experiments but using 5% SLS as the test irritant.

Results (see Table 4.5 and Fig 4.3)

The data for controls at Time 0h from NA irritation have been combined. There was no significant difference between the groups prior to SLS irritation. At 1h, 2/3 volunteers were inducing IL-8 secretion, which was being maintained at 6h and 24h. By 24h patients were also inducing IL-8.

Table 4.5 IL-8 secretion after SLS irritation

IL-8 pg/ml							
0h		1h		6h		24h	
Pt	Vol	Pt	Vol	Pt	Vol	Pt	Vol
n.d.	n.d.,n.d.		1153		1398	606	826
n.d.	n.d.,n.d		492		522	1109	315
n.d.	n.d.,n.d		n.d.		332	18515	222
	n.d.,n.d				314		251
	n.d.,n.d				548		1071
	n.d.,n.d				348		117
	n.d.,n.d				155		n.d.
	n.d.				n.d.		n.d.
	146						
	548						
	155						
	850						

Fig 4.3 IL-8 secretion in suction blister fluid with SLS irritation
*18515 pg/ml

IL-8	Time 0h v. Time 1h	p=0.10	n.s.
	Time 0h v. Time 6h	p=0.003	highly significant
	Time 0h v. Time 24h	p=0.02	highly significant

Conclusions

The limited number of samples from patients greatly affects the valid interpretation of the results. The trend, however, suggests there is little, if any, IL-8 in the uninvolved skin of patients and volunteers. IL-8 is then rapidly produced by 1h (2/3 volunteers) and found in most samples at 6h and 24h post SLS irritation, in both patients with ICD and volunteers.

If the data for SLS irritation is compared with NA irritation, there is a marked difference with SLS inducing IL-8 secretion in blister fluid in the first 24h of irritation, this being significant at both 6h and 24h in volunteers and at 24h in patients. This differential induction of IL-8 secretion would explain the presence of a neutrophilic infiltrate in the epidermis after SLS irritation seen in the earlier histopathology study.

CHAPTER 5. THE QUANTITATIVE AND QUALITATIVE ANALYSIS OF LANGERHANS CELLS IN CUTANEOUS NONANOIC ACID IRRITATION BY CONFOCAL MICROSCOPY

5.1 Introduction

The investigation of changes in epidermal Langerhans cells (LC) by conventional microscopy has been beset by problems of distortion of the tissue sample due to sectioning and fixation. It has been suggested that confocal laser scanning microscopy (CLSM) is a better method for investigating not only the morphology but also the numbers of epidermal LC (Scheynius, 1990; Van Oostveldt, 1990). In CLSM, a high intensity light source (usually a laser) is focused via an objective lens onto the specimen. The light emanating from the focal plane on the specimen, passes through a detector pinhole and the signal is amplified to produce a digital image. The optical system is used to scan the specimen in the x and y axis thereby building up an image, point by point i.e. an optical section (see Fig 5.1 below).

Fig 5.1 Confocal microscopy- a schematic diagram to show the physical principles.
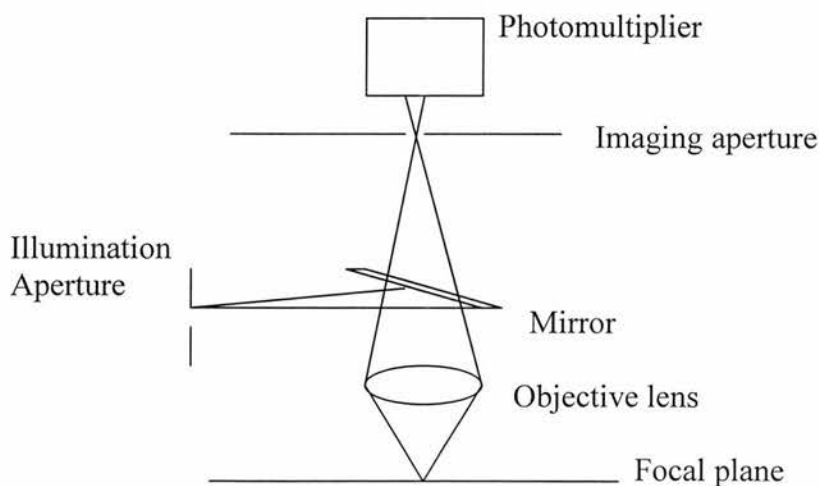
Optical sectioning has the following advantages:

- better contrast by rejecting light from out of focus planes (Hall, 1991)

- spatial relationships between cells to be examined by three-dimensional (3-D) reconstruction of such optical sections

- digital images may be stored.

In the present study, CLSM was used to determine qualitative and quantitative changes in the epidermal LC population during the first 24hours of induction of irritant reactions in both healthy volunteers and patients with chronic irritant dermatitis. Conventional immunohistochemistry was also used to quantify epidermal LC and to examine the direction of any movement of LC after irritation.

5.2 Materials and methods

Subjects

Forty six patients with chronic irritant dermatitis (for diagnostic criteria see Table 2.1, p.38) were recruited from the Contact Dermatitis clinic at the Royal Infirmary of Edinburgh. Twenty-three healthy volunteers with no past history of any skin disease or atopy were additionally recruited. There were 3-9 subjects in each group of patients and volunteers at all time points, for both quantitative and morphological analyses.

Experimental irritant reactions (see section 2.2.2 for details)

6 volunteers had no irritant applied. The remaining patients and volunteers had 120µl of
80% nonanoic acid in propan-1-ol applied under occlusion to normal looking skin of the
volar aspects of the forearms (with standard 8mm Finn chambers®) for either 1, 6 or 24
hours.

Patients having biopsies were divided into an "early"(n=16) and "late" (n=6) groups.
Three patch test chambers (one with 15µl of the vehicle, two with 15µl of nonanoic
acid) were applied to the forearms for 1-6 hours in the early group and for 24-48 hours
in the late group. All patients had a time zero biopsy taken from clinically normal
forearm skin.

Suction blisters and biopsies (see sections 2.5.1 and 2.5.2 for full details)

Suction blisters were raised at the site of irritant reactions using plastic chambers based
on the Dermovac® (Kiistala, 1968). Epidermal roofs were carefully excised and snap-
frozen in liquid Nitrogen and stored at $-196\,^{\circ}C$.

A 6mm punch biopsy from untreated normal forearm skin was taken (with 1%
lignocaine) as a control and the three remaining patch test sites were assessed visually
and similarly biopsied one hour after the various time points.

Immunofluorescence staining as described in section 2.7.1

Immunohistochemistry as described in section 2.7.2

Positively stained epidermal LC (cell body with a minimum of 2 dendrites) were counted simultaneously by three co-workers using a multi-headed microscope and expressed as the total number of positive cells/mm basement membrane.

Confocal laser scanning microscopy and statistical analysis

As described in section 2.7.3

5.3 Results

Confocal laser scanning microscopy (see **Tables** 5.1, 5.2 , 5.3 and **Figs**. 5.3, 5.4)

A representative low power field from a normal volunteer is shown in **Plate** 5.1. In patients one hour after irritation the number of LC fell (**Plate** 5.4) and continued to decrease at both 6 hours (**Plate** 5.5) and 24 hours. In contrast, in normal volunteers 1 hour after irritation, there was no significant change in the number of LC (**Plate** 5.2) and only started to fall 6 hours and 24hours post NA irritation (**Plate** 5.3). Statistical analysis of variance showed a highly significant difference between the LC numbers in patients and volunteers, $p < 0.001$ as well as a significant effect of time of NA irritation, $p < 0.001$. There was no significant interaction between time and group (i.e. patients or volunteers, $p = 0.152$).

Normal healthy volunteers had a mean of 6.0 dendrites per LC with a mean length of 15.4µm prior to application of NA. The morphology of a typical LC (high power field) is shown in **Plate** 5.6. Following irritation there was a reduction in the number of dendrites at all time points ($p < 0.05$). This effect was coupled with a decrease in the

mean length of each dendrite at 6 hours (p<0.001) and 24 hours (p<0.001). Patients

however, had a mean of 4.5 dendrites per LC with a mean length of 12.0μm prior to

application of NA. There was a significant fall in the number of dendrites in patients but

this did not occur until 6 hours (p<0.05,) and 24 hours (p<0.05). A closer inspection of

the cell showed a more rounded cell body and retraction of the remaining dendrites

(**Plate** 5.7). In addition there was a highly significant reduction in the length of

dendrites by 24hours.

Table 5.1 Langerhans cell counts and standard errors by confocal microscopy.

	Time-0h		Time-1h		Time-6h		Time-24h	
	Pts	Vol	Pts	Vol	Pts	Vol	Pts	Vol
Mean no. of LC/mm^2	421	323.6	225.7	116	389.8	444.2	334.3	156.4
SEM	3	17.4	25.2	14.8	53.5	33.9	14.8	26
n	3	9	6	6	10	9	6	5

For raw data see **Appendix 7**

Table 5.2 Number of dendrites per LC counts and standard errors by confocal microscopy.

	Time-0h		Time-1h		Time-6h		Time-24h	
	Pts	Vol	Pts	Vol	Pts	Vol	Pts	Vol
Mean no. of dendrites /LC	4.5	6.0	5.2	4.0	3.8	4.1	2.8	3.5
SEM	0.3	0.9	0.6	1.2	1.4	1.1	1.8	1.6
n	3	9	6	6	6	6	6	6

For raw data see **Appendix 8**

Table 5.3 LC dendrite lengths and standard errors by confocal microscopy.

	Time-0h		Time-1h		Time-6h		Time-24h	
	Pts	Vol	Pts	Vol	Pts	Vol	Pts	Vol
Mean length of dendrites /μm	12.0	15.4	10.6	14.3	10.3	5.5	5.7	5.6
SEM	0.8	1.2	2.6	0.8	3.5	6.6	6.7	7.3
n	3	9	6	6	6	6	6	6

For raw data see **Appendix 8**

Table 5.4 Numbers of CD1a+ve Langerhans cells/mm basement membrane after irritation with nonanoic acid.

Patient No.	Control	24h	48h
1	9.2	5.8	0.5
2	11.2	5	4.3
3	19.0	5.6	5.4
4	21.3	9	2.1
5	13.5	9	3.5
6	10.6	2.9	0.8
7	11.7	10.2	5
Median no. of LC	**11.7**	**5.8***	**3.5****
Normal healthy Volunteers (n=5)	**8.6**		

