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Modulation of leucocyte adhesion molecules, a T-cell chemotaxin (IL-8) and a regulatory cytokine (TNF-α) in allergic contact dermatitis (rhus dermatitis)

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Summary To understand the molecular events which are important in leucocyte trafficking in cutaneous inflammation, poison ivy/oak extract was applied topically to the skin, and the simultaneous assessment of a variety of clinical and immunopathological parameters performed. The clinical response of subjects was divided into three main groups: I, 2–24 h after application, before the onset of erythema; II, 48 h–1 week after application during maximal clinical changes; III, 2–3 weeks after application when the inflammation had subsided. Six different biopsies per subject were evaluated over the study period and the density of dermal cellular infiltrate, and the distribution of intercellular adhesion molecule-1, (ICAM-1), endothelial leucocyte adhesion molecule-1, (ELAM-1), vascular cell adhesion molecule-1, (VCAM-1), interleukin 8 (IL-8) and tumour necrosis factor-alpha (TNF-α), determined. Eight hours after exposure, before lymphocytes and monocytes had entered the dermal interstitium or epidermis, the keratinocytes expressed TNF-α and ICAM-1, whilst the endothelial cells expressed ELAM-1, VCAM-1 and ICAM-1. Group II biopsies revealed increasing keratinocyte expression of TNF-α and ICAM-1 with the appearance of IL-8, which correlated with the onset of epidermal T-cell trafficking. The endothelium was strongly positive for ELAM-1 and VCAM-1, but there was no influx of neutrophils. Group III biopsies showed a decrease in the expression of ICAM-1, VCAM-1 and ELAM-1 by both keratinocytes and endothelium with a reduction in epidermal/dermal inflammation, although the endothelial cell staining of VCAM-1 and ELAM-1 did not completely disappear. These results suggest that on exposure to poison ivy/oak, keratinocytes rapidly produce TNF-α which leads to an early autoinduction of ICAM-1, and later IL-8. There is also a paracrine-mediated induction and augmentation of underlying endothelial cell ELAM-1, VCAM-1 and ICAM-1.

The cellular events induced in human skin following epicutaneously applied allergens include early morphological changes in endothelial cells, within 4–8 h, which precede the migration of lymphocytes into the dermis.[1] However, after 24–48 h, lymphocytes are observed bound to endothelial cells of the superficial vascular plexus, and entering the dermis and epidermis.[1] These cellular alterations imply that resident cells, keratinocytes, endothelial cells and perivascular dermal dendrocytes, can become rapidly activated and produce molecular mediators for the recruitment of circulating mononuclear cells into the dermis/epidermis. We previously observed that urushiol (the active ingredient in poison ivy/oak) directly induces keratinocyte ICAM-1, prior to dermal T-cell infiltration, with dermal dendrocytes appearing as prominent early constituents of the inflammatory reaction.[2]

To define the molecular events responsible for the migration of inflammatory cells, we have applied a poison ivy/oak extract to normal skin and taken sequential biopsies.[2] A variety of leucocyte adhesion molecules (ICAM-1, ELAM-1, VCAM-1), a T-cell chemotactic factor (IL-8), and a cytokine (TNF-α), known to regulate adhesion molecule and IL-8 production in co-ordination,[3] were assessed immunohistochemically. At the same time the sequence of trafficking of inflammatory cells in the skin during the evolution of inflammation was determined.[3,4]

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Methods

Patients

Seven adult volunteers (four males, three females; age range 26–54 years) with a positive history for poison ivy dermatitis, who were otherwise in good health without any current skin disorder, and not taking any medication, were selected for study. All 4-mm punch biopsies were obtained after informed consent with the approval of the University of Michigan Human Subjects Committee. Portions of a tonsil were also obtained from a patient undergoing elective tonsillectomy.