* $p < 0.03$ ** $p < 0.02$

Basement membrane lengths were measured using the HOME microscope. the number of CD1a+ve LC (cell body plus a minimum of 2 dendrites) were counted and expressed as the median number of cells/mm basement membrane. The early and late time points were statistically compared with their respective non-involved controls using a 2-tailed Mann-Whitney U test.
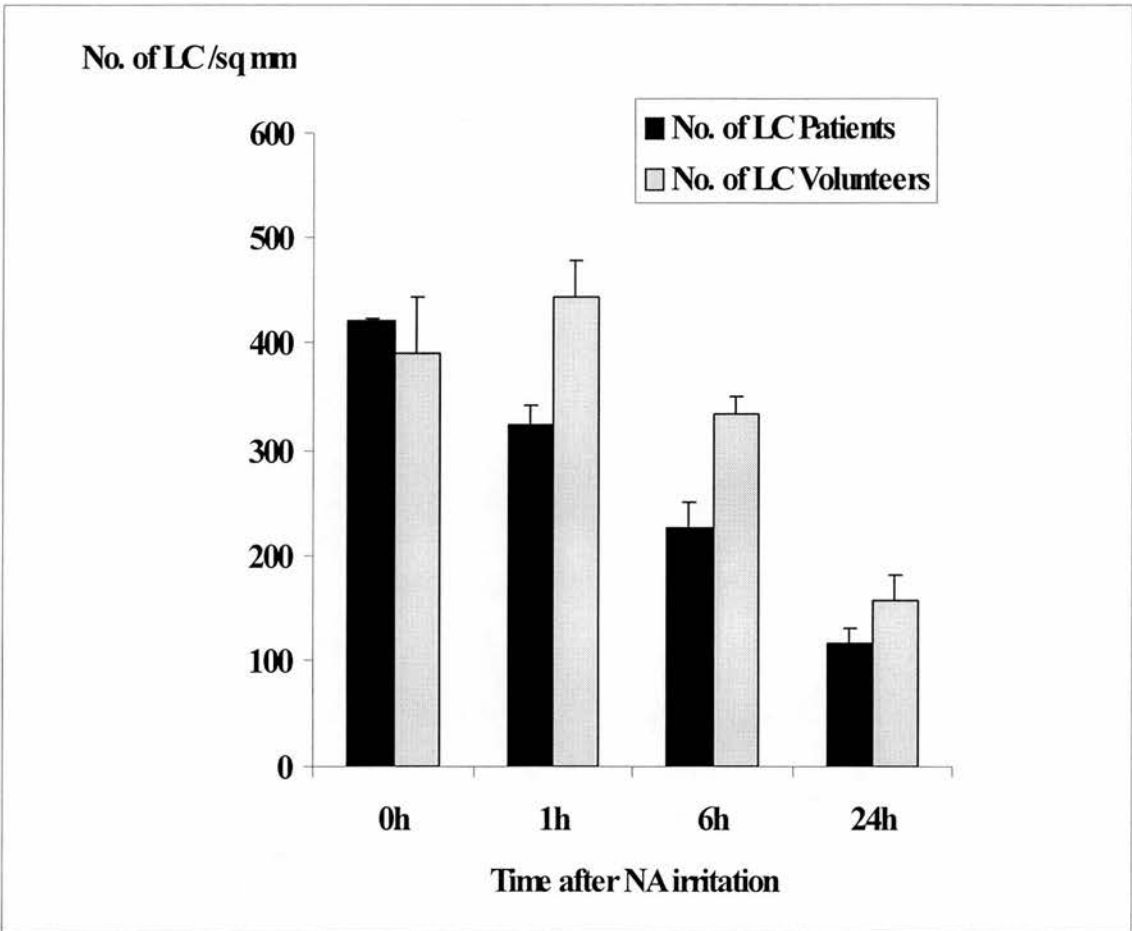
Fig. 5.2 Bar chart showing the number of CD1a+ve Langerhans cells (+ s.e.m) after irritation with nonanoic acid.
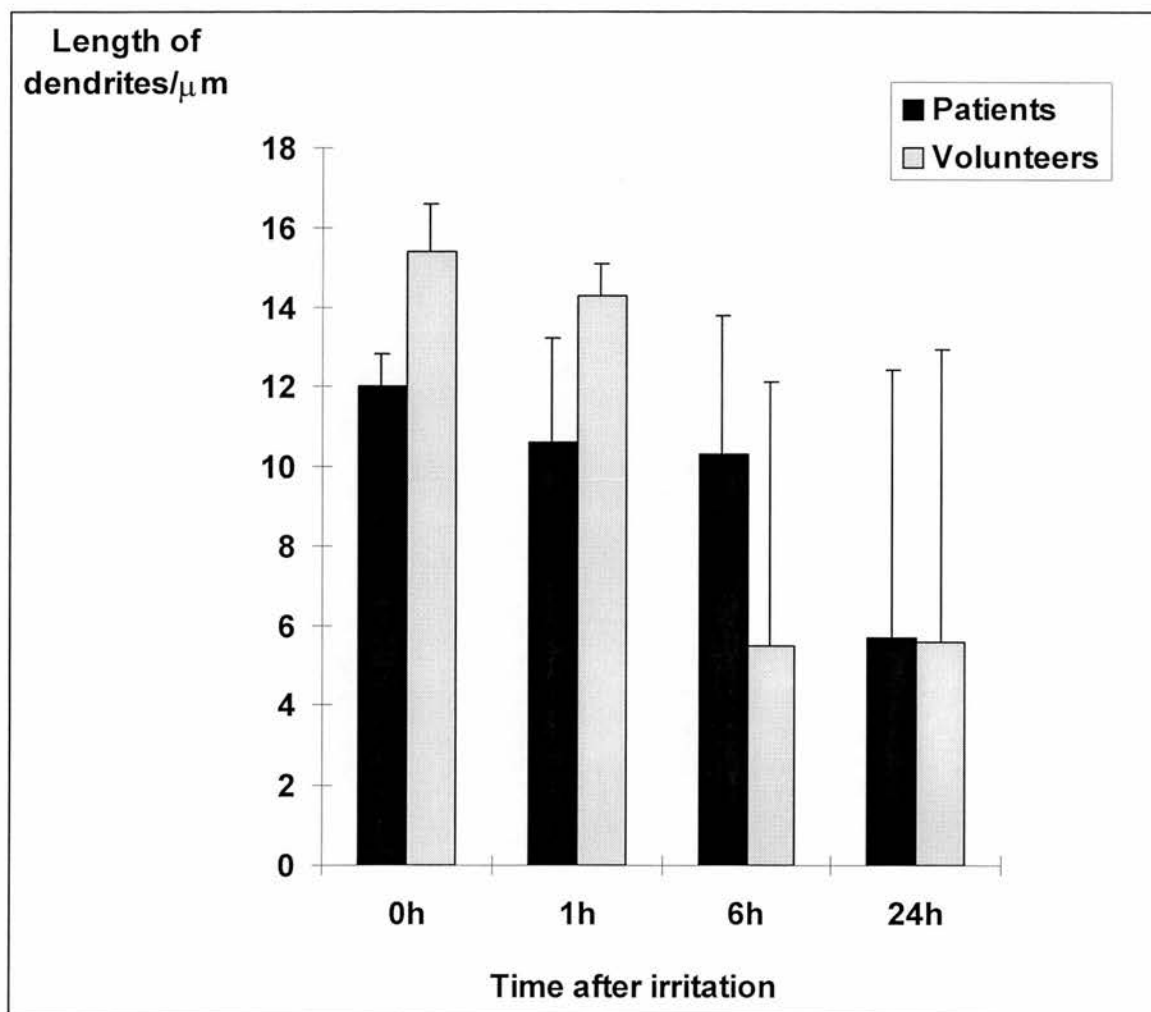
Fig. 5.3 Bar chart showing the changes in length of dendrites (+ s.e.m.) after irritation with nonanoic acid.
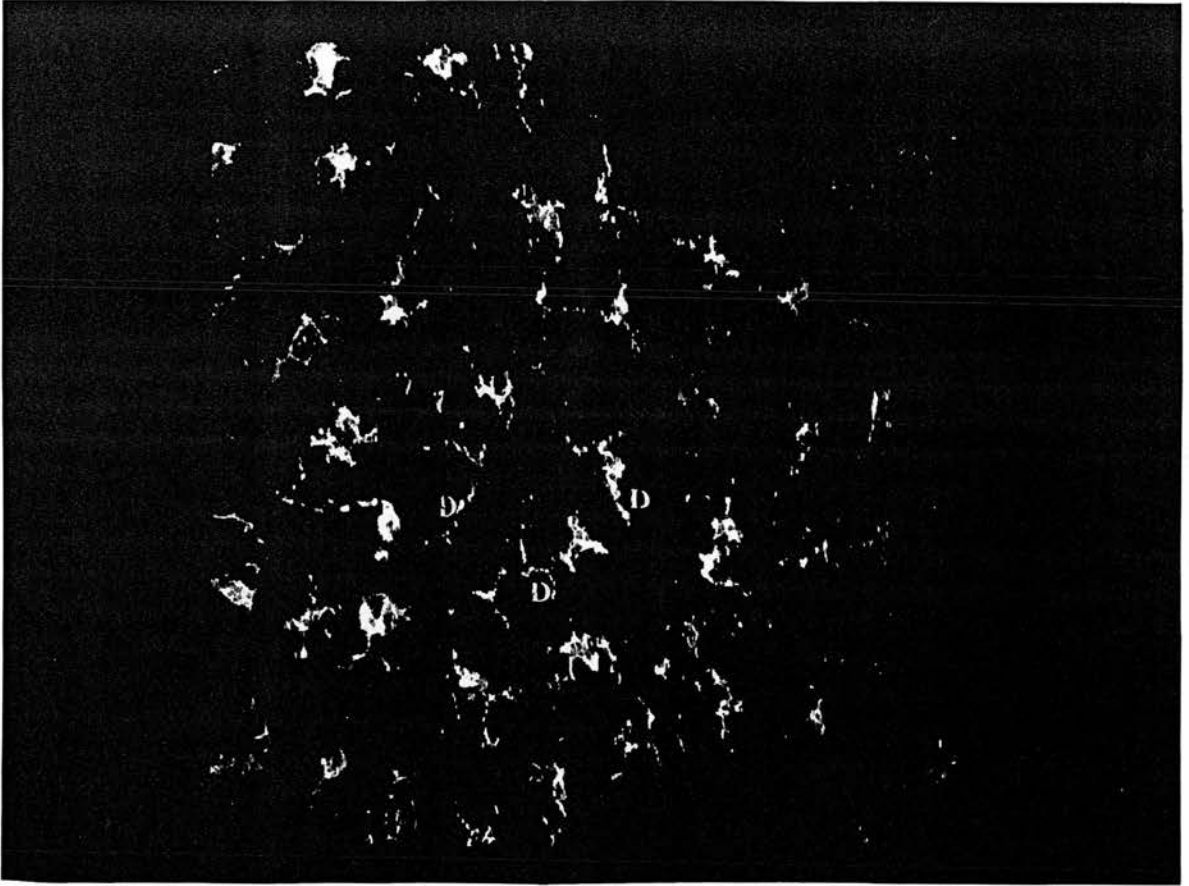
PLATE 5.1 Confocal microscopy image of CD1a+ LC in a **volunteer** on non-irritated skin (x 800) showing typical dendritic (D) appearance.
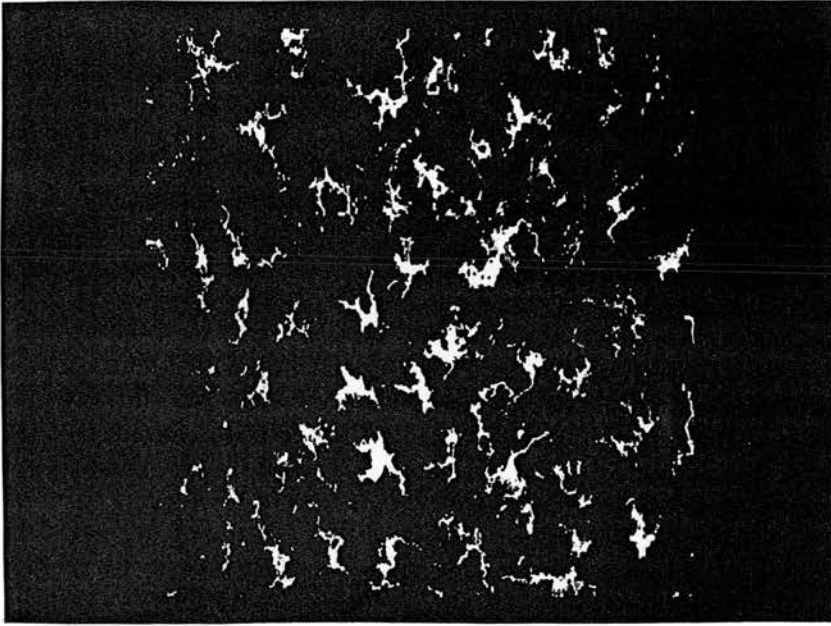
PLATE 5.2 CD1a+ LC **-volunteer** at 1h post NA irritation (x 800) showing little change in number or morphology of LC
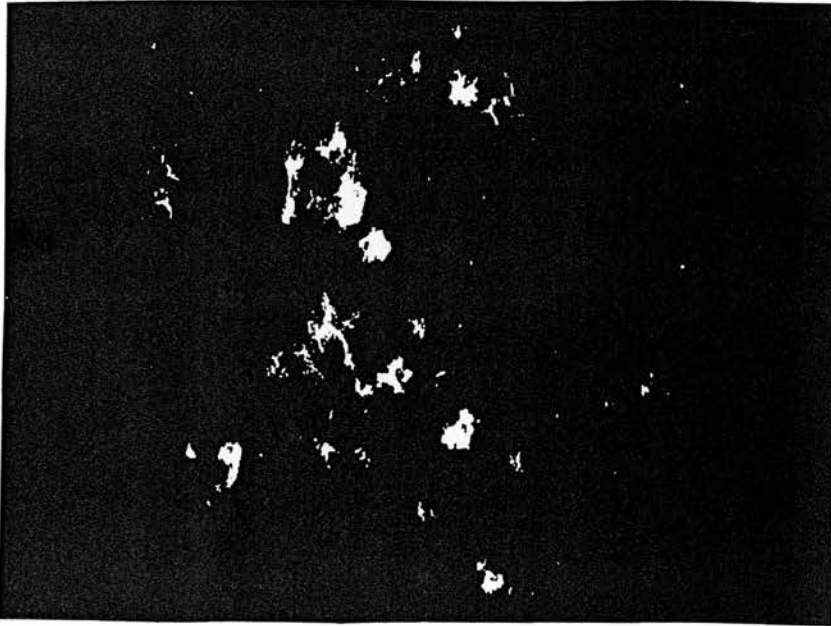
PLATE 5.3 CD1a+ve LC - **volunteer** at 24h post NA irritation (x800) showing marked reduction in number of LC
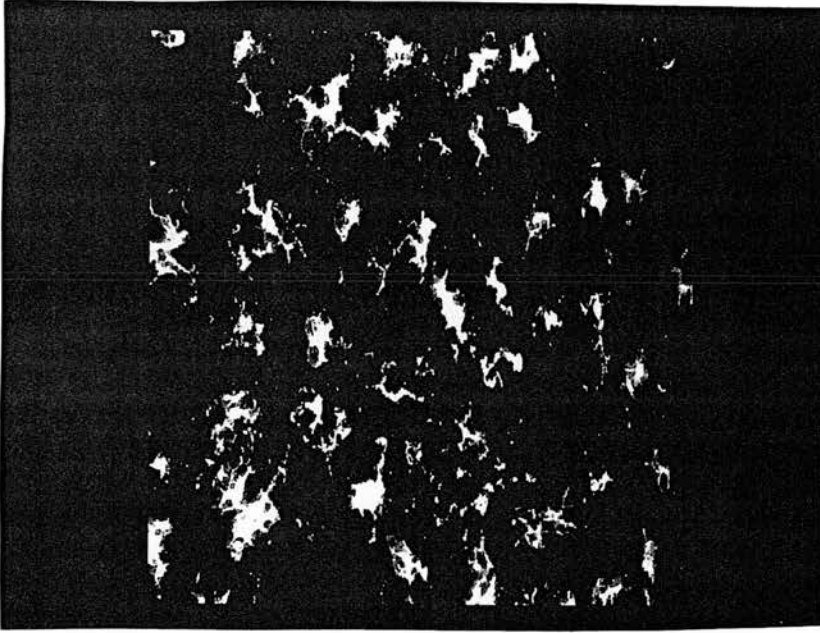
PLATE 5.4 CD1a+ LC – **patient** at 1h post NA irritation (x 800)
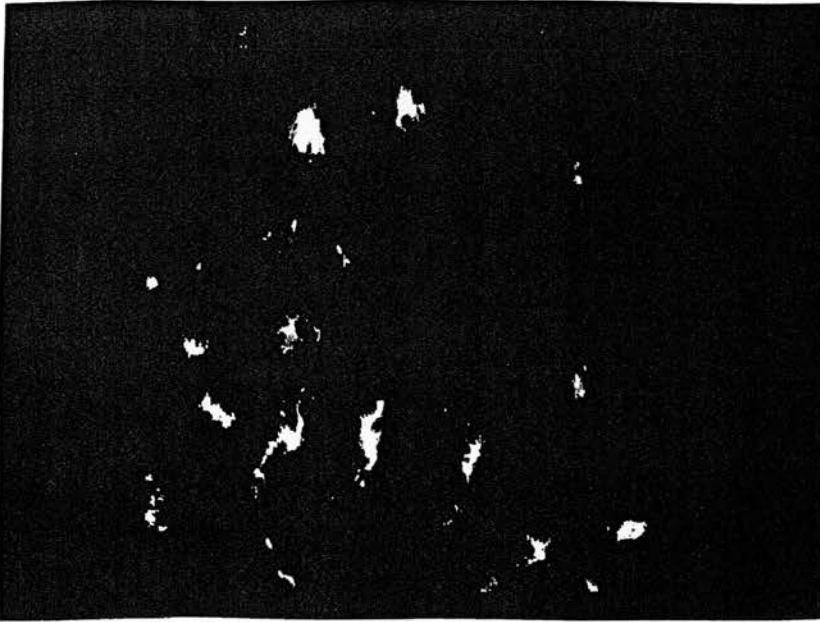
PLATE 5.5 CD1a+ve LC -**patient** at 6h post NA irritation (x800) showing reduction in number of LC
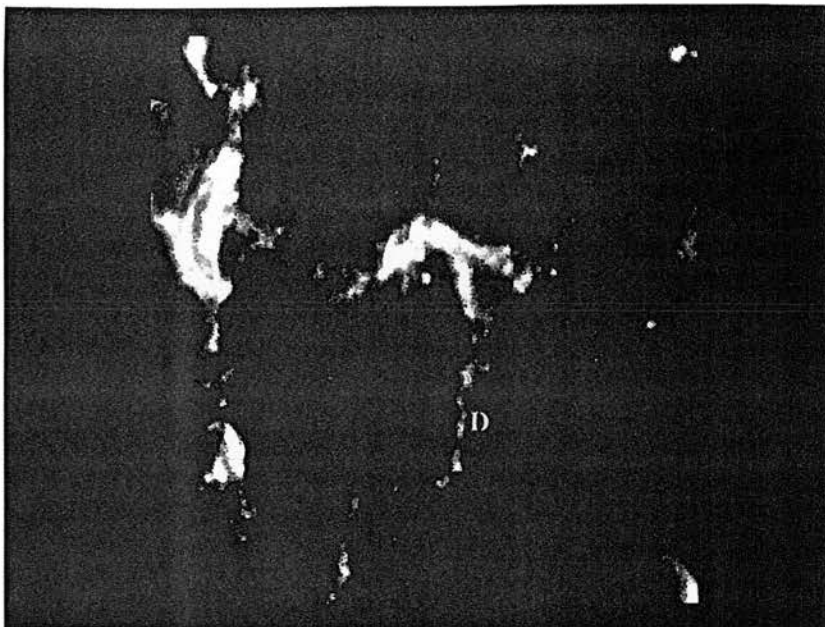
PLATE 5.6 High power confocal microscopy image clearly showing morphology of dendrites (D) of CD1a+ LC in **patient** prior to irritation (x 3200)
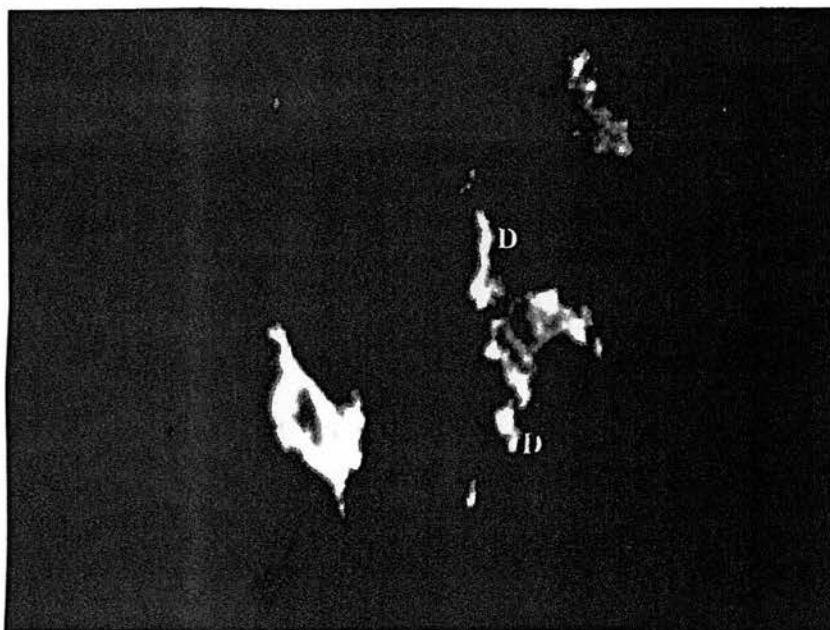
PLATE 5.7 CD1a+ LC in **patient** at 6h post NA irritation (x 3200) showing reduction in length and number of dendrites (D)

Immunohistochemistry for CD1a staining

The results of counting CD1a+ve cells in frozen sections are shown in Table 5.4 above.

The distribution pattern of CD1a+ve cells is shown in **Plates** 5.8 and 5.9.

The median number of CD1a+ve LC in untreated, uninvolved forearm skin from the

patient group was 11.7/mm basement membrane. The number of CD1a+ve LC in the

uninvolved forearm skin of volunteers was significantly lower at 8.6/mm basement

membrane, $p<0.01$. CD1a+ve LC in both control groups showed a normal even

distribution throughout the epidermis. The effect of NA at 6h was to induce a

redistribution of CD1a+ve LC to a basal pattern (**Plate** 5.9) which was still present at 24

and 48h. Vehicle alone at all time points had no effect on the number or distribution of

CD1a+ve LC.

5.4 Discussion

The function of Langerhans cells as antigen presenting cells in allergic contact

dermatitis has been studied extensively (Stingl et al, 1978; Toews et al, 1980; Braathen,

1980) but their role in irritant contact dermatitis is poorly understood. Previous data

using conventional immunohistochemical methods have shown increased (Scheynius et

al, 1984; Lindberg, 1986), unchanged (Kanerva et al, 1984; Avnstorp et al, 1987) and

decreased (Ferguson et al, 1985; Gawkrodger et al, 1986; Willis et al, 1990; Mikulowska,

1994) numbers of LC in experimental irritant contact dermatitis. The importance of the

chemical nature of the irritant itself was highlighted by examining the different changes

with several irritants (Willis et al, 1990). Willis and her colleagues, using standard light

microscopy and immunohistochemistry, found there was a significant reduction in
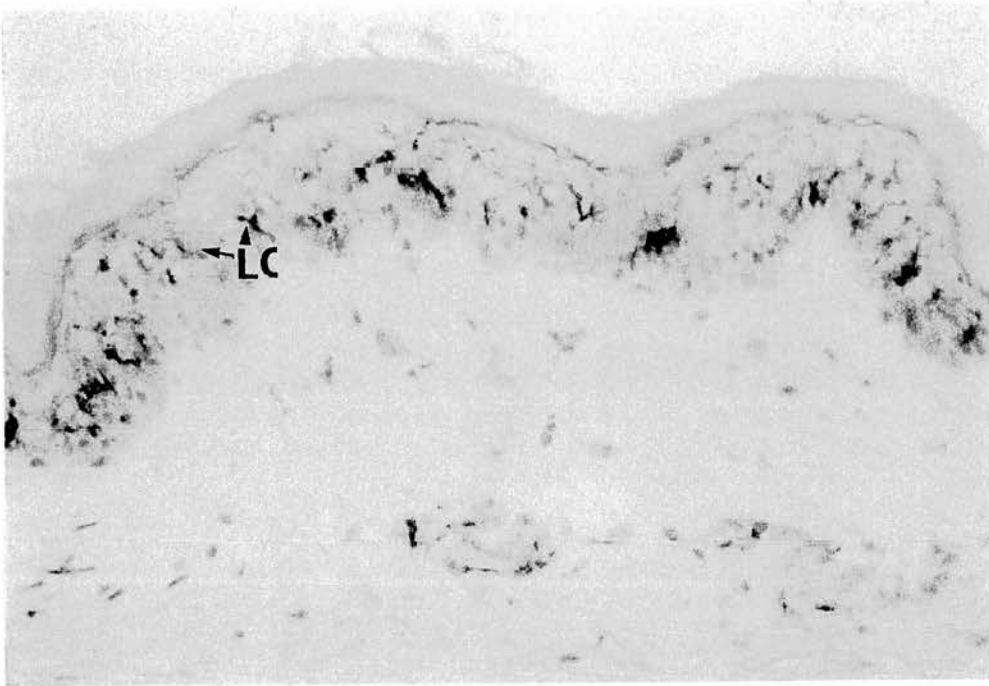
Plate 5.8 Normal distribution of epidermal CD1a+ LC (stained red) x216
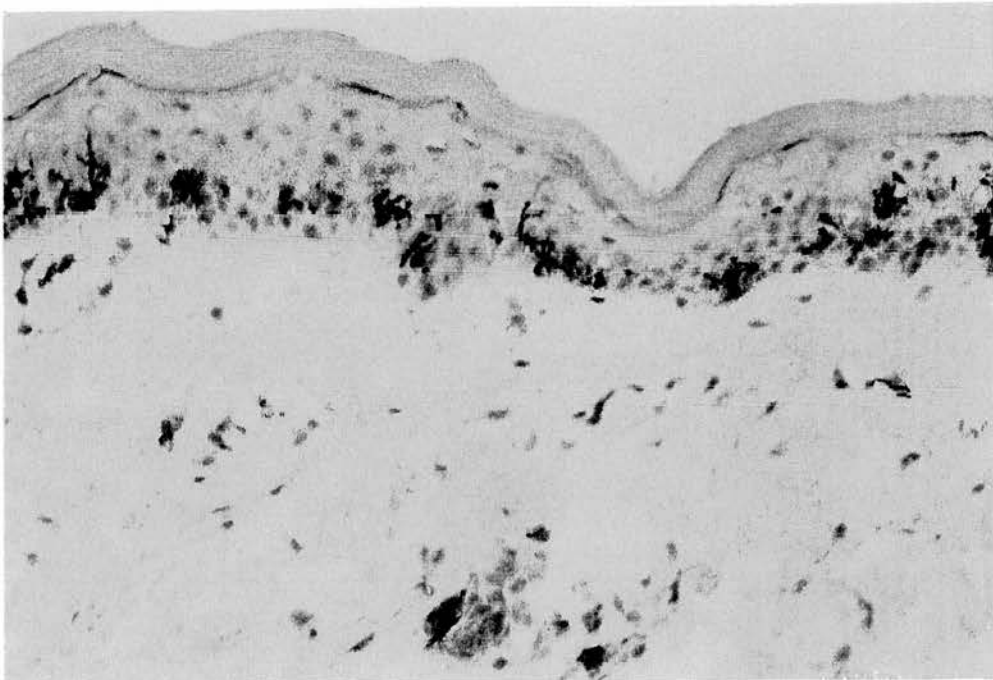
Plate 5.9 CD1a +LC moving to a basal distribution 6h post NA irritation x216

epidermal LC density with 80% NA but no difference with sodium lauryl sulphate or croton oil. The quantification of CD1a+ LC by light microscopy of perpendicular sections using morphometric methods is open to misinterpretation due to variations in the thickness of tissue sections (Yu et al, 1994). The use of CLSM showed that experimental irritation does induce a significant reduction in epidermal LC density and that this trend is in keeping with the immunohistochemical data. Unlike previous work this study has focused on the early time course of irritation i.e. the initial 24 hours after application of NA. This is also the first study to my knowledge, which has investigated patients with a clinical irritant dermatitis. This may be relevant because it has been noted that CD1a reactivity is different between patients and volunteers. This study showed that patients have increased numbers of epidermal LC in clinically normal skin compared with skin from normal healthy volunteers with a greater median number of CD1a +ve LC per mm basement membrane (see Table 5.4). The results show that this is not only a quantitative difference but that the LC also behave differently with an earlier and more rapid reduction in patients after NA irritation. Volunteers initially have an increased number of LC after 1 hour before exhibiting a similar stepwise fall. This increase at 1 hour may be through recruitment of LC. In patients, their LC may be more sensitive to external stimuli with a lowered threshold for activation and movement out of the epidermis. The question must be asked as to whether the reduction in CD1a+ LC is a true migration of LC out of the epidermis or simply due to loss of CD1a expression. The immunohistochemical staining of CD1a+ LC in perpendicular sections supports our belief that this is a true migration through the epidermis. This has been recently shown for sodium lauryl sulphate irritation (Mikulowska, 1994). An alternative hypothesis

based on our results suggest that NA not only causes migration of the LC out of the epidermis, but also induces apoptosis of LC which would not be picked up with CD1a staining (Forsey et al, 1998). The apoptosis has been seen on both on H&E staining (see Chapter 3) and confirmed by TUNEL immunostaining (Forsey et al, 1998).

In normal human epidermis LC form a monolayer of cells located suprabasally with 5-9 dendrites and the number of LC/mm^2 varies depending on the body site with a lower number in sun-exposed sites (Yu et al, 1994). Morphological changes in the dendrites of epidermal LC following NA irritation have been determined by semi-quantitative analysis by arbitrary grading (Willis et al, 1990). CLSM has allowed a more accurate definition of these changes in dendritic length. Other groups have looked at morphological alterations with CLSM in normal skin and after experimental irritation in volunteers (Scheynius et al, 1992). The latter study showed a marked reduction in the number of LC (CD1a fluorescence) after 80% NA but not SLS.

It has been shown that samples of lymph from vessels draining the region of a SLS- induced irritant dermatitis in human volunteers, contained an increase in LC, T and B cells (Brand et al, 1995) and cytokines (Hunziker et al, 1992), suggesting immunological activation. Conventional teaching defines irritant contact dermatitis as an inflammatory reaction pattern which follows non-immunological damage to the skin (Mathias, 1987). However, it is now believed irritants may activate keratinocytes non-specifically, inducing them to express adhesion molecules and to produce chemotactic factors and cytokines (Bos, 1993), including TNFα, which has been shown to promote LC migration (Cumberbatch, 1995).

These results confirm earlier reports that the number of epidermal LC are decreased in one type of experimental irritant reaction. CLSM provides a sensitive technique which allows both quantitative and structural analysis of tissue specimens. It gives that added third dimension of volume not possible with standard immunohistochemical techniques. The choice of the threshold value is important as this discriminates between the true CD1a staining and non-specific background. The analysis of stored images in one sitting enabled the same threshold to be used. The fading of staining is another possible source of error but antifadents such as Citifluor and p-phenyldiamine (Bieber et al, 1998; Horton et al, 1984) keep this to a minimum.

On the basis of this work alone it is not possible to comment on the degree of immunological activation of LC, but studies which investigate cytokine induction (see chapter 4), T cell recruitment and expression of adhesion molecules after the application of irritants (future studies) may help to gain a better insight into the mechanisms of inflammation which they produce.

CHAPTER 6. THE USE OF SKIN WINDOWS TO EXAMINE THE ACTIVATION OF NEUTROPHILS IN-VIVO FROM INFLAMED SKIN

6.1 Introduction

Human polymorphonuclear leucocytes (predominantly neutrophils) are the primary effector cells in acute inflammation. They are rapidly recruited from the bloodstream in large numbers and pass through the vascular endothelium to the inflamed site, where they are able to release a battery of toxic metabolites and enzymes with microbicidal activity. The resolution of inflammation is dependent on the balance between the need for clearance of the inflammatory stimulus and the protection of the host tissue. Neutrophils are short-lived cells which have been shown, both in-vivo and in-vitro, to die by apoptosis- a process that prevents inappropriate release of inflammatory mediators (Haslett, 1992) and provides signals which lead to phagocytic clearance from the inflamed site.

We were particularly interested in the role of neutrophils in cutaneous inflammation induced by irritants. Skin window chambers permit the in-vivo collection of extravasated neutrophils and thus allow the study of the fate of neutrophils in irritant-mediated inflammation. Analysis of surface molecule expression on neutrophils can provide important clues about their activation state and whether the neutrophils have undergone apoptosis. Upregulation of CD11b/CD18, CD35, and CD16 with loss of CD62L indicates neutrophil adhesion to vascular endothelium (See Ch1 p35). Neutrophil apoptosis is characterised by upregulation of CD11b/CD18 and marked downregulation of CD16 and CD62L.

6.2 Aims

1. To determine the leucocyte mobilisation rate in human forearm via skin windows.

2. To determine the expression of CD11b/CD18, CD11c/CD18 and CD62L (L-Selectin) on neutrophils in irritated and normal forearm skin by flow cytometry.

3. To determine if neutrophil apoptosis occurs in irritated skin.

6.3 *Experiment 1*: Leucocyte mobilisation rate

Materials and Methods

Subjects

Healthy normal volunteers were recruited and 20ml blood venesected and spun to remove the cellular component. The serum was removed and stored at 4°C.

Irritant reactions

Mild reactions were induced with SLS on the forearm as in section 2.2.2. The irritant patch was left in place for 24h. SLS was chosen because of the results from the histopathology study indicated a greater epidermal neutrophilic infiltrate with this irritant than with any other irritant.

Tape stripping

The patch was removed and the area sellotape stripped as detailed in section 2.7.2.

Skin windows

Skin window chambers were placed over the stripped site and filled with autologous serum and left in situ for different time periods. The chamber fluid was sampled and

normal saline used to wash out the chamber to ensure the greatest possible retrieval of leucocytes.

The variables tested included the following:

- Number of sellotape strips   : 90-150
- Duration of irritation   : 24h
- Volume of irritant   : 34-60μl
- Time chamber affixed to arm   : 24-51h

Flow cytometry-see Section 2.8.6 Materials and Methods

Results

The sellotape stripping technique yielded $0.25 \times 10^6 - 1 \times 10^6$ neutrophils (the latter value on one occasion only). This poor yield of neutrophils was insufficient for the immunoassays of neutrophil activation/apoptosis surface markers. An alternative technique was employed in an attempt to increase the yield of neutrophils. The experience from the suction blister studies suggested that removal of the epidermis would allow a more predictable contact of serum with the dermal vasculature than sellotape stripping.

6.4 *Experiment 2*: suction blister skin window

Method

The irritant patch was removed and a suction blister cup placed above the site. A

suction blister was raised as described previously (see section 2.5.2). The contralateral

forearm of each subject was used as a control without any irritant.

See **Plates** 6.1 and 6.2

Results

Table 6.1 Leucocyte (neutrophils) harvested from skin windows chambers following

SLS irritation

Subject No.	Chamber fixation time - No. of leucocytes (x 10^6)			
	6h		24h	
	Test	Control	Test	Control
1	-	-	1.74	5.64
2	0.28	1.29	0.39	2.64
3	1.14	0.78	1.15	1.55
4	1.37	1.56	1.71	1.02
5	0.85	2.14	3.02	2.50

Conclusion

The suction blister skin window yielded sufficient neutrophils for the immunoassays.

6.5 *Experiment* 3: Neutrophil activation and apoptosis in irritant reactions

Subjects and Methods – as in expt.1. The neutrophils were harvested and kept on ice.

Immunoassays and flow cytometry (for details see section 2.7.5)

Peripheral blood cells were prepared as described previously (see section 2.7.4).

Plate 6.1 SLS and control site irritant reaction after suction blister roof removed.

Plate 6.2 Close up of control site prior to skin window placement

The expression of the neutrophil surface markers CD11a, CD11b, CD62L, CD16 and CD35 was determined for test sites, control sites and from subjects' peripheral blood neutrophils. Flow cytometric analysis was performed by the EPICS Profile II (Coulter Electronics, Luton, UK).

Cytocentrifuge spins

Cell counts were performed manually with a haemacytometer and cytospins of leucocytes were stained with haematoxylin to look for morphological signs of apoptosis, i.e. nuclear condensation/vacuolation.

6.6 Results

The first flow analysis (Fig 6.1) showed that more than one cell population was present in the skin window chamber fluid, namely neutrophils and lymphocytes. The overlay shows that these groups have distinct profiles that allowed them to be distinguished easily for separate analysis.

Fig. 6.2 shows the analysis of neutrophil surface molecule expression from a volunteer following SLS irritation for 24hours. The non-binding mouse antibody (negative control) showed negligible fluorescence but there was an increase in both CD11a, CD11b and CD35 antibodies, indicating surface expression comparable to that on freshly isolated peripheral blood neutrophils. Together with a higher level of CD16 expression and lack of L-Selectin (CD62), these results suggest that the neutrophils collected have become activated. Somewhat surprisingly the neutrophil surface marker activation of the neutrophils from the control site (i.e. no irritant on contralateral
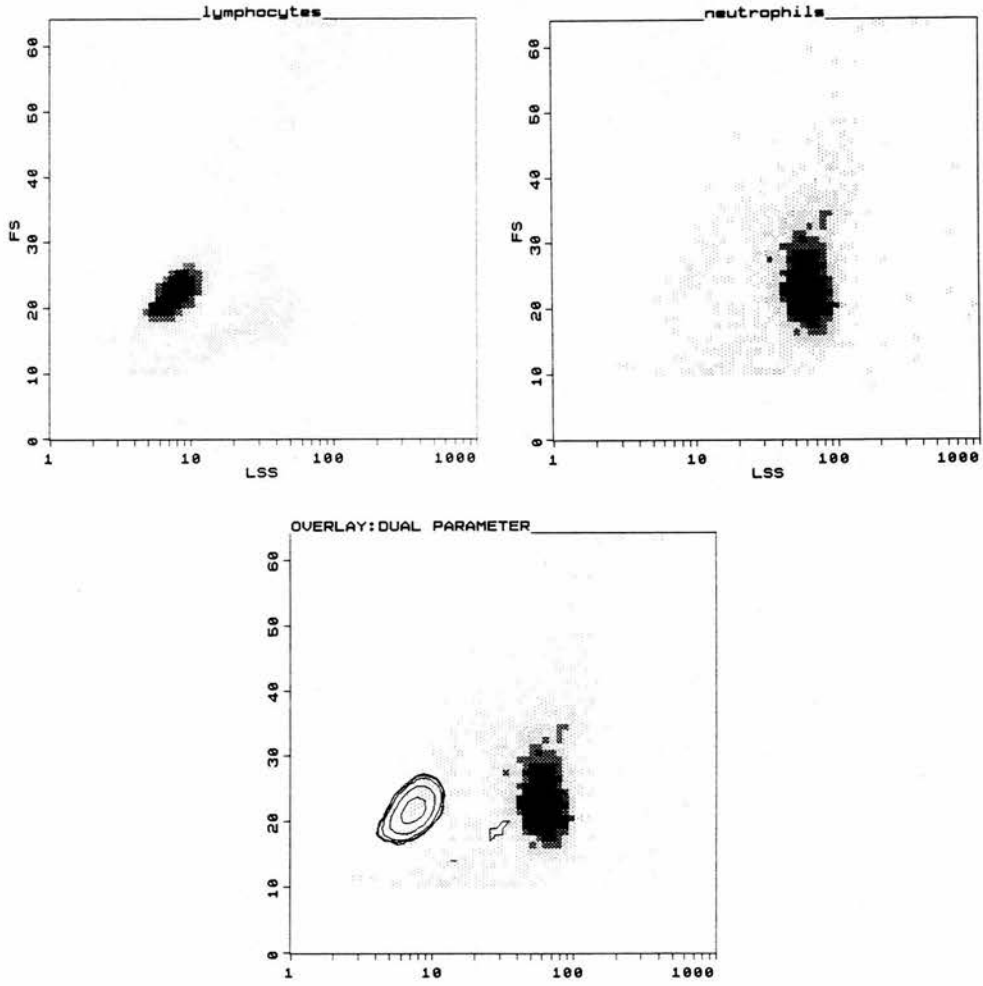
Fig 6.1 A representative histogram of the two populations of cells in a flow sample, lymphocytes and neutrophils, with overlay showing the clear separation between them.
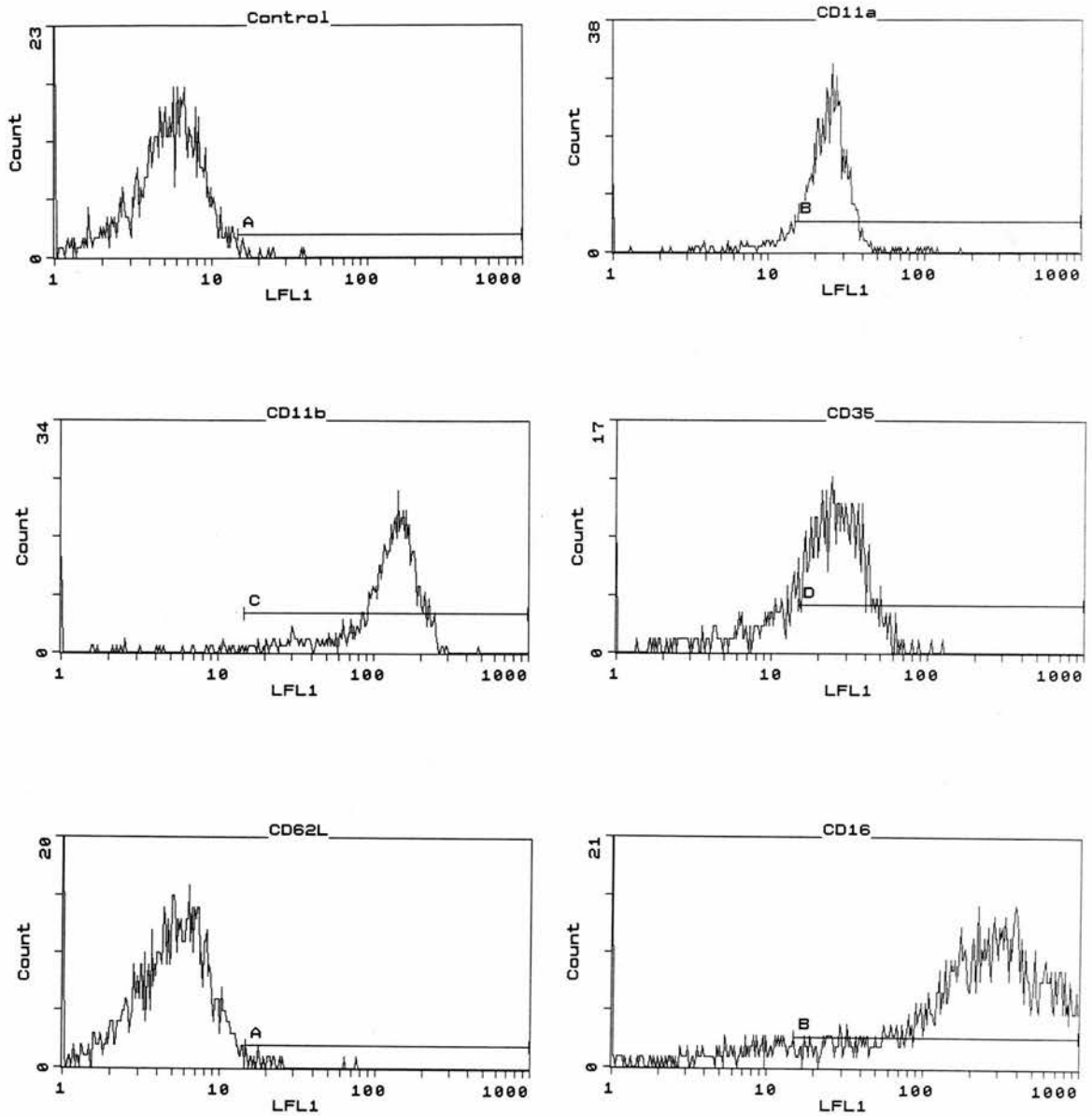
Fig. 6.2 24h SLS irritation
Fluorescence histograms showing the expression of neutrophil surface markers CD11a, CD11b, CD35, CD62L, CD16 and control for neutrophils from a volunteer. Data from one representative experiment of four that were performed.

117
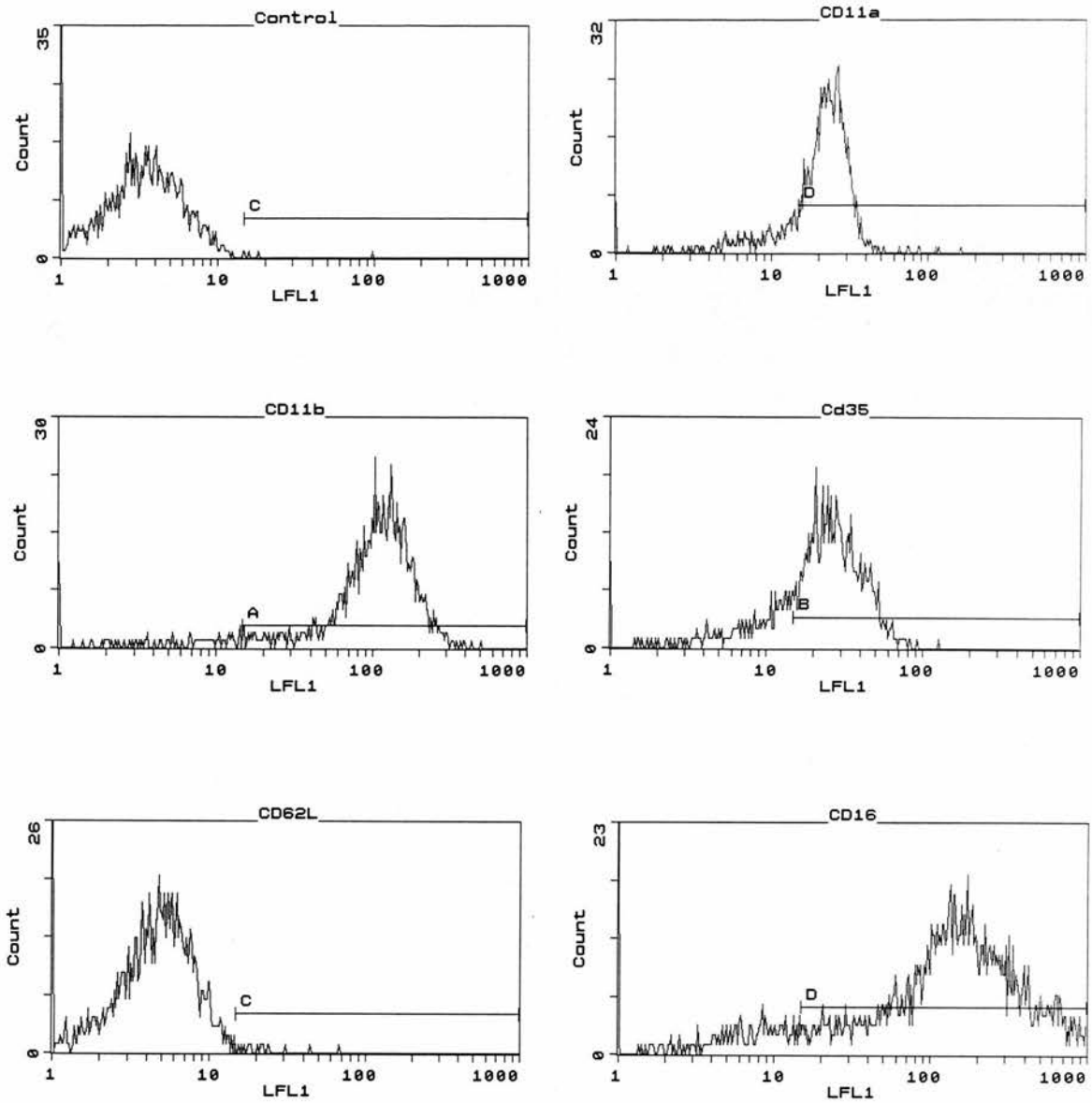
Fig. 6.3 24h control site

Fluorescence histograms showing the expression of neutrophil surface markers CD11a, CD11b, CD35, CD62L, CD16 and control for neutrophils from a volunteer. Data from one representative experiment of four that were performed.

forearm) were also virtually identical to the test site –Fig.6.3. The flow profiles for 6h

SLS irritant and controls were virtually identical and are not shown here.

To confirm that the L-Selectin antibody was working properly, peripheral blood T cells

were harvested in a similar fashion to the neutrophils and examined for L-Selectin

expression. Fig 6.4 shows high levels of L-Selectin expression when compared with the

T cell negative sample. This is typical of T cells and confirmed that the assay was

functioning adequately.

The unexpected similarity of the profiles for neutrophils from control and test

sites suggested that the irritation had little effect on neutrophil activation in the skin

window chamber. In order to test this hypothesis, a further experiment was performed.

Peripheral blood from a volunteer was withdrawn and the neutrophils harvested as

described by Haslett et al (1985). The neutrophils were placed in a skin window

chamber with autologous serum, overlying intact, non-irritated normal forearm skin.

Fig. 6.5 shows the profiles for the various markers. The results once again show high

levels of CD11a/CD18, CD11b/CD18 and CD16 and no detectable L-Selectin (CD62L)

expression.