Induction of inflammation and biopsy procedure

A poison ivy/oak mixture 1:50 (w:v) in alcohol (Hollister-Stier, Elkhart, IN, U.S.A.) was applied using the Altest System (Sodertalje, Sweden) to six different sites (5 mm diameter, 5 cm apart) on the flexor forearm of each volunteer. The patch-test reaction sites were divided into three different groups.[2] Group I biopsies were obtained 2–24 h after application of the poison ivy/oak mixture, before the onset of erythema or any other detectable clinical change. Group II biopsies were obtained 48 h–1 week after application when there were maximal clinical changes, and Group III biopsies were obtained 2–3 weeks after application when the inflammation had subsided, and there was only mild, residual erythema. Each volunteer had a baseline (pre-application) biopsy and then sequential biopsies of Group I, Group II and Group III sites were taken, with no more than six biopsies taken from each subject. A representative biopsy protocol would be as follows: baseline; 4, 8, and 24 h (Group I); 48 h or 1 week (Group II); 2 or 3 weeks (Group III).

Evaluation procedures

Clinical features. The patch-test sites were graded as follows: 0, no reaction; 1, erythema; 2, erythema and oedema; 3, erythema, oedema, and vesiculation.

Histopathology. The slides were assessed by two of the authors (CEMG, BJN). The dermal cellular infiltrate was composed primarily of lymphocytes and monocytes/macrophages (without neutrophils) and was graded semiquantitatively from 0–4 as follows: 0–1, no increase above the normal, pretreatment biopsy, or a few perivascular mononuclear cells; 2, a mild increase in perivascular mononuclear cells with rare scattered interstitial cells; 3, a moderate increase in perivascular cells accompanied by an easily recognizable dermal interstitial and epidermal influx of mononuclear cells and 4, an intense perivascular and interstitial mononuclear cell infiltrate with numerous intraepidermal cells.

Immunohistochemistry. The punch biopsies were orientated and mounted on gum tragacanth (Sigma Chemical Co., St Louis, MO, U.S.A.), snap frozen in isopentane chilled in liquid nitrogen, and then stored at −70°C until use. Cryostat sections (5 μm) were fixed in cold (4°C) acetone and incubated with primary monoclonal antibodies (MAbs) or antisera diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma Chemical Co.) for 45 min at room temperature. The sections were then washed in PBS, and an avidin–biotin immunoperoxidase technique was used (Vectastain, Vector Labs, Burlingame, CA, U.S.A.) as previously described,[2] with 3-amino 9-ethylcarbazole as the chromogen and counterstained with 1% haematoxylin.

The MAbs used were BB11 (anti-ELAM-1) and RR1/1 (anti-ICAM-1) obtained from Drs R. Lobb and C. Benjamin (Biogen Inc., Boston, MA, U.S.A.) and Dr T. Springer (Dana Farber Cancer Institute, Boston, MA, U.S.A.), respectively; MAb 4B9 (anti-VCAM-1) was produced as previously described.[5] Staining of human umbilical vein endothelial cells (obtained courtesy of Dr R. Marks, University of Michigan) before and after exposure to TNF-α (250 U/ml; Biogen Inc.) for 6 and 24 h using these anti-ELAM-1 and anti-VCAM-1 antibodies produced identical results to those previously published (data not shown).[6,7] The highly purified rabbit antisera against human TNF-α and IL-8 was provided by Dr S.Kunkel. The sensitivity and the specificity of the rabbit anti-TNF-α and IL-8 sera was confirmed by comparison to pre-immune rabbit sera, as well as neutralization pre-incubation studies with recombinant TNF-α and IL-8 as previously described.[8] Other MAbs used included Leu 6 (anti-CD1, Becton Dickinson, Burlingame, CA, U.S.A.), T11 (anti-CD2, a pan-T cell marker, Coulter Immunology, Hialeah, FL, U.S.A.), and EBM 11 (anti-monocyte/macrophage; Dako Corp, Santa Barbara, CA, U.S.A.). The specific dilution and antibody concentrations used were: BB11 (1:50; 20 μg/ml); 4B9 (1:50; 20 μg/ml); RR1/1 (1:10,000; 1 μg/ml); TNF-α and IL-8 (1:2000); Leu 6 (1:20; 2·5 μg/ml) and T 11 (1:50; 10 μg/ml); EBM 11 (1:100; 4 μg/ml).