Apoptosis

The cytocentrifuge spins of the collected chamber fluid showed the cells were nearly all

neutrophils and the occasional mononuclear cell (**Plates** 6.3 and 6.4). There was no

morphological evidence of apoptosis in the SLS or control site neutrophils. However,

the peripheral blood neutrophils did show some apoptotic cells with vacuolation and

nuclear condensation (**Plates** 6.5 and 6.6).
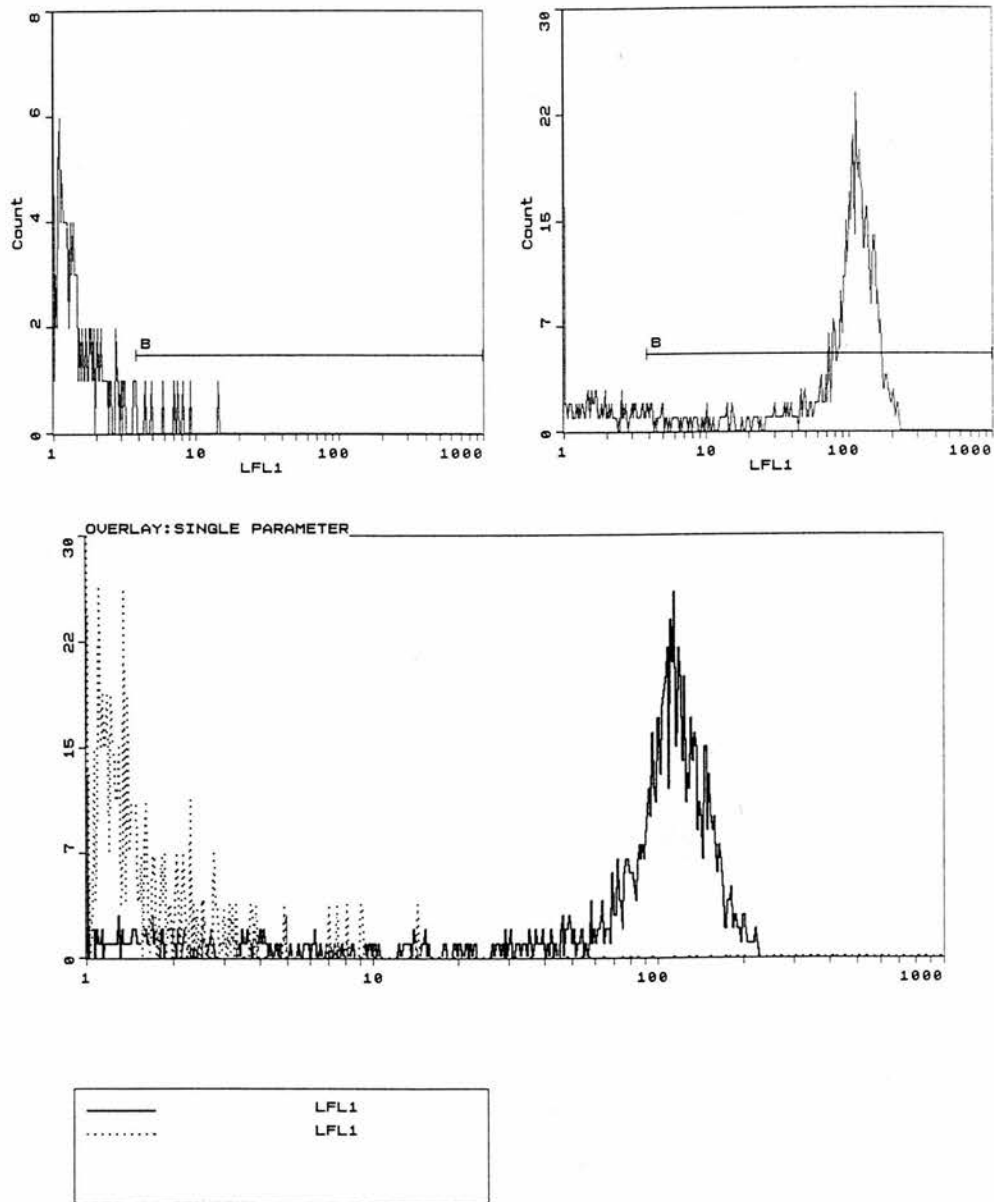
Fig. 6.4a Peripheral Tcells; 6.4b Tcell negative preparation
Fluorescence histograms (with overlay) showing the expression of the surface marker
CD62L from peripheral blood of a volunteer.
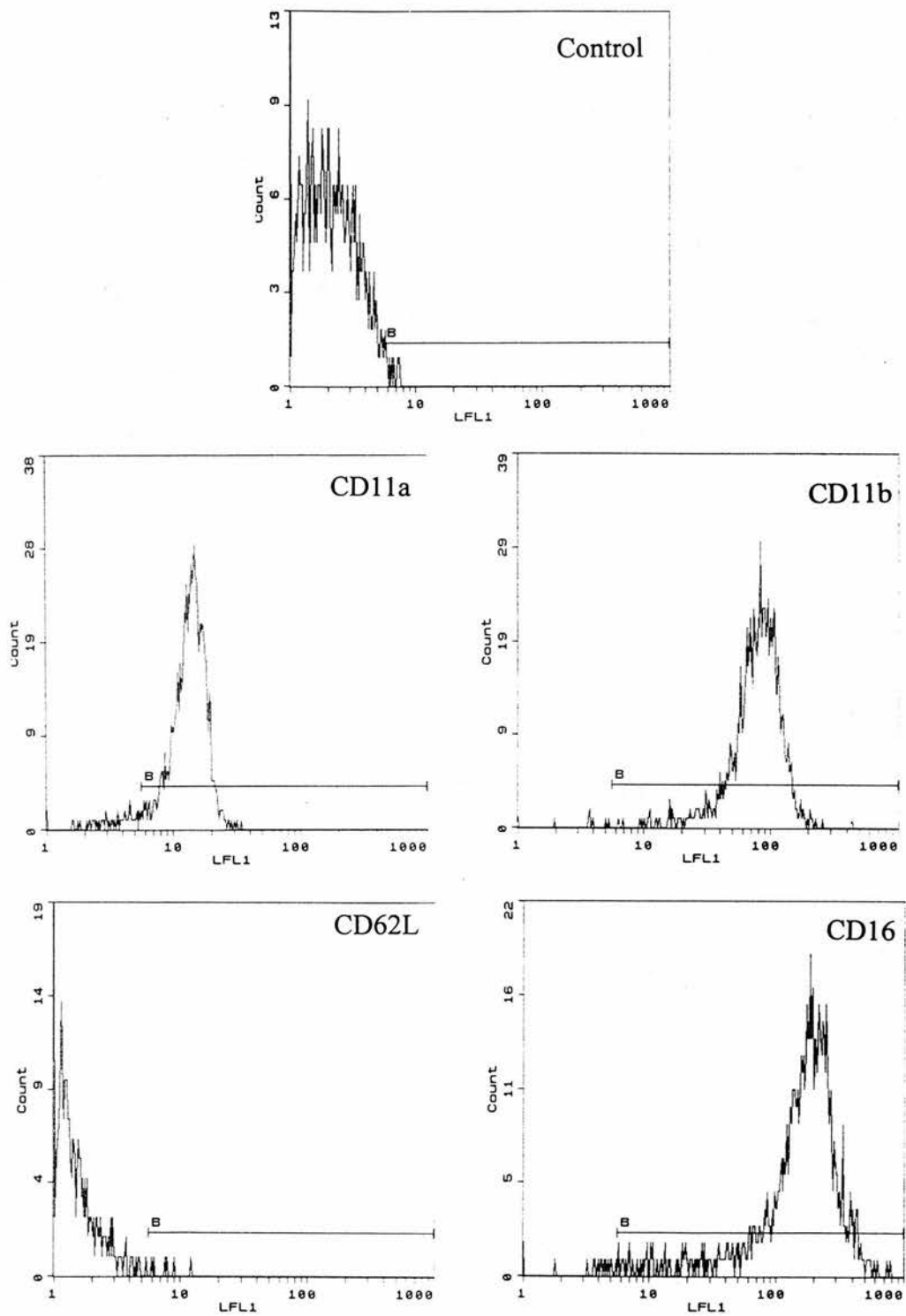
Fig. 6.5 Peripheral blood neutrophils placed on non-irritated site
Fluorescence histograms showing the expression of neutrophil surface markers from a volunteer. Data from one representative experiment of two that were performed.
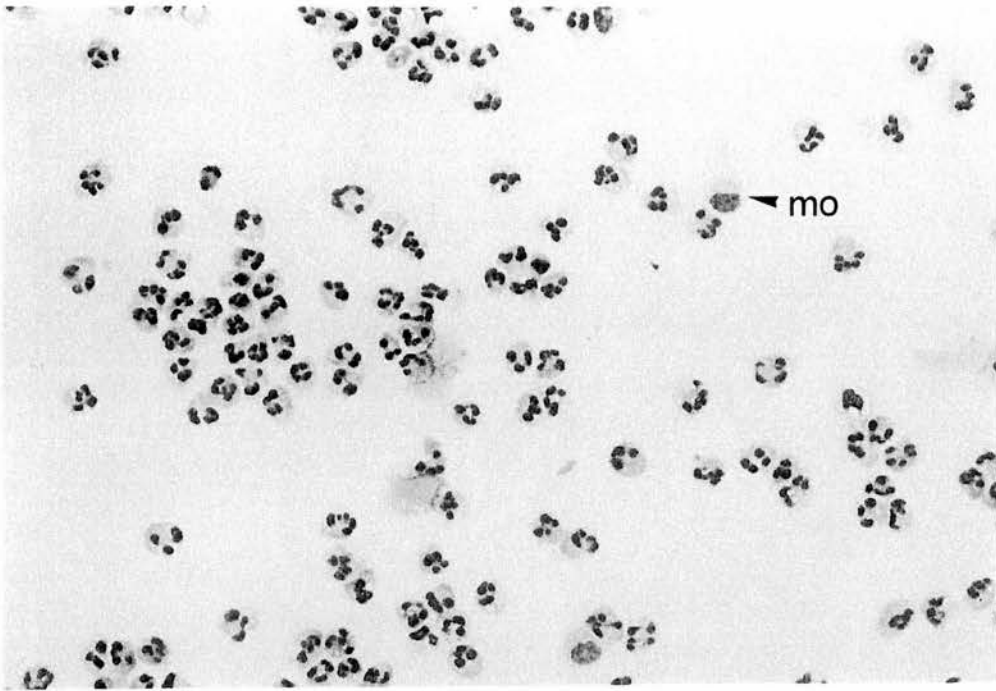
Plate 6.3 Cytospin of chamber fluid stained to show neutrophils with the occasional mononuclear cell (mo) – **control site at 24h**   x216
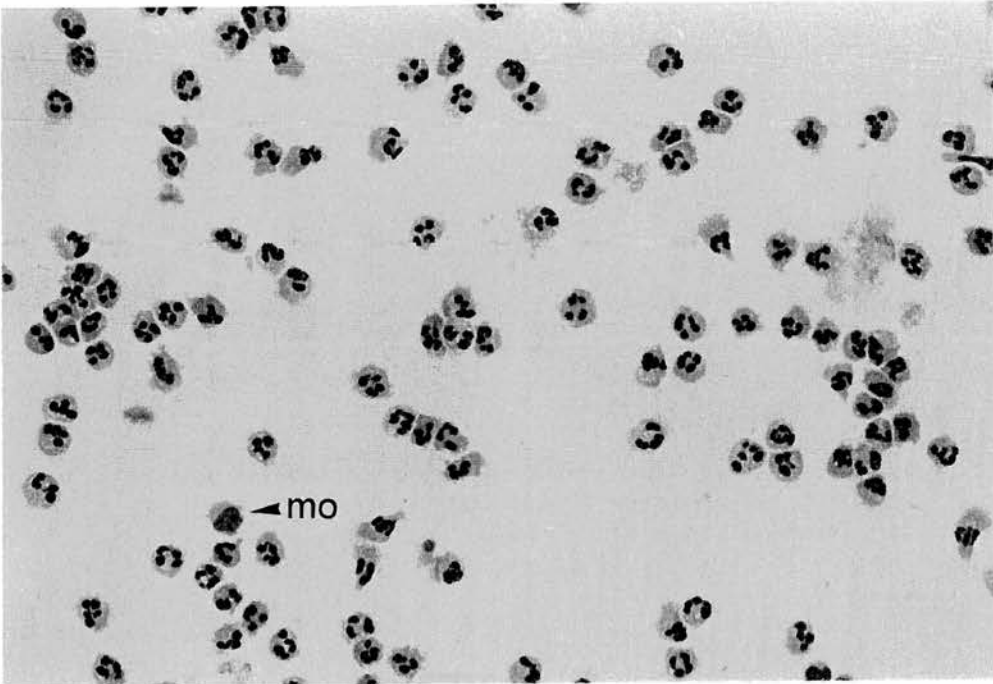
Plate 6.4 Similar preparation to above - **SLS irritated site at 24h** x216
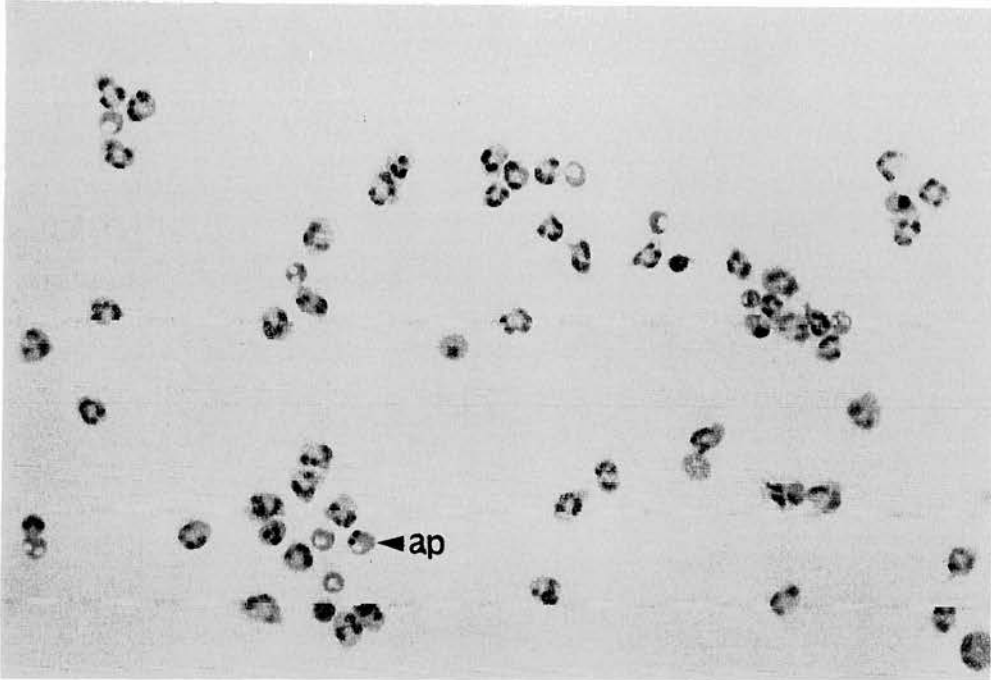
Plate 6.5 Cytospin of chamber fluid stained to show peripheral blood neutrophils with the occasional apoptotic cell (apop)   x216
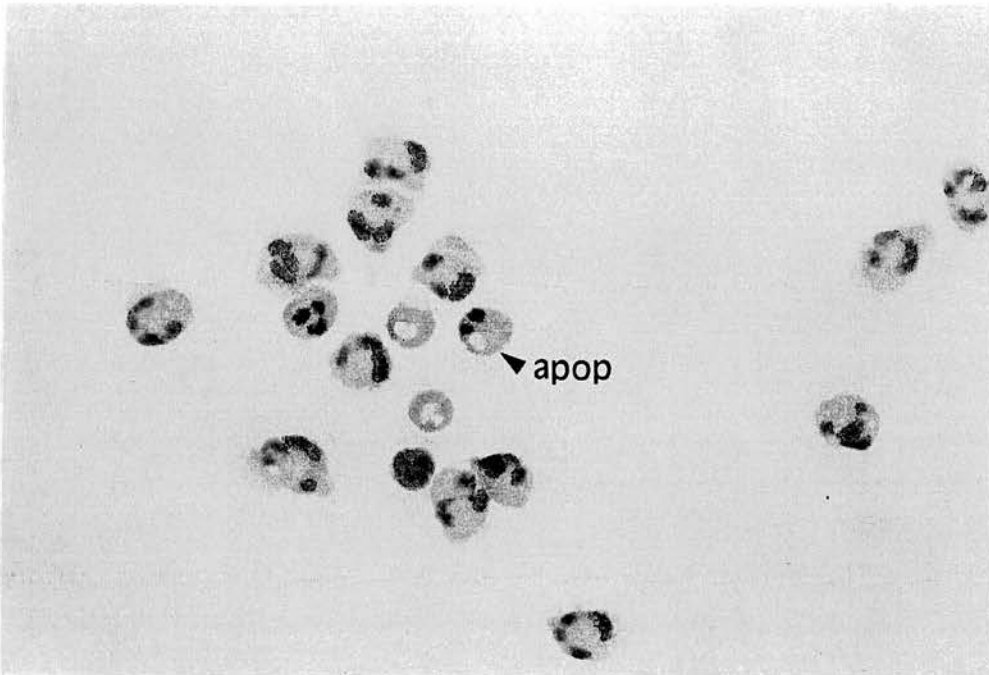
Plate 6.6 Same preparation as above at higher magnification showing apoptotic cell with nuclear condensation and vacuolation.   x512

Conclusions

The above experiments indicate that neutrophils collected in skin window chambers are activated at both control and SLS irritated sites. This activation appears to be independent of the suction blister or irritant, as a similar surface marker profile was observed with peripheral blood neutrophils placed on non-irritated skin. SLS induced no apoptosis in the samples examined.

6.7 Discussion

Neutrophils have been linked to the pathogenesis of a variety of diseases (Malech, 1988) but little is known about their role in irritant contact dermatitis. Neutrophil influx during inflammation is dependent upon the coordinated regulation of different adhesion molecules (McEver, 1992). Cytokines induce endothelial activation at an inflammatory site, which leads to an increase in a selectin-dependent adhesion (P- and E-selectin). Neutrophils are arrested by adhesion via integrins (mainly β_2 integrins and their ligands) and the expression and function of neutrophil adhesion molecules such as CD11b/CD18 and CD62-L determines whether or not neutrophils pass through the endothelial barrier (Condliffe et al, 1996). The above experiments demonstrate that in–vivo studies of neutrophils in irritant reactions is possible, allowing quantitation of the rate of influx into skin window sites and phenotypic analysis of recruited cells.

The initial study attempted to duplicate the results obtained by Morris et al (1985) by using sellotape stripping and skin window chambers. This failed to produce the numbers of neutrophils required for the neutrophil activation marker immunoassays,

despite altering variables such as the number of sellotape strips and volume of irritant. The reason for the lower yield may be related to the variability in the sellotape stripping. The pressure applied at each strip was not uniform and would have lead to variable removal of epidermal layers. The use of a suction blister prior to affixing a skin window chamber bypassed this problem and the subsequent increase in neutrophil yield confirmed the benefit of this method.

The apparent lack of difference between the neutrophil yield from SLS irritation and control (no irritant) implied that the irritant had little bearing on the influx of neutrophils (see Table 6.1). This could be that insufficient irritant was being applied or the method of application was incorrect. Previous work in the histopathology study (Chapter 3) had shown that a similar volume of SLS applied under patch test occlusion did induce an epidermal neutrophilic infiltrate, although this was in patients with irritant contact dermatitis. One may speculate that patients' neutrophils may be activated to migrate more readily than in volunteers. A previous study by Lerche et al (1981) had a different approach to contact of the skin with the test substance. This group mixed the nickel sulphate solution in with the autologous serum before injecting the resultant mixture in to the chamber. We were rather reluctant to do the same with SLS aqueous solution as the absorption characteristics and scarring possibilities were unknown and potentially hazardous.

The assessment of neutrophil activation using flow cytometric analysis in both test and control sites did not reveal significant differences. There was evidence of neutrophil activation with high levels of expression of CD11a/CD18, CD11b/CD18, CD35, CD16 and no expression of CD62L expression. One conclusion could be that neutrophil

migration into the chamber leads to activation. This, however, would be incorrect since the experiment using the neutrophils harvested from peripheral blood and applied to intact non-irritated skin, showed identical results. The importance of this experiment cannot be overemphasised. The removal of neutrophils from blood itself causes activation and activation is independent of epidermal removal and independent of SLS irritation.

There was little evidence of neutrophil apoptosis in the cells of both irritant and control samples. This may be due to the temperature at the point of contact i.e. the skin surface blister site. Dransfield et al (1994) showed that neutrophil apoptosis is associated with a reduction in CD16 expression . Unpublished data from the same group indicate that neutrophil apoptosis is inhibited at low temperatures. Further evidence of the importance of temperature in progression to apoptosis is shown in Fig. 6.6. It is clearly shown that the percentage of apoptosis increases (the higher left-sided peak) with the greater the time the neutrophils are left at 37°C. It would therefore be of interest to determine the skin surface temperature under the skin window chambers although one would expect it to be less than 37°C.

In summary, it was possible to harvest neutrophils in vivo by a combination of suction blisters and application of autologous serum over the eroded site. The activation of neutrophils, as determined by immunoassay of neutrophil surface markers, is a very easily triggered event. In our hands, at least, there was no difference in neutrophil activation between irritated and control sites. In addition neutrophil apoptosis was not detected after the induction of cutaneous irritant reactions.

a)

b)

overnight @ 37°

20h @ room temp, 60 min @ 37°

"   , 40 min @ 37°

"   , 20 min @ 37°

21h @ room temp

20h @ room temp
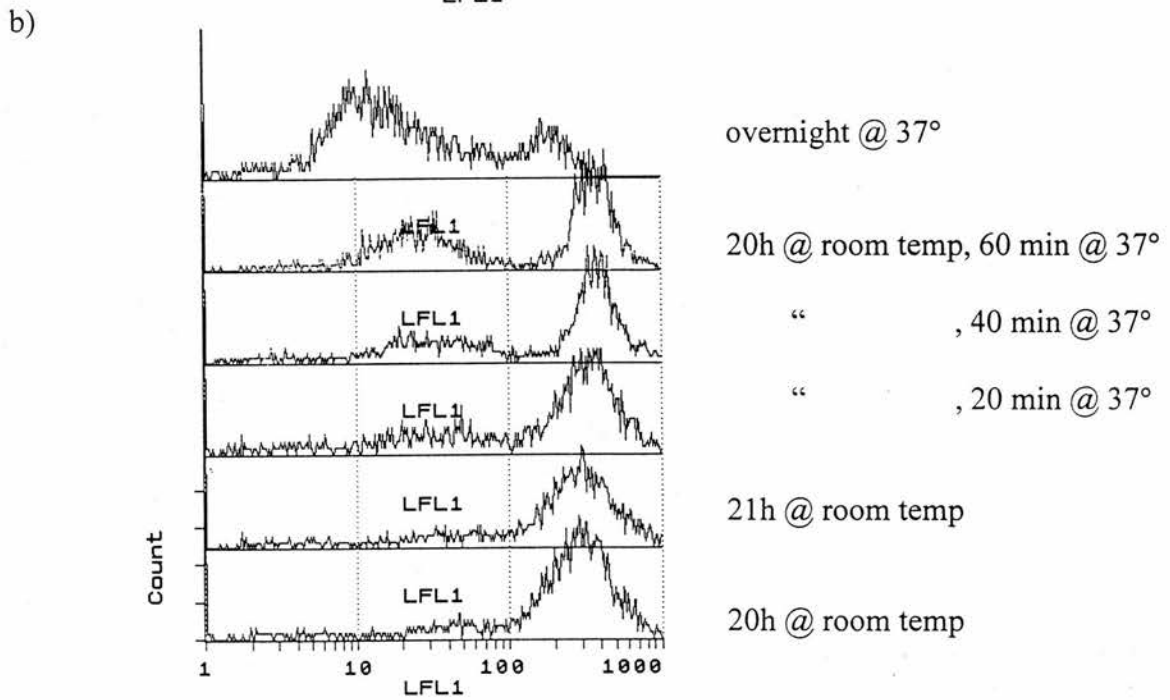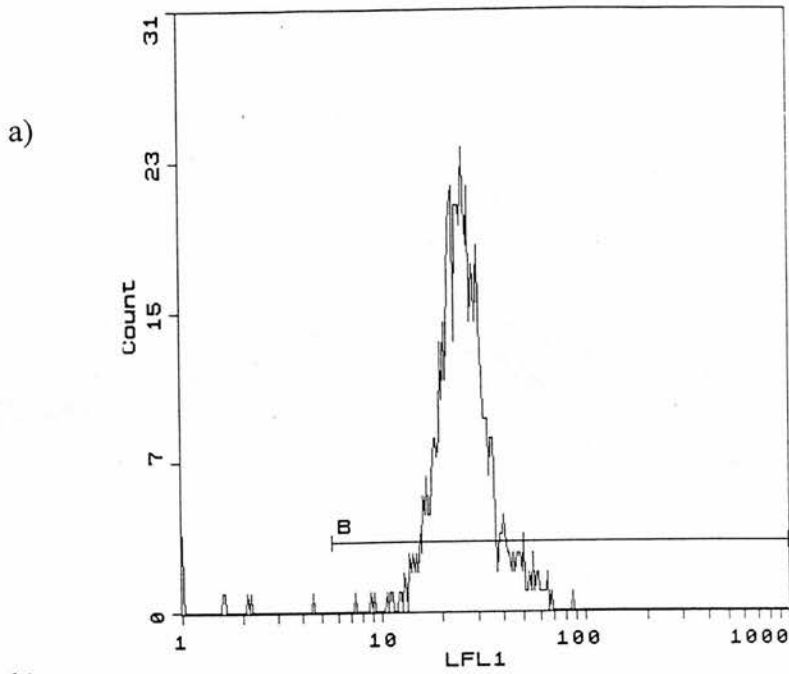
Fig. 6.6a Cd16 expression. Fluorescence histogram of CD16 expression of neutrophils left on the bench overnight.
Fig. 6.6b Temporal changes in expression of CD16 with temperature showing the increasing percentage of apoptotic neutrophils with an increasing temperature. (personal communication from Dr. I. Dransfield)

CHAPTER 7 : DISCUSSION

7.1 Histopathology of ICD

The histopathological changes associated with a primary irritant vary with the
chemical nature of the irritant (Bjorneberg, 1968; Willis et al, 1992). The latter
group systematically investigated the action of structurally unrelated irritants and
studied changes in volunteer's normal skin after 48hour patch test occlusion. They
found that SLS irritation induced characteristic epidermal changes with parakeratosis
and vesiculation. Nonanoic acid was noted to induce effects on epidermal
keratinocytes which was attributed to premature keratinisation or possibly
irreversible cellular injury (Willis et al, 1992). The results presented in Chapter 3
have differed from previous work in two ways. Firstly the uninvolved skin of
patients with ICD and volunteers has been biopsied. Secondly the early events in
irritant reactions have been examined. The latter has allowed a clearer understanding
of the evolution of histological changes in experimental ICD.

The findings of the study have confirmed certain findings but also highlight
the differential effects of SLS and NA. The induction of parakeratosis by SLS was
confirmed in the later stages of irritation (24 and 48h). In addition SLS induced
spongiosis which proceeded to frank vesiculation. NA by contrast, induced minimal
spongiosis, which is in accord with Willis et al. A new finding was the effect of NA
on apoptosis. NA induced basal cell apoptosis from the early phases of irritation and
although SLS did induce apoptosis in a few biopsies, the proportion never reached
that of NA (see Table 3.2). Further work from our research group has now defined
many of these apoptotic cells as apoptotic LC (Forsey et al, 1998). This suggests that

this is a direct toxic effect of NA since it occurs early in the development of the irritant response.

The dermal changes associated with experimental ICD were also analysed in some detail. SLS induced a dermal neutrophilic infiltrate of a greater magnitude than NA by 48 hours. Some of these neutrophils had migrated to the epidermis being located in the infiltrate in SLS induced epidermal vesicles. Both irritants induced a mononuclear infiltrate on routine haematoxylin and eosin sections, and like previous work this has been the commonest finding in the histopathological studies of experimental ICD (Nater, 1976; Willis et al, 1992). The most interesting aspect of this infiltrate is that it is common to not only the model irritants but also to the vehicle control biopsies (water and propanol). This implies that patch test occlusion itself affects normal skin homeostasis without necessarily being apparent on clinical examination.

Dithranol irritation in this study did not produce any significant clinical irritation despite the encouraging pilot study. The only consistent finding due to dithranol was epidermal intracellular oedema which occurred throughout the time course of irritation.

7.2 Cytokines and inflammation

The mechanisms of inflammation in ICD are poorly understood. The analysis of cellular infiltrates in skin biopsies from subjects who have had an experimental ICD has shed some light on the above. Nonanoic acid has previously been shown to induce minimal exocytosis of inflammatory cells into the epidermis (Willis et al, 1993). SLS, however, induces a marked neutrophilic infiltrate (see Chapter 3). In

clinical practice one of the major unresolved issues is how to differentiate allergic from irritant contact dermatitis. The in-vivo assay of cytokines may provide an answer. In Chapter 4 an attempt was made to study the early and therefore primary pro-inflammatory cytokine profiles in experimental ICD.

IL-1 and TNF-α production can be induced by stimulating keratinoctyes. TNF-α has already been shown to provide one signal for LC migration during cutaneous immune and inflammatory responses (Kimber, 1992). Similarly, in immunological responses, IL-1 appears to be involved in the process of antigen presentation to T lymphocytes by LC and other antigen presenting cells (Kurt Jones, 1985). More recently Enk (1992) investigated IL-1 mRNA in both ACD and ICD in mice. They found an upregulation of IL-1β mRNA only in irritant reactions. The extrapolation of such a result to humans must be done with caution. Brand et al (1996) assayed IL-1β in skin lymph from volunteers. They induced an ICD (n=6)by applying 10% SLS for 48 hours and ACD (n=5) using either diphencyprone or DNCB. Two control volunteers had no irritant or allergen applied. Lymph samples were collected by cannulating the draining lymphatics on the lower limb. They found IL-1β protein (assayed by ELISA) in lymph from both ACD and ICD volunteers, although the amount varied widely between volunteers. In mice the IL-1β mRNA signal was believed to be almost exclusively produced by epidermal LC (Enk, 1992). In humans epidermal, dermal and circulating lymph cells may all contribute to IL-1β protein production.

The importance of keratinocytes in the initiation of an inflammatory response was demonstrated by Nickoloff et al (1994). This group used a non-immunological stimulus- tape stripping. This was chosen for its inherent reproducibility compared

with delipidisation, which is often used in mice. Tape stripping induces epidermal hyperplasia with removal of non-viable stratum corneum and minimal damage to the underlying keratinocytes. Polymerase chain reaction was used to amplify RNA from the epidermal layer of skin sections. An increased message was found for TNF-α, IL-8, IL-10, IFN-γ and ICAM-1. These molecular events preceded any movement of inflammatory cells from the circulation into the epidermis or dermis and probably represent changes that occur in normal human skin. Nickoloff et al (1994) concluded that epidermal keratinocytes have an important contributory role in the cytokine milieu following the abrogation of the epidermal barrier.

The results presented in Chapter 4 mainly refer to the two pro-inflammatory cytokines, IL-1 and IL-8. NA was initially chosen for this study because of its even and predictable clinical irritant response. There appeared to be a heterogeneous response to NA irritation in both patients and volunteers. The lack of data at time 0h makes it difficult to be conclusive. The most likely explanation is that IL-1α production is intrinsic to both patients and volunteers and independent of irritation. The IL-1α protein detected is likely to represent constitutively produced IL-1α by epidermal keratinocytes. This returns to the argument put forward by Nickoloff et al (1994) stating that disruption of the epidermal barrier was sufficient to stimulate the cytokine cascade. In the present studies IL-8 and IFN-γ was essentially unaffected by NA irritation up to 6hours post irritation. Upon changing the irritant stimulus to SLS, IL-8 was detected, consistent with the neutrophilic infiltrate in SLS biopsies (Chapter 3) and the previous report of the neutrophil chemoattractant effect of SLS (Frosch, 1987). To the best of my knowledge only one group has published data (carried out after the experiments in this thesis) on in-

vivo cytokine profiles taken from biopsies of skin following SLS-induced ICD (Hoefakker et al, 1995). This group applied 10% SLS in 5 volunteers under patch test occlusion for 72 hours, describing the clinical reactions as intense erythema and moderate infiltration (equivalent to grade 3, see page 41). They examined the expression of IL-1α, IL–2, TNF-α, and IFN-γ by mRNA in-situ hybridisation and direct immunohistochemistry with monoclonal antibodies. The results showed an upregulation of both IL-2 and IFN-γ (i.e.protein) in the mononuclear dermal infiltrates compared with uninvolved normal skin. There was a significant increase in IL-1α producing cells and TNF-α producing cells in irritated skin. The localisation of these cells in the epidermis by in-situ hybridisation revealed that IL-1α protein was found in all epidermal cell layers whereas the TNF-α protein was located in the lower epidermis and basal layer adjacent to the dermal infiltrates. Our research group is currently undertaking in-situ hybridisation on the skin biopsies from the histopathology study which should provide an opportunity to compare the results with the above.

In summary the cytokine blister fluid assays have shown that in NA irritation, IL-1α is detected and most probably produced from epidermal keratinocytes and dermal mononuclear cells (predominantly T cells). IFN-γ was undetectable at 6 hours following NA irritation, which may be due to induction of milder reactions than in experiments carried out by Hoefakker et al (1995). IL-8 protein was not detected after NA irritation but was found after SLS irritation.

7.3 Langerhans cells in ICD

The function of LC in ICD is relatively unknown compared with ACD. The study presented in Chapter 5 has shown changes in human epidermal LC during cutaneous irritation with NA. The results from a number of different groups have revealed conflicting data. In SLS irritation for example, the density of epidermal LC during experimental ICD has been shown to be increased (Scheynius et al, 1984), unchanged (Kanerva et al, 1984) or decreased (Ferguson et al, 1985; Mikulowska, 1994). The reasons for these differences could be due to basic inter-individual variation in SLS-induced ICD. Another source of error could result from using skin sections instead of epidermal sheets. Bieber et al (1988) highlighted the wide variation in counting of LC in vertical sections. A very important source of error is due to the pharmacokinetics of SLS irritation. The rate of absorption of SLS and total dose penetrating the skin barrier has not been fully determined. A problem common to all of these studies has been the small numbers of volunteers willing to have skin biopsies. In this thesis even though a large number of patients were recruited, the number at each time point (especially time 0h) was small.

If one examines studies with similar methods to our own, some interesting findings emerge. Mikulowska et al (1994) showed that 1% SLS only lowered LC counts after the area of irritation (from 8 to 12 mm Finn chambers) and volume of irritant was increased. Willis et al (1990) had already found that the chemical nature of the irritant is a major determinant of any effect on LC. Moreover, a recent study by Proksch et al (1996) has developed this concept further. They disrupted the skin barrier by acetone (rolling with impregnated cotton wool balls), tape stripping or SLS. Biopsies of the skin revealed no evidence of cell damage or spongiosis. At

24h, there was an increase in epidermal LC with a concomitant rise in TEWL (confirming barrier disruption) with both SLS and tape stripping. Thus a mild irritant stimulus may lead to an increase in LC. Our study used NA, which has many characteristics of a model topical irritant, and the confocal laser microscopy findings did highlight some important points. Firstly there were significant morphological changes in LC after 6 and 24 hours of NA irritation, with a reduction in the number of dendrites/LC and secondly a reduction in the mean lengths of dendrites. The initial assumption was that these LC were migrating to the dermis. The immunohistochemical CD1a staining in sections from irritated skin (**Plates** 5.8 and 5.9) showed a very definite redistribution to a basal location within the epidermis. This was backed up by data from Brand et al (1995) who analysed skin lymph at the site of SLS-induced ICD in human volunteers. They found, both during and after irritation, an increase in the number of CD1a+ LC in skin lymph.

Willis et al (1990) performed an electron microscopic (EM) study of LC after irritation with NA and commented that 'some LC had evidence of cellular injury including damaged organelles, condensed nuclear chromatin and lipid accumulation'. Our research group (in work carried out subsequent to the experiments in Chapter 5) has now put forward an alternative hypothesis regarding the fate of these LC (Forsey et al, 1998). The histopathology study had revealed the presence of basal apoptotic cells on H&E staining after NA irritation. These sections were stained for CD1a and combined with TdT-mediated dUTP-biotin nick end-labelling (TUNEL) which proved that many apoptotic cells were CD1a+ (85 –100%). In contrast, SLS irritation revealed no CD1a+ apoptotic cells. This effect of NA on CD1a+ cells was restricted to the epidermis with the dermal CD1a+ population remaining unaffected,

even after 48 hours exposure to NA. Therefore NA irritation causes a reduction of CD1a+ epidermal LC not only through their migration out of the epidermis but also through induction of apoptosis. The EM changes observed by Willis et al (1990) probably reflect morphological signs of apoptosis.

Confocal microscopy has been criticised for its expense and problems with thick sections. It is a useful method for quantifying labelled structures and has the added benefit of allowing structural analysis because of its ability to section in 3-D, but there are a few points which ought to be borne in mind of undertaking CLSM work:

- single person analysis reduces the chance of bias

- the threshold at which cells are deemed positive should be stated clearly

- analysis of cell numbers and morphological data can be done at leisure if the images are stored in one sitting.

Unfortunately the technique is not suitable for routine use as it is labour intensive and prone to hardware breakdown. The former relates to morphology e.g. dendrite lengths whereby 40 optical sections are taken for each LC. Our microscope was temperamental with valuable time lost waiting for the company engineer to fix the hardware.

The confocal results indicated patients with ICD had a higher number of epidermal CD1a+ LC in unaffected skin than volunteers, a finding substantiated by immunohistochemistry of identical sections. The exact reason for this is unclear, since it appeared to be unrelated to age, sex or atopic status. One explanation would be that the development of ICD leads to an intrinsically higher number of epidermal

LC. Our experiments may be criticised on two accounts. Firstly the lack of data points at time 0h and secondly the measurement of LC in affected dermatitic skin. These points are addressed later.

7.4 Irritant susceptibility and atopy

Patients with atopic dermatitis are generally considered to be more sensitive to irritants than normal individuals (Van der Valk et al, 1990; Agner, 1990b; Tupker et al, 1990). Despite this body of evidence, Gollhausen et al (1991) believe the concept of a lowered threshold to irritancy in AD remains putative, based on clinical impressions rather than on hard data. Nassif et al (1994) proposed that irritancy in atopics is due to inflammatory cell hyper-reactivity and that the impaired skin barrier (reflected by an increased TEWL) may be a secondary feature. This group demonstrated this by applying increasing concentrations of SLS under patch test occlusion to both atopic and non-atopic subjects. They had two groups, one normal (n=19) and an atopic group (n=20) and calculated the concentration of SLS which would induce an irritant reaction in at least 50 % of that group. They found it was 0.6 % SLS for normals compared with 0.28% SLS for atopics, a result which reached statistical significance. They concluded that the lowered irritancy 'threshold' in atopics may be due to increased cytokines and other mediators present in peripheral blood, mucosal tissue and skin, although they presented very indirect evidence for this hypothesis. Berardesca (1994), however, found an increased TEWL in the uninvolved skin of atopic individuals, suggesting that atopics have a damaged barrier which is not clinically apparent. The findings in Chapter 3 have shown that there were no detectable differences between atopics and non-atopics with ICD when

analysing the various histopathological parameters except for apoptosis. This may imply insufficient numbers of patients to detect any real differences between the two groups.

A novel hypothesis for explaining irritant susceptibility has recently been described by Wakelin et al (1997). This group studied the promoter region polymorphisms in the TNF-α gene. TNF-α is a pro-inflammatory cytokine and any predisposition to switching on this gene may induce irritant inflammation. The volunteers who had a low threshold (i.e. those who showed clinical erythema with a low concentration of SLS) had a statistically significant higher allele frequency of the polymorphism which favoured TNF-α production. They suggest that heterogeneity of this cytokine production may be an important determinant of susceptibility to ICD, a feature which may also be applicable to other cytokines.

7.5 Problems in this project

The following points apply to all aspects of the studies in this thesis.

1.Numbers of subjects

The initial submission for the grant application to support the study of the molecular and cellular mechanisms of ICD envisaged greater numbers of patients with ICD. 66 patients were eventually recruited for the histopathology and immunohistochemistry study (target 120). For the suction blister cytokine study, 35 patients were recruited (target 60). The full complement of volunteers was recruited for the latter. Does this imply unrealistic or overambitious figures? These numbers were chosen to allow comparisons between the different subgroups e.g. atopics versus non-atopics. The

final figures were such that some of these comparisons were done with too few in a group, resulting in a lower statistical power.

It was certainly not easy to persuade patients to undergo four punch biopsies and the group felt relieved that there were no complaints following the procedures. However, when some patients were retraced to see if they would volunteer for the Time 0h suction blister and LC studies, none were forthcoming.

2. Dithranol

One of the major problems and therefore disappointments was the lack of clinical irritation from dithranol. Despite the results from the pilot study and use of a concentration similar to other groups, it was not possible to elicit any significant irritant reaction (see Fig 3c). From clinical experience, it is known that dithranol causes staining as a delayed response and perhaps investigating early changes i.e. 6-24h did not take sufficient account of the pharmacokinetics of this compound.

3. Langerhans cell study

The qualitative study of LC (chapter 5) with irritation used an approach with different patients for each time from 0-24h. The results were pooled for each time point. An alternative and statistically more powerful design, would have been to study subjects with suction blisters raised at all four time points. This of course may not have been permissible as a greater amount of discomfort and time would have been required.

4. Cytokine assays

The major drawbacks in this study were the faulty IFN-γ kit (something beyond our control) and lack of material. The yield of suction blister fluid ranged from 10-240μl. These volumes allowed at best no more than three cytokine assays but realistically two cytokine assays per subject. This automatically reduces the data points on the subsequent graphs (see Figs 4.1, 4.2 and 4.3). In retrospect the volumes required for the assay should have been calculated prior to raising suction blisters. This might have encouraged us to apply four suction blister cups although it seems unlikely that patients and volunteers would have consented to this extra inconvenience.