Keratinocyte ICAM-1 expression was graded as: 0, no epidermal staining; 1, less than 25% of all epidermal keratinocytes/section were positive; 2, 25–50%; 3, 50–75%; 4, >75%. The same scoring system was used to measure the degree of epidermal keratinocyte TNF-α and IL-8 positivity.

Endothelial cell adhesion molecule (ELAM-1, ICAM-1 and VCAM-1) expression was assessed by the following scoring system: 0, negative or no vessel reactivity; 2, weak reactivity that was generally detectable on less than five vessels per section; 4, moderate reactivity easily detectable on 6–10 vessels per section; 6, strong reactivity with easily detectable and diffuse staining of more than 10 vessels per section. It should be noted that the positively scored vessels were primarily those of the superficial vascular plexus and not larger vessels (arteriolar) or lymphatic endothelium.

Results

Normal skin and tonsil

The baseline biopsies of normal skin revealed no reactivity for ELAM-1 and VCAM-1 on endothelial cells and only weak ICAM-1 staining of endothelial cells as previously described.[2,6,7] Perivascular dermal dendritic cells, and occasional perivascular lymphocytes were positive for VCAM-1. The perivascular dendritic cells which were VCAM-1 positive were in the same position as those labelled by the macrophage antibody EBM 11, but there was no labelling of epidermal $CD1^+$ Langerhans cells (data not shown). The follicular dendritic cells within the central portion of the lymphoid follicles, and the interdigitating dendritic cells in the interfollicular zones were strongly VCAM-1 positive, as found by Rice *et al.* using the INCAM-110 MAb that recognizes VCAM-1.[7] Fewer than 10% of the lymphocytes in the T-cell and B-cell zone of the tonsillar tissue were VCAM-1 positive, and only occasional venular endothelial cells were VCAM-1 positive. The venular endothelium of the tonsil was focally ELAM-1 positive, but there was no staining of arterioles, or any other cell types (data not shown). Perivascular cutaneous dermal dendrocytes and eccrine