7.6 Experimental ICD as a model for clinical chronic ICD

Unlike many previous studies of chronic ICD an attempt was made to define the criteria for a diagnosis of ICD. One of the novel aspects of this work was to examine the effects of different irritants in patients with ICD rather than volunteers. The cytokine responses seemed to differ little but some differences were observed in the number of LC in epidermal sheets. The irritant SLS had little bearing on the outcome of neutrophil collection and activation in the skin window experiments.

Experimental ICD is by definition supposed to mimic chronic ICD. The once only patch test application has been the most widely used method for inducing irritation, but Allenby has advocated the repetitive arm immersion model (1993) as a more appropriate model for the clinical situation. Our results showed that the irritants produced different and distinctive effects, which also means that extrapolation to the patient with ICD must be done with caution. SLS has been the

standard irritant for a number of decades because of its detergent effect and because it is found in many products that are likely to come into contact with the skin of affected individuals (e.g. cleaners and other wet work occupations). The variables in any experimental model such as the volume of irritant, time of application and area to which it is applied must be kept constant if studies are to be of any benefit. Thus the diversity of effects of different irritants e.g. epidermal changes, barrier disruption implies that experimental irritant contact reactions are only a very basic model for chronic ICD in humans.

Summary

This work has shown that irritants have different pathological effects despite producing a similar clinical endpoint i.e. erythema.

Nonanoic acid induced a reduction in epidermal LC and alteration in morphology of LC. This was probably due to LC apoptosis- a result of direct toxicity of the irritant and increased migration out of the epidermis. NA irritation stimulated the production of IL-1α but had no effect on IL-8 production.

Sodium lauryl sulphate induced epidermal oedema and parakeratosis. It also had effects on the dermis with induction of a neutrophilia. IL-8 production was a feature of SLS irritation but activation of neutrophil surface molecules appeared to be independent of irritation.

7.7 Suggestions for future work

1. The problem of studying unaffected skin of volunteers could be remedied by biopsying skin from affected sites of ICD and control sites from the same

patients. These biopsies could then be examined for pro-inflammatory cytokine profiles by immunohistochemistry and monoclonal antibodies. The LC numbers as well as their function e.g. class II MHC expression, could be studied in the same biopsies.

2. The control mechanisms which prevent inflammation may also provide clues about chronic ICD. For example, one could investigate the expression or production of inhibitory molecules at irritated sites such as

   - IL-10- which inhibits IFN-γ induced MHC Class II expression

   - TGF-β - a molecule which reduces KC proliferation.

3. The confirmation of apoptosis induced by NA by electron microscopy would be further evidence of the mode of action of this topical irritant.

4. The concept of an autoantigen being responsible for chronicity in ICD needs further examination. Do specific T cells recognise this autoantigen? Is this putative autoantigen a structural protein or even a bacterial antigen located in the epidermis? Work has already commenced to answer these questions.

5. The use of an arm immersion test (Allenby, 1993) prior to patch testing with irritants should prove to be a clinically relevant model. The forearm is pre-irritated by immersion in a solution of SLS for ten minutes. This is repeated 2-3 times before patch tests are applied. Biopsies and suction blisters could then be undertaken on the affected and normal forearms.

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SUBJECT INFORMATION SHEET

As you know we are trying to unravel the complicated ways in which skin irritants cause dermatitis. This involves the application of three well-known irritants (sodium lauryl sulphate, dithranol and nonanoic acid) of which we have considerable experience. These irritants have been chosen because their action is confined to skin and they have no longterm ill-effects.

Weak concentrations of the irritants will be applied on a small area of your skin to either your forearm or abdominal wall for a period of up to 24 hours. They will produce slight redness and possibly some discomfort. After removal of the irritant your skin will be sampled using *skin biopsy/suction blister/skin windows.

* Elliptical Skin Biopsies (not exceeding 1.5cm in length), or 4-6mm punch biopsies will be carried out under local anaesthetic (2% lignocaine) - a routine procedure in the Department of Dermatology. Biopsies will be taken from the test sites exposed to the irritants (or controls) on your forearm or abdominal wall. The small wound will be stitched with one or two sutures. No more than 4 such biopsies will be performed and the number will, of course, be discussed with you. Small scars (not exceeding 1.5cm in length) will result from this procedure.
OR
* Suction Blisters - the Department of Dermatology has considerable experience of this procedure. A suction blister device is attached to the test site on your forearm or abdominal wall and a negative pressure applied for one hour. Five small suction blisters are raised. After removal of the device blister fluid can be removed with a small syringe and the roofs snipped without local anaesthesia. The denuded area will be dressed with an anti-bacterial impregnated tulle-gras dressing. The procedure is virtually painless and there is no residual scarring, but some temporary pigmentation may be anticipated after it.
OR
* Skin Windows - the test site is gently abraded with a scalpel or by sellotape stripping until there is slight oozing. A small chamber is then applied and is filled with sterile salt water solution. The fluid in the chamber is aspirated and replaced at regular intervals up to 72 hours. The procedure is uncomfortable rather than painful. There is no residual scarring but temporary pigmentation may occur at the site.

These samples will enable us to study ways in which irritants cause dermatitis and, hopefully, may eventually lead to better treatment of this common condition.

We must emphasise that, if you do not wish to continue at any stage of the investigation, you are fully free to withdraw. Should you choose to do so, it would, in no way, alter your future management in our department.

Dr. H. Shahidullah,
Clinical Research Fellow in Dermatology.

APPENDIX 2. **Diagnoses of patients used in histopathology and suction blister studies. (see Chapter 3)**

Histopathology study

Subject No.	Age	Sex	Diagnosis	Atopic/Non-atopic
TI	48	M	Hand dermatitis	non
T2	44	M	Leg dermatitis	non
T3	19	F	Hand dermatitis	non
T4	26	F	Facial dermatitis	At
T5	20	M	Hand dermatitis	At
T6	29	F	Hand +face derm	At
T7	20	F	Hand dermatitis	At
T8	31	F	Hand dermatitis	At
T9	25	F	Hand dermatitis	non
T10	61	M	Hand dermatitis	non
T11	23	M	Facial dermatitis	At
T12	18	M	Hand dermatitis	At
T13	57	M	Hand dermatitis	non
T14	46	F	Hand dermatitis	non
T15	38	M	Hand dermatitis	non
T16	44	F	Leg dermatitis	At
T17	29	M	Hand dermatitis	non
T18	46	F	Eyelid dermatitis	non
T19	58	F	Hand dermatitis	non
T20	39	M	Facial dermatitis	At
T21	28	F	Hand dermatitis	At
T22	53	M	Hand dermatitis	At
T23	35	F	Hand dermatitis	non
T24	56	M	Hand dermatitis	non
T25	42	F	Hand dermatitis	non
T26	47	F	Eyelid dermatitis	non
T27	22	M	Eyelid dermatitis	non
T28	55	F	Hand dermatitis	non
T29	18	F	Hand dermatitis	non
T30	65	M	Hand dermatitis	non
T31	59	M	Hand dermatitis	non
T32	35	M	Hand dermatitis	non
T33	40	F	Hand dermatitis	non
T34	21	F	Hand dermatitis	At
T35	20	F	Hand dermatitis	non
T36	24	M	Hand dermatitis	non
T37	48	M	Hand dermatitis	At
T38	24	F	Hand dermatitis	At
T39	28	F	Hand dermatitis	non
T40	52	M	Hand dermatitis	non

Subject No.	Age	Sex	Diagnosis	Atopic/Non-atopic
T41	32	F	Hand dermatitis	non
T42	24	M	Hand dermatitis	At
T43	43	F	Hand dermatitis	non
T44	21	F	Hand dermatitis	non
T45	59	M	Hand dermatitis	non
T46	24	F	Hand dermatitis	At
T47	47	M	Hand dermatitis	non
T48	34	F	Hand dermatitis	non
T49	59	M	Hand dermatitis	non
T50	32	M	Hand dermatitis	At
T51	58	F	Hand dermatitis	non
T52	51	M	Hand dermatitis	non
T53	31	M	Hand dermatitis	At
T54	55	F	Hand dermatitis	At
T55	25	M	Hand dermatitis	non
T56	29	M	Hand dermatitis	non
T57	44	F	Hand dermatitis	At
T58	58	F	Hand dermatitis	At
T59	39	F	Hand dermatitis	At
T60	46	F	Hand dermatitis	non
T61	55	M	Hand dermatitis	non
T62	19	M	Hand dermatitis	At
T63	28	M	Hand dermatitis	At
T64	22	M	Hand dermatitis	At
T65	61	M	Hand dermatitis	non
T66	51	M	Hand dermatitis	non
SUMMARY				
Total no. of pts		66		
Mean Age		38.5	(18-65)	
Male:Female	34/32			
Atopic :Non atopic	25/41			

Suction blister study

Subject No.	Age	Sex	Diagnosis	Atopic/Non-atopic
B1	27	M	Hand dermatitis	At
B2	26	F	Ear dermattis	non
B3	26	F	Facial dermatitis	At
B4	59	F	Hand dermatitis	non
B5	35	F	Hand dermatitis	At
B6	52	M	Hand dermatitis	non
B7	19	F	Hand dermatitis	At
B8	28	F	Hand dermatitis	At
B9	50	F	Hand dermatitis	non
B10	24	F	Hand dermatitis	non
B11	60	F	Hand dermatitis	non
B12	23	M	Hand dermatitis	non
B13	31	F	Hand dermatitis	At
B14	58	F	Hand dermatitis	At
B15	26	F	Hand dermatitis	At
B16	30	F	Hand dermatitis	non
B17	22	F	Hand dermatitis	At
B18	41	M	Hand dermatitis	non
B19	63	F	Hand dermatitis	non
B21	24	F	Hand dermatitis	At
B22	26	F	Hand dermatitis	At
B23	57	F	Hand dermatitis	non
B24	18	F	Hand dermatitis	non
B25	20	M	Hand dermatitis	At
B26	47	M	Hand dermatitis	non
B27	18	F	Hand dermatitis	non
B28	54	M	Hand dermatitis	non
B29	58	F	Hand dermatitis	non
B30	31	F	Hand dermatitis	non
B31	47	F	Facial dermatitis	At
B32	32	F	Hand dermatitis	non
B33	23	F	Hand dermatitis	At
B34	35	F	Hand dermatitis	non
B35	23	F	Hand dermatitis	non
B36	46	F	Hand dermatitis	non
B37	34	F	Hand dermatitis	At
SUMMARY				
Total no. of pts		36		
Mean Age		35.9	(18-63)	
Male:female		7:29		
Atopic :Non atopic		15:21		

APPENDIX 3 : Histopathology proforma used to record details of sections

(see Chapter 3)

Date   Name of assessor   Section code

Photograph Y/N   Review Y/N

Redo section Y/N

Comments:

Appendages   yes=1   none=0

Stratum corneum   0-compact   parakeratosis(yes)-1

                    1-basketweave   "   (none)- 0

Granular layer   0-absent   1-present   2-thickened

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)

	Necrosis/ Apoptosis nil = 0 1-5 =1 5-10 = 2 * if v. high	Oedema i.c. ves spon	Infiltrate	Cells in infiltrate(if +ve) G M Neut Eos
Prickle cell				
Basal layer				

i.c.= intracellular

	Infiltrate	Cells in infiltrate(if +ve) G M Neut Eos	
Localised-papillary dermis			
reticular dermis			
perivascular			
peri-appendageal- pilo-sebaceous			
peri-appendageal- sweat gland			
Diffuse			

APPENDIX 4.   Erythema scores for SLS, NA and DL(see Chapter 3)

Erythema values for Nonanoic acid irritation

	Erythema /Δa^* value arbitrary units					
	Normal	vehicle	**1h**	**6h**	**24h**	**48h**
	7.96	-1.18	6.05	4.38	5.57	3.5
	8.29	2.67	-0.09	4.66	4.59	8.95
	8.43	1.83	0.45	3.54	1.42	5.5
	7.3	1.47	2.21	4.44	1.47	1.04
	9.92	-0.18	1.48	3.42	5.98	6.76
	10.79	-0.86	-0.07	2.08	2.47	3.37
	7.66	0.37	1.64	5.05	4.97	5.61
	9.04	0.65				6.59
	5.84	0.21				5.51
	8.68	-0.19				5.22
	8	0.72				4.1
	6.58	1.13				
	8.68	3.56				
	5.88	-0.37				
Mean	**8.1**	**0.67**	**1.67**	**3.94**	**3.78**	**5.10**
S.E.M.	0.38	0.36	0.80	0.38	0.74	0.63

Erythema values for Sodium lauryl sulphate irritation

	Erythema /Δa^* value arbitrary units					
	Normal	**vehicle**	**1h**	**6h**	**24h**	**48h**
	7.44	5.33	0.78	4.5	3.55	6.4
	6.95	-0.26	0.53	2.88	1.49	1.29
	13.4	-0.96	1.35	2.83	6.01	7.36
	8.73	1.74	2.39	3.9	5.94	9.87
	7.76	1.44	0.31	4.6	1.68	5.71
	9.9	-1.89	-1.06	7.29	8.73	0.28
	9.6	-1.99	-1.54	6.18	0.27	1.07
	8.33	0.1			4.91	6.53
	8.5	0.08				16.53
	4.45	0.85				6.25
	8.24	1.41				
	5.62	1.08				
	7.6	-1.53				
	9.77	-1.37				
	12.21	2.27				
Mean	**8.57**	**0.42**	**0.39**	**4.60**	**4.07**	**6.13**
S.E.M.	0.59	0.50	0.50	0.62	1.00	1.52

Erythema values for Dithranol irritation

	Erythema /Δa^* value arbitrary units					
	Normal	**vehicle**	**1h**	**6h**	**24h**	**48h**
	5.47	-0.78	-0.42	0.07	-0.06	-0.08
	6.68	-0.34	0.83	1.86	0.94	1.18
	8.68	-1.26	0.48	0.19		1.37
	10.44	1.18	-0.85	0.41		2.00
	6.16	-0.95	1.89	-0.23		-0.08
	6.5	0.51	0.88	0.01		0.14
	7.53	-0.84	0.57	-1.83		
	3.89	0.34				
	6.93	0.47				
Mean	**6.92**	**-0.18**	**0.48**	**0.07**	**0.44**	**0.76**
S.E.M.	0.62	0.27	0.34	0.41	0.50	0.36

APPENDIX 5

Raw data tables and statistical analyses for histopathology study (see Chapter 3, Table 3.5)

1. Parakeratosis

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	1	1	1	7
Absent	31	13	31	9
Total	**32**	**14**	**32**	**16**

Late NA v. Late SLS Fishers exact test p=0.059 not significant (n.s.)

2. Basal apoptosis

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	14	9	3	5
Absent	18	5	29	11
Total	**32**	**14**	**32**	**16**

Early NA v. Early SLS $\chi^2 = 8.01$ p<0.005**   significant
Late NA v. Late SLS $\chi^2 = 2.08$ p<0.1   n.s.

3. Spongiosis

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	5	6	1	11
Absent	27	8	31	5
Total	**32**	**14**	**32**	**16**

Early NA v. Early SLS $\chi^2 = 2.72$ p<0.1   n.s.
Late NA v. Late SLS $\chi^2 = 3.23$ p<0.1   n.s.

4. Vesiculation

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	3	0	3	5
Absent	29	14	29	11
Total	**32**	**14**	**32**	**16**

Late NA v. Late SLS Fishers exact test p=0.036* significant

5. Intravascular neutrophilia

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	12	1	3	7
Absent	20	13	29	9
Total	**32**	**14**	**32**	**16**

Early NA v. Early SLS $\chi^2 = 5.7$ p<0.025*   significant
Late NA v. Late SLS   Fishers exact test p=0.059 n.s.

6. Papillary dermal neutrophilia

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	12	6	4	8
Absent	20	8	28	8
Total	**32**	**14**	**32**	**16**

Early NA v. Early SLS $\chi^2 = 4.08$ p<0.05*   significant

7. Intravascular eosinophilia

No. of biopsies	NA		SLS	
	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	4	0	2	3
Absent	28	14	30	13
Total	**32**	**14**	**32**	**16**

Late NA v. Late SLS   Fishers exact test p=0.138 n.s.

8. Papillary dermal eosinophilia

No. of biopsies	Early (1& 6h)	Late (24 &48h)	Early (1& 6h)	Late (24 &48h)
Present	5	1	4	6
Absent	27	13	28	10
Total	**32**	**14**	**32**	**16**

Late NA v. Late SLS $\chi^2 = 5.73$ $p<0.025*$ significant

Vehicle controls

1. Propanol (for NA) No. of biopsies

Parakeratosis   0/24
Intracellular oedema   20/24
Spongiosis   2/24
Basal apoptosis   5/24
Basal infiltrate   0/24
Papillary derm. Infil 16/24   all mononuclear

2. Water (for SLS)   No. of biopsies

Parakeratosis   0/24
Intracellular oedema   20/24
Basal apoptosis   3/24
Basal infiltrate   2/24
Papillary derm. Infil 14/24

APPENDIX 6

Tables of clinical reaction grades and assays performed in suction blister cytokine ELISA study (Chapter 4)

1. Volunteers

Volunteer No.	Volume of blister fluid/μl	Time after irritation	Irritant reaction grade	Assays performed		
				IFN-γ	IL-1α	IL-8
BV2	80	1h	1	+		
BV3	120	1h	1		+	+
BV4	100	1h	1			
BV7	120	1h	1	+	+	
BV10	80	1h	1		+	+
BV11	80	1h	1	+		
BV13	60	1h	1		+	
BV22	40	1h	1			+
BV23	60	1h	1	+		
BV30	Lost	1h	1			
BV1	100	6h	2	+	+	
BV5	70	6h	2	+		
BV6	80	6h	2	+		
BV8	80	6h	2	+		
BV12	40	6h	2			+
BV17	150	6h	2	+		+
BV20	100	6h	2	+		+
BV21	75	6h	2		+	+
BV27	80	6h	2		+	+
BV28	70	6h	2		+	
BV9	100	24h	2		+	+
BV14	80	24h	2			+
BV15	140	24h	2	+	+	
BV16	180	24h	2	+	+	+
BV18	140	24h	2	+		+
BV19	180	24h	2	+	+	+
BV25	180	24h	2	+	+	+
BV26	150	24h	2	+		+
BV29	100	24h	2	+	+	
BV24	180	24h	2	+	+	+

2. Patients with ICD

Patient No.	Atopic/ non-atopic	Volume of blister fluid/ μl	Time post irritation	Reaction grade	Assays performed		
					IFN–γ	IL-1α	IL-8
B1	Atopic	nil	1h	0			
B3	Atopic	nil	1h	2			
B5	Atopic	50	1h	0			+
B7	Atopic	60	1h	2			+
B8	Atopic	50	1h	1		+	
B13	Atopic	250	1h	0	+	+	+
B14	Atopic	100	1h	1	+		+
B15	Atopic	150	1h	2	+	+	
B17	Atopic	200	1h	2	+	+	+
B2	Non-atopic	70	1h	0		+	
B4	Non-atopic	50	1h	1	+		
B6	Non-atopic	100	1h	2	+	+	
B9	Non-atopic	40	1h	1			+
B10	Non-atopic	90	1h	2	+		+
B11	Non-atopic	130	1h	1	+	+	
B12	Non-atopic	100	1h	1	+	+	
B18	Non-atopic	10	1h	2			
B19	Non-atopic	130	1h	1		+	
B24	Non-atopic	100	1h	1	+		+
B21	Atopic	120	6h	2	+	+	
B22	Atopic	100	6h	2		+	+
B25	Atopic	150	6h	2	+	+	
B16	Non-atopic	110	6h	2		+	+
B23	Non-atopic	100	6h	2	+		+
B26	Non-atopic	140	6h	2	+	+	
B31	Atopic	180	24h	2	+	+	+
B33	Atopic	120	24h	2	+		+
B37	Atopic	240	24h	2	+	+	+
B27	Non-atopic	200	24h	2	+	+	+
B28	Non-atopic	150	24h	2	+		+
B29	Non-atopic	140	24h	2	+	+	
B30	Non-atopic	180	24h	2	+	+	+
B32	Non-atopic	130	24h	2	+		+
B34	Non-atopic	150	24h	2	+	+	
B35	Non-atopic	140	24h	2		+	+
B36	Non-atopic	200	24h	2	+	+	+

APPENDIX 7 Langerhans cells/mm^2 raw data for confocal study (Chapter 5)

1. Patients

prefix b= patient with ICD ; suffix a=1h b=6h c=24h nonanoic acid irritation
suffix n=patient with blister at non-irritated site

Patients-0h	LC/ mm^2	Patients-1h	LC/ mm^2
b2n	418	b10a	224
b4n	427	b11a	326
b9n	418	b12a	382
		b2a	407
		b4a	335
		b6a	326
		b7a	318
		b8a	300
		b9a	294
n	3	n	9
mean	421	mean	323.6
sd	5.19	sd	52.31
sem	3	sem	17.44