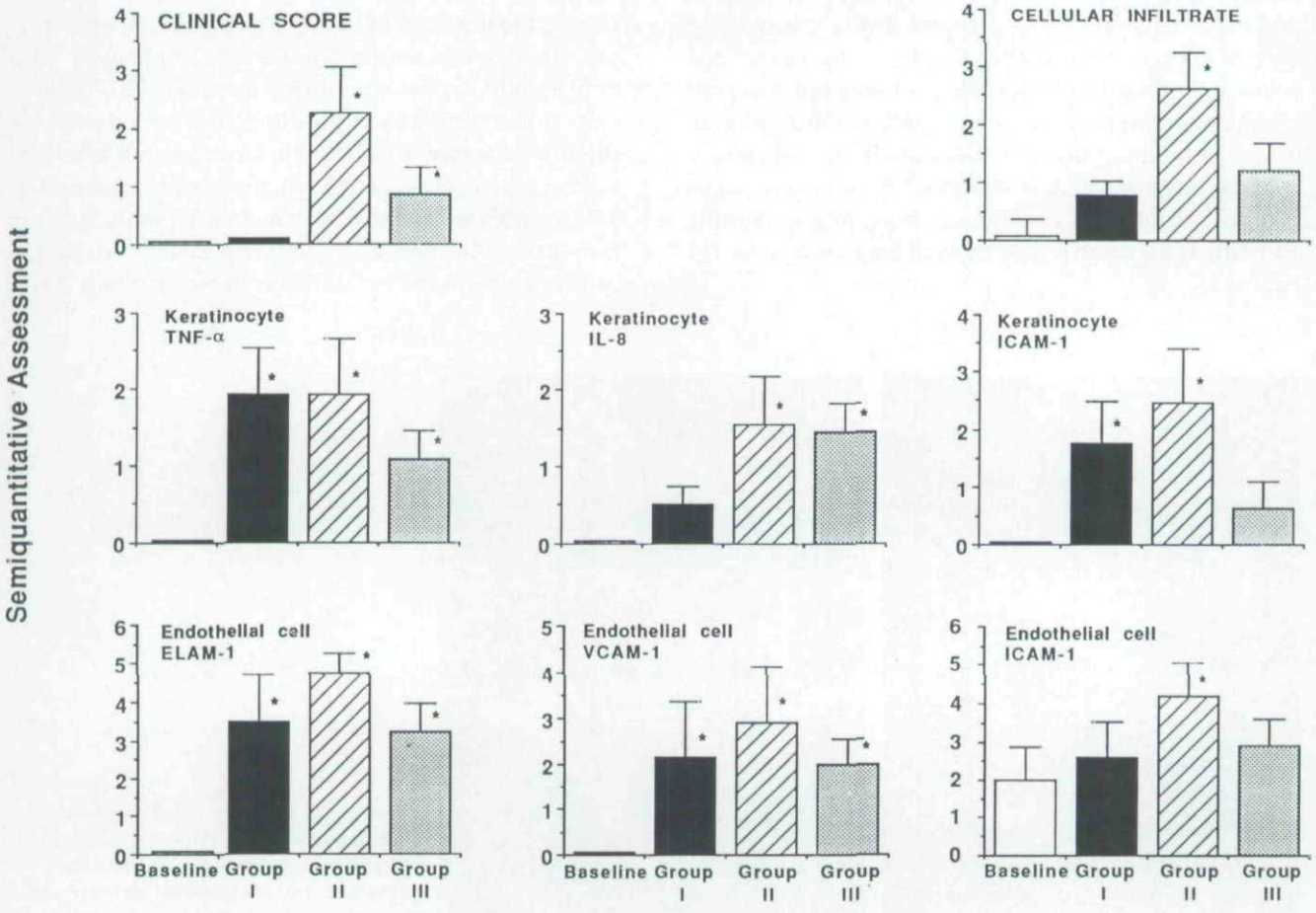

Figure 1. Summary of clinical, pathological, and immunohistochemical results including a semiquantitative assessment of staining of TNF-α, IL-8, ICAM-1, ELAM-1 and VCAM-1 (means+SD). * $P<0{\cdot}05$ when compared to baseline valves using the non-parametric sign test.

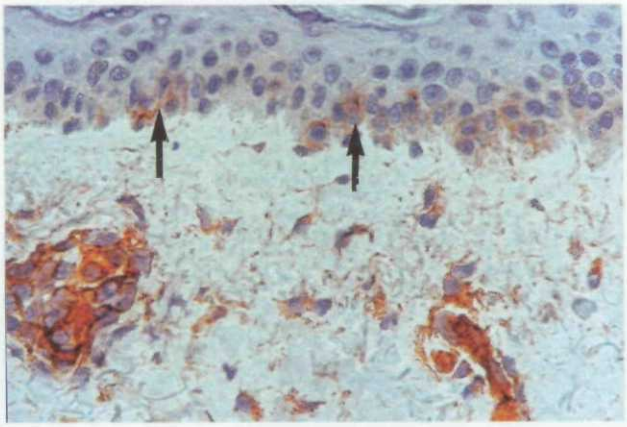

Figure 2. ICAM-1 expression within 24 h of application of poison ivy/oak extract by basal keratinocytes (straight arrows) and by dermal dendritic cells located in the upper dermis.

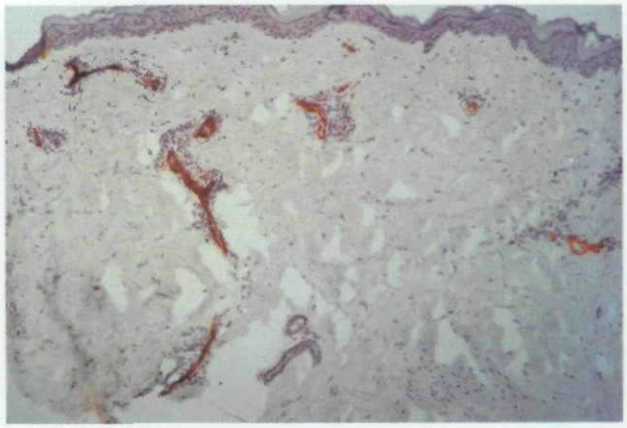

Figure 3. ELAM-1 expression on endothelial cells in both the superficial and deep vascular plexus 8 h after treatment.

sweat duct epithelium were positive for TNF-α. IL-8 positive cells were confined to eccrine duct epithelium and acrosyringium.[8] This IL-8 and TNF-α reactivity in normal skin, as well as the staining pattern described below was judged to be specific as it was not observed using pre-immune rabbit serum, and pre-absorption of the anti-serum with recombinant IL-8 and TNF-α resulted in an absence of staining.[8] A summary of the clinical, pathological and immunohistochemical results for pretreatment and post-treatment biopsies is shown in Figure 1.

Group I: pre-erythematous skin reactions to poison ivy/oak

During the first 24 h following application of poison ivy/oak, the skin was essentially clinically unchanged, with only a slight degree of erythema detected in one patient (Fig. 1) The cellular infiltrate during this period was only slightly increased over baseline levels, with a marginal increase in the perivascular lymphocyte population (Fig. 1) but with no detectable movement of lymphocytes into the surrounding dermis.[1,2] However, there was a significant increase in the keratinocyte expression of ICAM-1

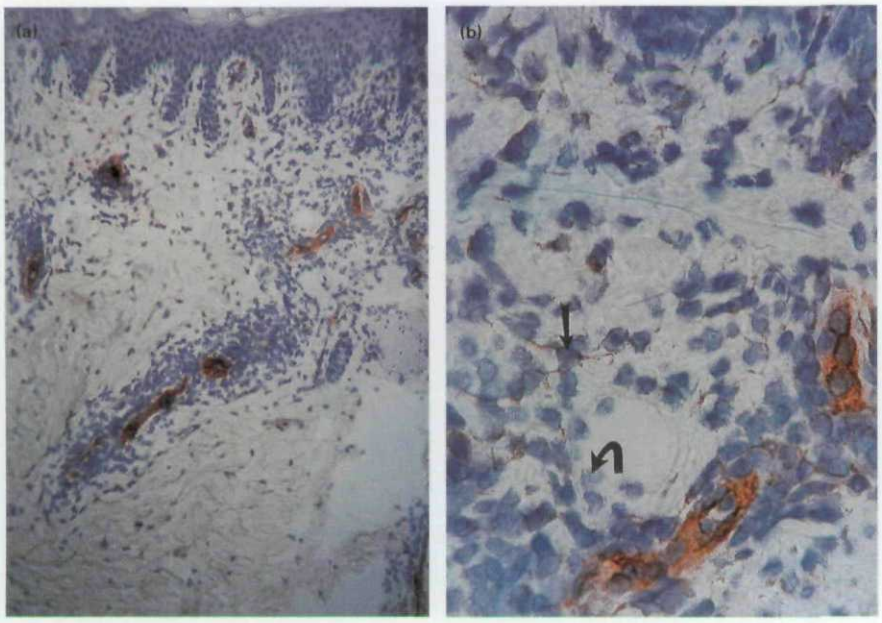

Figure 4. VCAM-1 expression on vascular endothelial cells and perivascular dendritic cells (straight arrow) 18 h after treatment (a) low-power magnification; (b) higher power magnification. Note the absence of VCAM-1 expression by lymphatic endothelium (curved arrow).

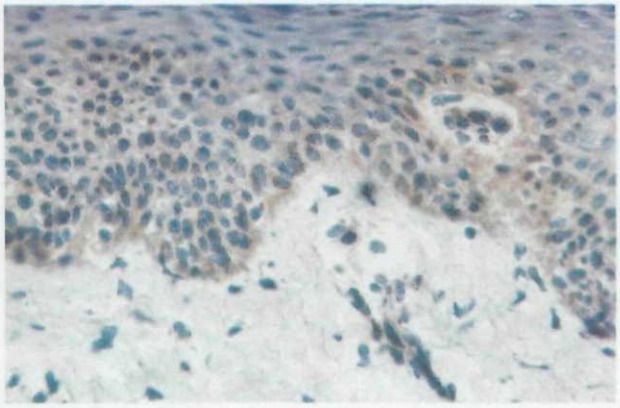

Figure 5. Expression of TNF-α by epidermal keratinocytes as early as 4 h after treatment, with enhancement along the basement membrane zone.