Patients-6h	LC/ mm^2	Patients-24h	LC/ mm^2
b16b	153	b27c	97
b25b	247	b28c	76
b26b	318	b29c	132
b21b	189	b30c	174
b22b	182	b31c	129
b23b	265	b32c	88
n	6	n	6
mean	225.7	mean	116
sd	61.7	sd	36.15
sem	25.21	sem	14.76

2. Volunteers

prefix BV, VN, JS =normal volunteers
suffix A=1h B=6h C=24h nonanoic acid irritation

Volunteer-0h	LC/ mm^2	Volunteer-1h	LC/ mm^2
BV31	162	BV2A	420
BV32	259	BV3A	569
BV33	338	BV4A	450
VN0	652	BV7A	388
VN2	429	BV10A	418
VN4	444	BV11A	247
JS1	612	VN1	597
JS2	312	VN3	456
JS3	508	VN5	453
JS4	182		
n	10	n	9
mean	**389.8**	**mean**	**44.2**
sd	169.14	sd	101.5
sem	53.49	sem	33.85

Volunteer-6h	LC/ mm^2	Volunteer-24h	LC/ mm^2
BV1B	285	BV14C	118
BV5B	371	BV15C	144
BV6B	300	BV16C	241
BV8B	359	BV18C	185
BV12B	326	BV9C	94
BV17B	365		
n	6	n	5
mean	**334.3**	**mean**	**156.4**
sd	36.3	sd	58.1
sem	14.8	sem	25.98

APPENDIX 8. Raw data for LC dendrite lengths and no. of dendrites /LC (Chapter 5)

Key

B= Patient with ICD
BV= Volunteer
B–number-N= patient with ICD, non-irritated skin
Number prefix= no. of LC in specimen examined at high power
Letter suffix: A=1hour B=6hours C=24hours Nonanoic acid irritation
0=non-atopic 1=atopic

Specimen code	No. of dendrites/LC	length of dendrites/μm	atopic/ non atopic
1-BV31	4	18.75	0
		9.58	0
		16.04	0
		11.97	0
2-BV31	5	18.68	0
		22.7	0
		16.23	0
		14.94	0
		5.94	0
1-BV32	11	12.95	0
		8.85	0
		10.98	0
		31.61	0
		6.14	0
		6.4	0
		22.23	0
		26.03	0
		16.99	0
		14.64	0
		8.83	0
2-BV32	5	35.03	0
		9	0
		25.88	0
		27.06	0
		31.78	0
1-BV33	7	13.44	0
		22.55	0
		16.48	0
		10.53	0
		16.62	0
		11.37	0
		15.11	0

2-BV33	4	9.17	0
		8.76	0
		10.22	0
		10.06	0
3-BV33	6	11.6	0
		14.59	0
		14.2	0
		6.59	0
		6.65	0
		21.25	0
1-B2A	5	7.41	0
		5.5	0
		5.75	0
		8.4	0
		4.94	0
2-B2A	5	24.19	0
		11.03	0
		10.65	0
		6.93	0
		15.78	0
3-B2A	9	17.19	0
		12.28	0
		16.22	0
		10.34	0
		12.23	0
		2.85	0
		5.67	0
		7.13	0
		8.52	0
1-B5A	3	7.71	1
		5.44	1
		32.64	1
2-B5A	6	9.91	1
		10.89	1
		14.04	1
		7.11	1
		10.94	1
		8.97	1
1-B6A	5	9.46	0
		16.4	0
		4.09	0
		9.03	0
		10.31	0
2-B6A	4	11.25	0
		15.89	0
		4.56	0
		6.06	0
1-B7A	4	9.39	0
		5.26	1

			14.6	1
			9.05	1
2-B7A		5	9.54	1
			18.31	1
			6.93	1
			4.6	1
			4.5	1
1-B8A		5	15.74	1
			5.54	1
			5.63	1
			15.86	1
			8.82	1
2-B8A		7	16.13	1
			11.7	1
			12.07	1
			17.43	1
			18.29	1
			12.49	1
			9.41	1
3-B8A		7	6.59	1
			6.15	1
			12.26	1
			4.35	1
			9.32	1
			14.65	1
			7.94	1
1-B9A		5	11.28	0
			14.19	0
			8.06	0
			7.06	0
			19.09	0
2-B9A		4	14.59	0
			6.84	0
			6.12	0
			7.44	0
1-B10A		6	13.29	0
			12.12	0
			18.52	0
			3.49	0
			4.67	0
			3.1	0
2-B10A		4	20.12	0
			9.29	0
			16.15	0
			7.98	0
1-B11A		3	18.15	0
			8.61	0
			16.77	0
2-B11A		5	22.06	0

			14.26	0
			3.53	0
			22.39	0
			10.3	0
1-B12A		4	11.14	0
			10.02	0
			7.62	0
			1.95	0
2-B12A		6	4.3	0
			5.49	0
			10.28	0
			4.99	0
			6.38	0
			11.64	0
1-BV2A		6	11.51	0
			15.37	0
			9.1	0
			10.01	0
			5.02	0
			11.74	0
2-BV2A		4	25.52	0
			10.16	0
			12.43	0
			20.2	0
3-BV2A		4	10.33	0
			6.87	0
			8.62	0
			14.22	0
1-BV3A		3	23.73	0
			14.09	0
			29.56	0
2-BV3A			30.58	0
			24.72	0
			4.38	0
			4.02	0
			32.82	0
1-BV4A		4	20.93	0
			10.06	0
			23.18	0
			14.22	0
2-BV4A		3	12.8	0
			9.59	0
			9.02	0
3-BV4A		4	20.53	0
			11.19	0
			30.06	0
			22.14	0
1-BV7A		4	15.87	0
			12.32	0

			11.54	0
			7.78	0
2-BV7A		4	11.7	0
			7.3	0
			15.04	0
			16.24	0
3-BV7A		3	12.2	0
			7.82	0
			10.39	0
1-BV10A		5	10.45	0
			10.24	0
			7.68	0
			13.96	0
			4.4	0
2-BV10A			18.15	0
			17.16	0
			11.94	0
			18	0
			17.38	0
1-BV11A		3	25.38	0
			15.32	0
			13.61	0
2-BV11A		5	11.22	0
			11.35	0
			6	0
			11.25	0
			13.9	0
1-B16B		3	10	0
			11.81	0
			3.63	0
2-B16B		3	10.96	0
			12.64	0
			14.54	0
3-B16B		5	8.82	0
			10.09	0
			6.38	0
			8.02	0
			8.14	0
1-B21B		4	10.86	1
			7.38	1
			3.46	1
			11.33	1
1-B22B		6	9.94	1
			8.58	1
			15.13	1
			11.96	1
			12.35	1
			20.22	1
2-B22B		4	6.49	1

			6.45	1
			12.97	1
			9.45	1
1-B23B		2	14.07	0
			14.59	0
2-B23B		3	7.62	0
			15.31	0
			11.1	0
3-B23B		4	10.02	0
			10.72	0
			5.14	0
			6.11	0
4-B23B		4	13.24	0
			10.76	0
			9.41	0
			11.21	0
1-B25B		5	18.51	1
			20.04	1
			11.01	1
			14.65	1
			11.17	1
2-B25B		2	4.74	1
			6.22	1
1-B26B		3	6.41	0
			3.62	0
			8.26	0
2-B26B		3	5.47	0
			4.76	0
			8.36	0
3-B26B		3	17.25	0
			9.93	0
			5.95	0
1-BV1B		4	4.29	0
			4.61	0
			8.1	0
			2.78	0
2-BV1B		7	8.41	0
			4.72	0
			11.41	0
			4.15	0
			7.62	0
			4.7	0
			2.89	0
1-BV5B		5	6.61	0
			7.78	0
			4.33	0
			5.33	0
			5.26	0

2-BV5B	3	5.87	0
		8.98	0
		7.91	0
1-BV6B	3	8.99	0
		7.37	0
		12.18	0
2-BV6B	3	3.2	0
		4.42	0
		3.01	0
1-BV8B	3	4.02	0
		3.39	0
		2.19	0
2-BV8B	3	3.32	0
		7.38	0
		3.58	0
1-BV12B	5	6.79	0
		3.65	0
		2.54	0
		4.12	0
		5.67	0
2-BV12B	6	6.55	0
		4.28	0
		5.58	0
		3.87	0
		1.3	0
		3.54	0
3-BV12B	4	6.29	0
		2.37	0
		2.83	0
		4.07	0
1-BV17B	2	5.19	0
		2.12	0
2-BV17B	5	2.12	0
		8.67	0
		11.54	0
		12.21	0
		5.4	0
1-B27C	3	6.81	0
		7.1	0
		5.33	0
2-B27C	3	3.37	0
		3.03	0
		4.04	0
3-B27C	3	4.39	0
		3.55	0
		2.42	0
1-B28C	4	4.85	0
		3.31	0
		3.64	0

			2.27		0
2-B28C		2	2.53		0
			3.61		0
1-B29C		2	3.22		0
			3.39		0
2-B29C			7.88		0
			7.51		0
			2.52		0
			5.13		0
1-B30C		4	7.65		0
			5.31		0
			4.26		0
			5.98		0
2-B30C		2	7.07		0
			1.81		0
1-B31C		2	2.49		1
			5.05		1
2-B31C		3	6.08		1
			8.87		1
			7.57		1
1-B32C		4	7.49		0
			14.12		0
			5.29		0
			8.53		0
2-B32C			10.58		0
			3.85		0
			7.01		0
			3.56		0
			3.38		0
3-B32C			7.24		0
			2.62		0
			2.7		0
			7.15		0
			8.95		0
			8.66		0
1-BV9C		2	6.46		0
			5.25		0
2-BV9C		2	2.35		0
			2.53		0
1-BV14C		4	2.89		0
			5.39		0
			2.92		0
			3.84		0
2-BV14C		3	3.69		0
			3.61		0
			3.32		0
1-BV15C		5	4.55		0
			5.61		0
			2.31		0

			3.18	0
			3.22	0
2-BV15C		4	3.3	0
			3.33	0
			5.9	0
			3.97	0
1-BV16C		4	8.67	0
			15.98	0
			4.92	0
			7.36	0
2-BV16C		4	15.12	0
			5.47	0
			6.56	0
			8.2	0
1-BV18C		5	3.23	0
			4.81	0
			7.37	0
			6.76	0
			7.66	0
2-BV18C		2	7.58	0
			7.54	0
1-B2N		4	11.57	0
			17.5	0
			21.51	0
			6.11	0
2-B2N		6	23.47	0
			12.67	0
			8.56	0
			10.43	0
			11.53	0
			6.31	0
3-B2N		4	18.84	0
			14.76	0
			6.55	0
			14.24	0
4-B2N		4	11.69	0
			10.38	0
			5.94	0
			6.81	0
1-B4N		5	8.12	0
			8.04	0
			13	0
			11.14	0
			10.73	0
2-B4N		5	15.89	0
			18.27	0
			10.19	0
			15.16	0
			12.98	0

1-B9N		5	10.52	0
			3.96	0
			12.83	0
			5.15	0
			9.62	0
2-B9N		3	18.95	0
			16.29	0
			12.63	0

Epidermal Langerhans cell apoptosis is induced *in vivo* by nonanoic acid but not by sodium lauryl sulphate

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Summary   Exposure to irritants may cause chronic irritant contact dermatitis (ICD), characterized by irregular epidermal thickening and a predominantly dermal mononuclear cell infiltrate. The mechanisms involved, and why only certain individuals are affected, are not clearly understood. Different irritants may trigger different cellular and molecular interactions between resident skin cells and recruited inflammatory cells. In some individuals these interactions may become self-perpetuating resulting in persistent inflammation in the absence of continued exposure. This study examined Langerhans cell (LC) density in clinically normal skin of 46 patients with chronic ICD and 10 healthy individuals, and compared the action of the two irritants nonanoic acid (NA) and sodium lauryl sulphate (SLS) on the LCs and keratinocytes of clinically normal skin in patients with chronic ICD. There was a higher number of LCs/mm basement membrane in patients compared with controls, although there was no difference in the number of dendrites/LC nor in dendrite length. SLS induced keratinocyte proliferation after 48 h exposure, had no effect on LC number or distribution, and induced keratinocyte apoptosis after 24 and 48 h exposure. In contrast, NA decreased keratinocyte proliferation after 24 h exposure but this returned to basal levels after 48 h, and induced epidermal cell apoptosis after only 6 h exposure. NA dramatically decreased LC number after 24 and 48 h exposure, which was accompanied by basal redistribution and decreased dendrite length. Most significantly, NA induced apoptosis in over half of the LCs present after 24 and 48 h exposure.

Chronic irritant contact dermatitis (ICD) is a common clinical problem which affects people in a variety of occupations and results in a significant loss of time at work. Chronic ICD is a non-antigen-mediated multifactorial condition characterized clinically by erythema, oedema and dryness[1,2] and histologically by epidermal thickening and a mononuclear, predominantly dermal inflammatory cell infiltrate. Chronic ICD may follow contact with a diverse range of irritant substances. The differing chemical nature of these irritants is considered to influence the type of epidermal damage such as denaturation of epidermal keratins and stripping of surface lipids.[3] Considerable information is available concerning the influence of factors such as age, race and exposure site on the development of chronic ICD,[4] but less is known about the effects of irritants on epidermal cells directly and how these may relate to severity and chronicity of the condition. In particular the reasons why chronic ICD only occurs in some individuals exposed to irritants remain unclear.

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The histology of chronic ICD suggests that, even in the absence of a known antigen, the immune system is being driven by epidermal and dermal cells, or their products, to recruit and maintain activated inflammatory cells at a localized site over a prolonged time. This suggests interaction between components of the skin immune system[5] particularly Langerhans cells (LCs), activated keratinocytes (KCs) and lymphocytes, resulting in the recruitment of inflammatory cells to the affected area.

Previous studies have attempted to model ICD by studying epidermal and dermal changes after the application of irritants under standard patch test conditions to normal volunteers for 24 h or longer. The most commonly used irritants have been nonanoic acid (NA), sodium dodecyl (lauryl) sulphate (SLS) and dithranol.[6–9] It has been reported that NA and SLS can cause increased epidermal cell proliferation.[4,6] Experimental NA irritation has been shown to decrease numbers of epidermal LCs[10–14] and to decrease dendrite length.[10,11] SLS also variably affects LCs in experimental irritant reactions with decreased cell

Table 1. Additional criteria used for the diagnosis of chronic irritant dermatitis

History	Occupational exposure, affected areas relatively static
Examination	Erythema, hyperkeratosis or fissuring, glazed, scalded appearance of skin, hand dermatitis (dorsa): ring pattern, pulpitis
Patch test result	Negative (or no relevant positive reaction)

numbers,[15] increased epidermal cell numbers,[16] decreased dendrite length[10] or minor differences[17] all being reported. SLS 10% applied to the foot has been reported to induce migration of LCs from the skin to the lymph.[18]

We investigated the effects of NA and SLS on LCs and on epidermal proliferation and apoptotic cell death 1, 6, 24 and 48 h after their application to clinically normal skin of patients with chronic ICD.

Materials and methods

Ethical approval for the study was obtained from the Lothian Research Ethics Committee.

Subjects

Only patients with chronic ICD of > 6 weeks duration were recruited from the contact dermatitis clinic. Of these patients, 91% had chronic hand dermatitis only. Additional criteria for diagnosis are laid out in Table 1: a minimum of one criterion from each category had to be fulfilled before any patient was recruited for the study. Approximately half of the recruited patients were atopic as defined by the U.K. Working Party's diagnostic criteria for atopic dermatitis.[19] Volunteers with no history of skin disease were recruited from the academic, medical and technical staff of the Departments of Pathology and Dermatology at the Edinburgh University Medical School.

Irritants

For this study it was important to compare clinically similar irritant reactions. A pilot study was undertaken to establish the concentrations of both irritants which would give moderate erythema in over 75% of subjects tested after 24 h application as measured by Minolta Chroma Meter CR-300 (Minolta, U.K.). There was no significant difference in the reactions measured on

patch tested skin of normal individuals and non-involved skin of patients. The concentrations selected were NA 80% in propan-1-ol and 5% aqueous SLS. All irritant solutions were prepared by the Royal Infirmary Pharmacy.

Biopsies

Six-millimetre punch biopsies (up to a maximum of four per person) were taken under local anaesthetic with 1% lignocaine. Patients (25 men, 21 women, age range 18–62 years) were randomized into two groups and all had two irritant patches applied for either 1 or 6 h (group 1, $n = 32$), or 24 and 48 h (group 2, $n = 14$), and one vehicle control patch. A control 6-mm punch biopsy (normal uninvolved skin) was taken from the forearm at time zero. The irritant and vehicle control (15 μL) were applied to the volar aspect of the forearms (two per arm), under 8 mm Finn chambers. One hour after removal of the Finn chamber the degree of erythema was measured using a Minolta Chroma Meter CR-300 and each patch site biopsied. Half of each punch biopsy was snap frozen in liquid nitrogen and stored at −70 °C; the remainder was formalin fixed, dehydrated and embedded in wax. Single volar forearm punch biopsies from 10 normal volunteers (three men, seven women, age range 23–58 years) were processed in the same manner.

Blisters

Twenty-one patients (five men, 16 women) with chronic ICD, and 22 healthy volunteers (six men, 16 women) with no past history of any skin disease or atopy, were additionally recruited. NA was applied under 8 mm Finn chambers to normal appearing volar forearm skin as above. Seven volunteers had blisters raised with no irritant application; three volunteers and three patients had blisters raised after both no irritant application and NA application; 18 patients and 17 volunteers had NA applied for 1, 6 or 24 h.

Determination of visible apoptosis in haematoxylin and eosin stained sections

Three-micrometre paraffin sections stained with haematoxylin and eosin (H&E) were examined by two of three observers (R.J.S., C.S. and S.E.M.H.) simultaneously on a multiheader microscope at ×400 magnification. The concordance rate between observer pairs was > 95%. All sections were enumerated along their

whole length for the presence of visibly apoptotic cells in the basal and prickle layers. An apoptotic cell was defined by the presence of two or more apoptotic bodies.[20,21] The length of basement membrane (BM) was determined on a Zeiss HOME computer-assisted microscope (Zeiss, U.K.).[22] Apoptotic cells were then expressed as number of visibly apoptotic cells in the basal and prickle layers per mm BM. Cells in the stratum granulosum were not counted as their keratohyaline granules made it difficult to distinguish them from apoptotic cells.

TdT-mediated dUTP-biotin nick end-labelling

The Boehringer Mannheim '*In Situ* Cell Death Detection Kit' (Boehringer Mannheim, Mannheim, Germany, Cat. no. 1684809) was also used to detect apoptosis on paraffin sections. The manufacturer's protocol was followed and *in situ* DNA nicks visualized with fast red. This method is not apoptosis-specific and detects all forms of cell death involving DNA nicks (including apoptosis, necrosis and KC terminal differentiation). In the epidermis this poses particular problems when assessing the terminally differentiating cells in the stratum granulosum as some of the cells stain positive even in normal skin. This method was therefore used in conjunction with apoptosis detected morphologically in the prickle and basal layers on parallel H&E stained sections.

Immunohistochemical staining and analysis

Three-micrometre paraffin or 6-μm frozen sections were stained in a Sequenza semiautomatic staining system (Shandon Laboratories, Life Sciences International, Basingstoke, U.K.) using a standard protocol and visualized with either 3,3'-diaminobenzidine tetrahydrochloride or fast red. Proliferating cells were detected using monoclonal antibody (mAb) to the Ki67 nuclear antigen expressed by cycling cells (Dako, Glostrup, Denmark, code M0722); CD1a+ LCs were detected using mAbs (Dako, code M721 on frozen sections and Immunotech 1590 from Beckman-Coulter, Luton, U.K., on paraffin sections). Cells with Ki67+ nuclei and CD1a+ cells (epidermal cell bodies with at least two attached dendrites) in the epidermis were counted over the entire section and the length of BM measured on the HOME microscope; the results were expressed as numbers of positive cells/mm BM. For CD1a+ cells the epidermal and dermal distribution pattern was also noted. The distribution patterns were analysed in the epidermis as

'normal' or 'upper epidermis' or 'basal' and in the papillary dermis as 'perivascular' or 'diffuse'.

Immunofluorescence staining and confocal laser scanning microscopy

Intact suction blister roofs were incubated in mouse monoclonal anti-CD1a antibody (Dako, code M721) overnight at 4 °C. The roofs were washed, incubated in fluorescein isothiocyanate-conjugated sheep anti-mouse IgG (Sigma Chemical Co., Poole, U.K.) for 1 h at room temperature, washed again and mounted in antifadent solution (Citifluor Ltd, Canterbury, U.K.) under glass coverslips sealed with nail varnish. The Zeiss LSM 10 confocal laser scanning microscope was used to examine the CD1a+ LCs. Blisters were optically sectioned in the z-plane and images (512×341 pixels) were collected at a rate of 2 s/scan. The whole intensity of the laser was used without a neutral density filter. One investigator (H.S.) examined all sections. For morphological assessment, CD1a+ LCs were examined at high power using a $\times 40$ objective lens and $\times 80$. For each LC, 40 optical sections were scanned, each of $0.5 \mu m$ thickness. The first section was always positioned immediately above the cell and scanning continued until no further fluorescence was visible. The stored images were analysed by a Kontron IBAS 20 image analysis system. A threshold of $32-35$ was set for image data (arbitrary scale $0-255$), this being the level above which no staining was seen on the negative controls. The number of dendrites/LC (defined as processes visibly attached to the cell body on the composite image) and lengths of dendrites were measured on each composite high power field image, after calibrating the field using a graticule of $10 \mu m$ length.

Statistical analysis

Overall changes within groups were investigated using the Friedman test. Any significant differences were then further qualified using the McNemar/Sign test. Analysis of results between groups was carried out using χ^2 or Wilcoxon signed rank 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.

Results

Gender, atopic status and vehicle controls

No significant difference was found between men and
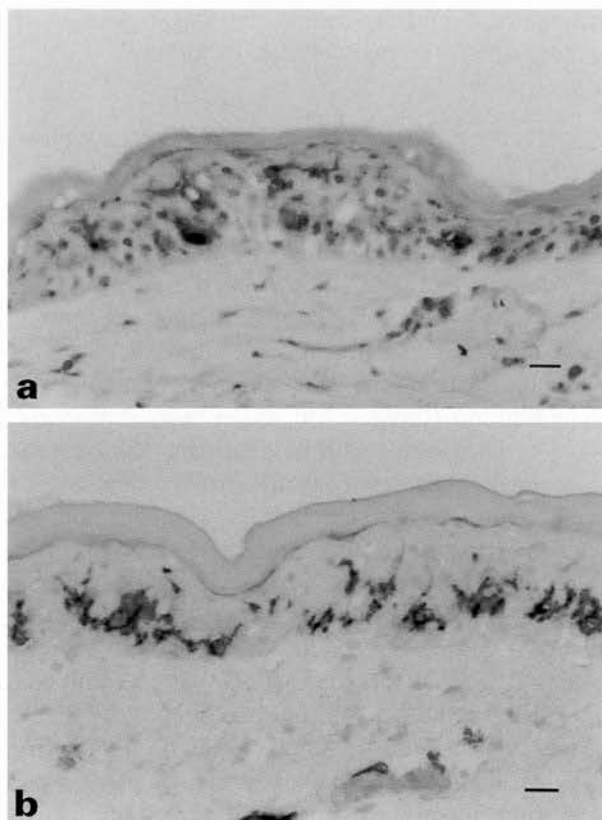
Figure 1. Distribution of CD1a+ Langerhans cells in (a) control volar forearm skin from a patient with chronic irritant dermatitis and (b) volar forearm skin after 24 h exposure to nonanoic acid in the same patient. Bar = 50 μm.

women or between atopic and non-atopic patients, at time 0, or at any time point, for any measurement after either irritant; thus data have been pooled. Vehicle controls had no effect on any parameter measured.

Patients with chronic irritant dermatitis have more epidermal CD1a + dendritic cells than normal volunteers

The median number of epidermal dendritic CD1a+ cells in sections of uninvolved forearm skin from all 46 patients was 12·4/mm BM (range 5·6–30·0). CD1a+ cells showed a normal even distribution throughout the epidermis. This number was significantly higher than the median number of epidermal dendritic CD1a+ cells in the forearm skin from 10 normal volunteers: 9·8/mm BM (range 4·8–15·5), $P < 0.01$. A similar trend was seen by confocal microscopy of blister roofs which demonstrated that the untreated, unaffected skin of patients had increased numbers of LCs/mm^2 (median value 418, range 224–427) compared with healthy

Table 2. Number of CD1a + dendritic cells/mm basement membrane after 24 and 48 h exposure to nonanoic acid

Patient no.	Control	24 h	48 h
1	·9·2	5·8	0·5
2	11·2	5	4·3
3	19·0	5·6	5·4
4	21·3	9	2·1
5	13·5	9	3·5
6	10·6	2·9	0·8
7	11·7	10·2	5·0
Median	11·7	5·8*	3·5**

$*P < 0.02$, $**P < 0.03$, two-tailed Mann–Whitney U-test.

volunteers (median value 390, range 162–429), although this was done on fewer individuals and did not reach significance.

Nonanoic acid rapidly causes basal redistribution of epidermal CD1a + cells followed by a decrease in their number

By 6 h after exposure to NA, the epidermal CD1a+ LCs had redistributed to a basal pattern in nearly all biopsies. This pattern was maintained at 24 and 48 h ($P < 0.001$ at all time points, χ^2-test; Fig. 1a,b). There was no change in the number of CD1a+ LCs/mm BM in the group of patients exposed to NA for 1 and 6 h, but there was a significant decrease after 24 h and a further drop at 48 h (Table 2).

Confocal microscopy of blister roofs confirmed these findings in patients with the number of LCs decreasing after 24 h NA exposure from a median of 418 to 116/mm^2. A similar effect was seen in blister roofs of normal volunteers with a decrease from a median of 390 to 156/mm^2. In the dermis, all biopsies showed a perivascular distribution of CD1a+ cells, which was not changed by NA application. There was no significant change between the median number of 28 CD1a+ cells/mm^2 papillary dermis at time 0 and any other time point.

The effect of nonanoic acid on Langerhans cell dendrite number and length

The confocal microscope was used to assess the effect of NA on the number and length of dendrites on epidermal LCs in blister roofs. The typical appearance of a CD1a+ LC from a healthy individual is shown in Figure 2(a). Normal healthy volunteers had a median number of 5·6 dendrites/LC with a median length of 14·5 μm prior to
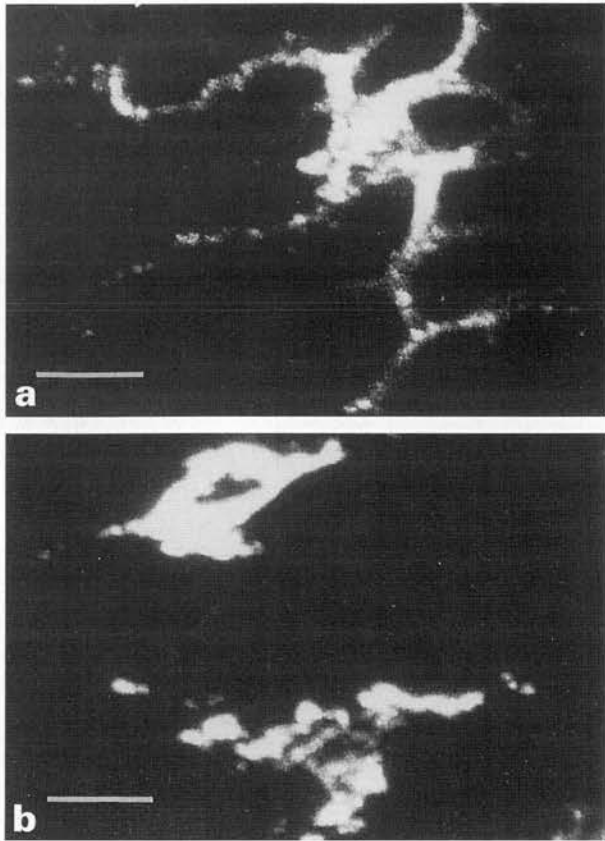
Figure 2. Confocal microscopy of CD1a+ Langerhans cells in epidermal sheets from suction blister roofs. (a) Normal volunteer at time 0; (b) normal volunteer after 24 h exposure to nonanoic acid. Bar = 5 μm.

application of NA; patients with chronic ICD had a median of 4·5 dendrites/LC with a median length of 11·5 μm, but neither of these parameters was significantly different between the two groups (Mann–Whitney *U*-test).

Volunteers showed a significantly decreased number of dendrites compared with the time 0 controls at 24 h (median 3·5 dendrites/LC, $P < 0.03$), whereas patients did not show a significant decrease in number of dendrites at any time point. In volunteers the dendrite length was significantly decreased at 6 h (median 4·5 μm, $P < 0.001$) and at 24 h (median 5·03 μm, $P < 0.001$) after treatment. Patients showed no difference from control values at 6 h, but the dendrite length was significantly decreased at 24 h (median 5·0 μm, $P < 0.001$). Comparison between patients and volunteers revealed that at 6 h the decrease in dendrite length in volunteers was significantly greater than that seen in patients ($P < 0.001$) but that at 1 and 24 h the two

groups were not significantly different from each other. Figure 2(b) shows the typical appearance of an LC from a patient after 24 h exposure to NA.

Sodium lauryl sulphate does not significantly decrease epidermal CD1a+ dendritic cells

There was a slight reduction in the number of epidermal CD1a+ LCs after 24 and 48 h exposure to SLS, but this was not significant (Table 3), nor was there any consistent alteration in their distribution pattern at any time point after SLS application. The difference between the group of patients treated with NA and the group treated with SLS was significant at both 24 h ($P < 0.01$) and 48 h ($P < 0.01$).

Epidermal proliferation decreases and then recovers after nonanoic acid but increases after sodium lauryl sulphate irritation

There was no significant difference between time 0 control biopsies and 1 and 6 h exposure to either irritant in the numbers of proliferating (Ki67+ nuclei) cells in the basal epidermis. Table 4 shows that there was a significant increase in basal layer KC proliferation after 48 h SLS exposure. In contrast, significantly decreased proliferation was seen at 24 h after irritation with NA, which recovered to control levels by 48 h. Figure 3(a,b) illustrates the Ki67 staining on time 0 control and 48 h biopsies from the same patient.

Both nonanoic acid and sodium lauryl sulphate cause apoptosis of epidermal cells

Visibly apoptotic cells were extremely rare in time 0 control biopsies, but were occasionally detected in the prickle and basal layers. After exposure to NA, a significant increase in prickle layer apoptosis was

Table 3. Number of CD1a+ dendritic cells/mm basement membrane after 24 and 48 h exposure to sodium lauryl sulphate

Patient no.	Control	24 h	48 h
1	17·6	11·5	10·7
2	20·2	11·1	5·7
3	9·3	2·4	8·9
4	10·5	9·6	5·5
5	8·6	10·0	11·2
6	30·0	31·0	19·2
7	12·1	6·2	13·5
Median	12·1	10·0	10·7

Table 4. Median (range) values of cells with Ki67+ nuclei/mm basement membrane in biopsies taken at time 0 and 24 and 48 h after exposure to sodium lauryl sulphate or nonanoic acid

Irritant	Time 0 control	24 h	48 h
Sodium lauryl sulphate ($n=6$)	28·2 (12·8–45)	28·6 (8·7–46·7) NS	56·2 (30·2–212·1) $P<0.02^*$
Nonanoic acid ($n=6$)	30·5 (13·3–128·8)	13·4 (6·9–25·1) $P<0.02^*$	45·9 (38·6–82·7) NS

NS, not significant. *P-values calculated by two-tailed Mann–Whitney U-test against time 0 control values from the same patients.

detected at 24 and 48 h ($P<0.03$) and basal layer apoptosis at 6 h ($P<0.04$), 24 h ($P<0.01$) and 48 h ($P<0.01$). Prickle layer apoptosis induced by SLS showed a similar pattern with a significant increase at 24 and 48 h ($P<0.01$). In contrast, there was no significant increase in basal layer apoptosis until 24 h after SLS application ($P<0.025$), but this was never as strong a response as that induced by NA. Comparison of NA and SLS treatment revealed a significantly higher incidence of basal layer apoptosis after NA exposure at 6 h ($P<0.01$) and 24 h ($P<0.05$). The appearance of apoptotic cells after 24 h exposure to NA is shown in Figure 4 in an H&E-stained section, and by TdT-mediated dUTP-biotin nick end-labelling.

Nonanoic acid, but not sodium lauryl sulphate, induces apoptosis in CD1a+ Langerhans cells

To determine whether the drop in CD1a+ LC numbers seen after NA irritation was related to the increase in apoptosis, paraffin sections were stained with anti-CD1a mAb. Figure 4(c) shows that many of the apoptotic cells were CD1a+. Overall, CD1a+ epidermal cells accounted for 85–100% of the total number of apoptotic cells seen. No CD1a+ apoptotic cells were seen after exposure to SLS.

Discussion

Chronic ICD is an important industrial concern and is responsible for major loss of working hours per annum. Many substances are known to induce ICD, but the mechanisms by which they do so and why only some individuals develop persistent disease remain poorly understood. To model the condition previous studies have investigated irritant responses after patch testing of normal volunteers. We reasoned that patients susceptible to chronic ICD might have some predisposing feature in their skin and thus examined patients with a history of the disease.

We found that patients with chronic ICD had a higher median number of epidermal CD1a+ LCs in unaffected skin than normal healthy volunteers biopsied at the same site and that this was independent of age, gender or atopic status. This may indicate either that individuals with a high number of epidermal LCs have a
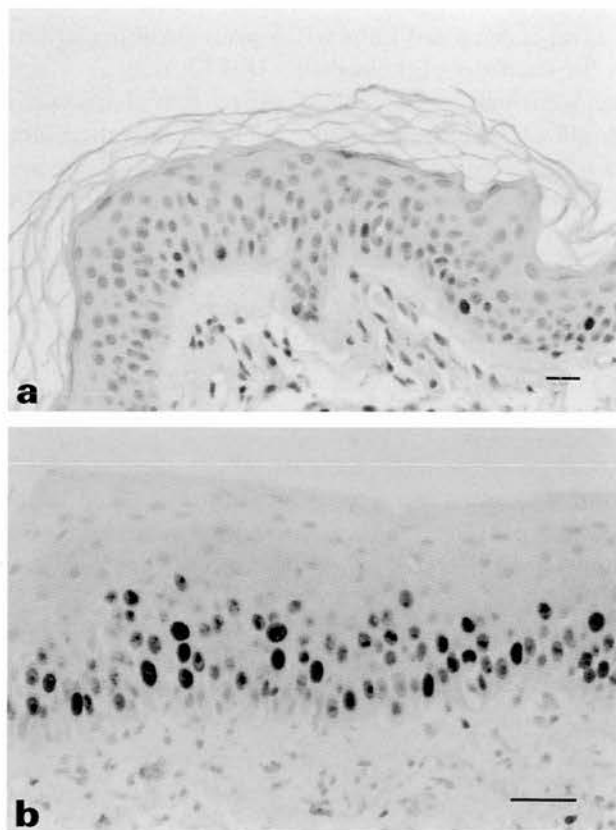
Figure 3. Immunohistochemistry of proliferating cells stained with antibody to Ki67 nuclear antigen in (a) control volar forearm skin from a patient with chronic irritant dermatitis and (b) volar forearm skin after 24 h exposure to sodium lauryl sulphate in the same patient. Bar = 50 μm.
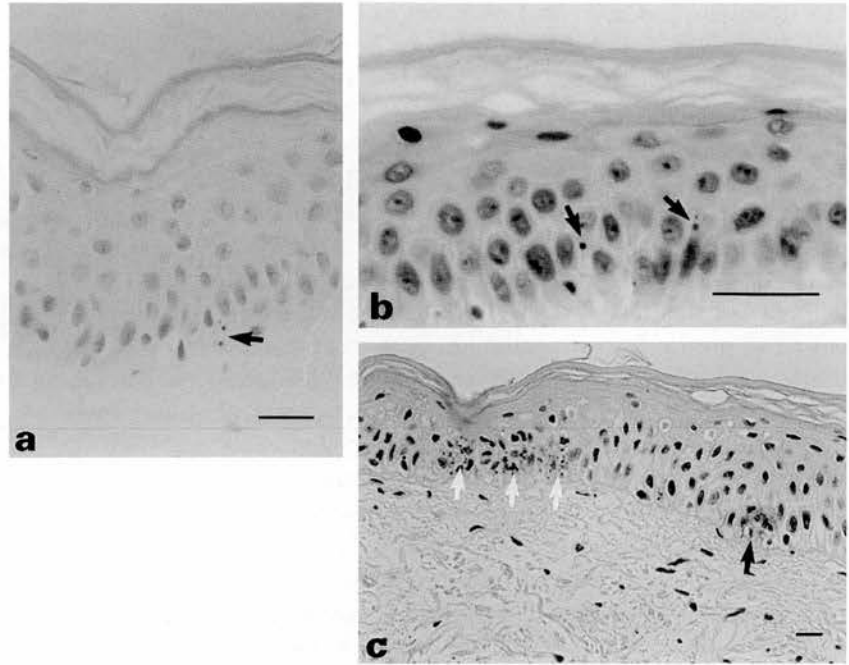
Figure 4. Apoptosis in sections from a biopsy of volar forearm skin from a patient with chronic irritant dermatitis after 24 h exposure to nonanoic acid. (a) Haematoxylin and eosin stained section, apoptotic cell arrowed; (b) TdT-mediated dUTP-biotin nick end-labelling stained section, apoptotic cells arrowed; (c) anti-CD1a stained paraffin section. CD1a+ apoptotic cells are indicated by white arrows; note the non-apoptotic, CD1a+ cell in the basal layer indicated by the black arrow. Bar = 50 μm.

predisposition to develop chronic ICD after irritant exposure or that the development of chronic ICD itself upregulates the number of epidermal LCs. We cannot distinguish between these possibilities at present. However, this finding may explain the phenomenon of hyperirritability of the skin associated with chronic ICD[23] as patients may be quicker to respond to any antigenic challenge as a consequence of higher local numbers of epidermal LCs.

We then examined the patch test response to two well-characterized skin irritants[6-9,24-28] in punch biopsies from patients with ICD. NA is a C9 free fatty acid found in lacquers, plastics, synthetic flavours and odours, turbojet lubricants and gasoline; SLS is a ubiquitous anionic detergent used in a variety of cosmetics, cleaning products and pharmaceuticals. Despite the fact that atopic individuals may be more likely to suffer from chronic ICD than non-atopics[29,30] we found that there was no difference between the response of atopic and non-atopic patients with chronic ICD to either irritant. This may indicate that atopy *per se* does not affect the outcome of chronic ICD but that there may be a greater chance of developing the condition in the first place if one is atopic.

The work presented here demonstrates that NA and SLS have very different effects on the KCs and LCs of patients with chronic ICD, which result in clinically similar irritant reactions at 48 h. SLS induces KC proliferation in the basal layers, whereas NA initially decreases the number of cycling cells, although these appear to return to control levels after 48 h. Both irritants induced a degree of KC apoptosis, which presumably balanced the proliferation. SLS had no effect on the numbers or distribution of LCs at any time point. In contrast, NA had a dramatic effect on LCs, inducing them to undergo apoptosis *in situ* with a peak 24 h after irritant application. A basal redistribution and a three- to fourfold reduction in numbers of epidermal LCs accompanied this. The dermal CD1a+ population appeared unaffected even after 48 h exposure of the skin to NA. Studies on suction blister roofs raised over NA patch tests on unaffected skin from patients confirmed the decrease in LC number and showed that NA decreased LC dendrite length but did not affect the number of dendrites per LC. Suction blisters from healthy volunteers treated with NA also showed a decrease in the number of LCs and in dendrite length but additionally a decrease in the number of dendrites per cell, indicating that patients not only have a higher baseline number of LCs but that their LCs react differently to an irritant stimulus.

Previous work has produced conflicting results regarding the response of LCs in irritant skin reactions in normal volunteers. Increased,[16] decreased[31] and unchanged[10,17] numbers have all been reported after

SLS irritation and decreased numbers of LCs together with decreased dendrite length[10] have been reported after NA application. As epidermal LCs represent a major signalling interface between cells of the immune system and the skin,[5,32,33] damage to this compartment may have profound effects on the subsequent inflammatory response generated, both in terms of its cellular composition and its duration.

The finding that NA can deplete the epidermis of LCs by inducing their apoptosis without having any apparent effect on the dermal population is of great interest from a number of points of view. First, this may indicate that NA is unlikely to induce activation of the immune system immediately after exposure. Secondly, this finding might be exploited clinically and experimentally in situations where it would be desirable to deplete the epidermis of LCs. Thirdly, NA must act specifically on LCs to activate their apoptotic pathway directly. This property can now be exploited to investigate skin responses to irritants and antigens in the absence of LCs.

Acknowledgments

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