and more prominent ICAM-1 expression of dermal dendritic cells in the upper dermis, particularly in the area between the tips of the dermal vascular plexus and the base of the keratinocytes (Fig. 2). It appeared that these dermal dendritic cells were more strongly ICAM-1 positive than dermal dendritic cells in the normal and untreated skin.[9] The prominent expression of ICAM-1 by basal keratinocytes, dermal dendrocytes, and the endothelial cells seemed to produce an almost continuous network of cellular adhesion molecules in the upper portion of the treated skin, which probably comprised a dermal microvascular reactive unit.[10] The endothelial

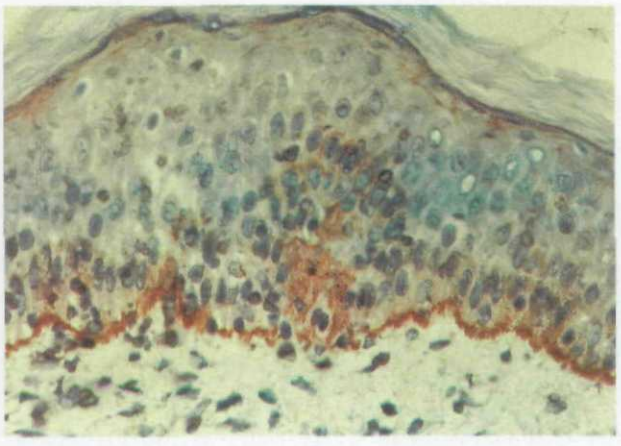

Figure 6. IL-8 expression by epidermal keratinocytes 48 h after treatment, accompanied by the movement of lymphocytes into the epidermis.

cells of the superficial vascular plexus became positive for both ELAM-1 (Fig. 3) and VCAM-1 (Fig. 4) during this period and this could be detected as early as 4 h after application of the poison ivy/oak extract.

Because there was a near-simultaneous induction of the expression of ICAM-1 by keratinocytes and of ELAM-1 and VCAM-1 by endothelial cells, suggesting the local production of an activating cytokine,[11] we sought to identify TNF-α in these sections. TNF-α is the only known cytokine which can induce all three adhesion molecules on these different cell types *in vitro*.[12–14] The epidermal keratinocytes showed an increased expression of TNF-α during this time period with little or no staining of any other cell type (Fig. 5). There was only a slight increase in keratinocyte IL-8 expression, which became more noticeable 48 h after treatment (Fig. 6).

Group II: inflammatory phase of poison ivy/oak reaction

Two days to 1 week following application of poison ivy/oak, the skin became erythematous with oedema and vesiculation. There was variation between patients in the degree and time of onset for each of these parameters,[2] but all patients eventually reached a clinical score of at least 2·0. The cellular infiltrate was greatly increased with prominent perivascular and interstitial dermal collections of lymphocytes, monocytes and macrophages, but no neutrophils. Mononuclear cells also began entering the epidermal compartment, with concomitant spongiosis and microvesiculation in four cases. The dermal and epidermal lymphocytes were negative for ELAM-1 and VCAM-1. Epidermal keratinocyte ICAM-1 expression gradually increased, as did endothelial staining for ICAM-1, ELAM-1 and VCAM-1. There was a slight decrease in keratinocyte TNF-α staining, but increased keratinocyte IL-8 staining as noted above (Fig. 6).

Group III: post-inflammatory phase

After 2–3 weeks, clinical examination of the skin revealed only a slight degree of residual erythema and scaling with no oedema or other changes. The cellular infiltrate had also substantially decreased compared to the Group II samples, and was approaching the baseline/group I values. Epidermal keratinocyte ICAM-1 was only focally present in three of our patients, and undetectable in the other four. However, the endothelial cell expression of ICAM-1, ELAM-1 and VCAM-1, while diminished, was still elevated over baseline values indicating persistent expression of both ELAM-1 (Fig. 7a) and

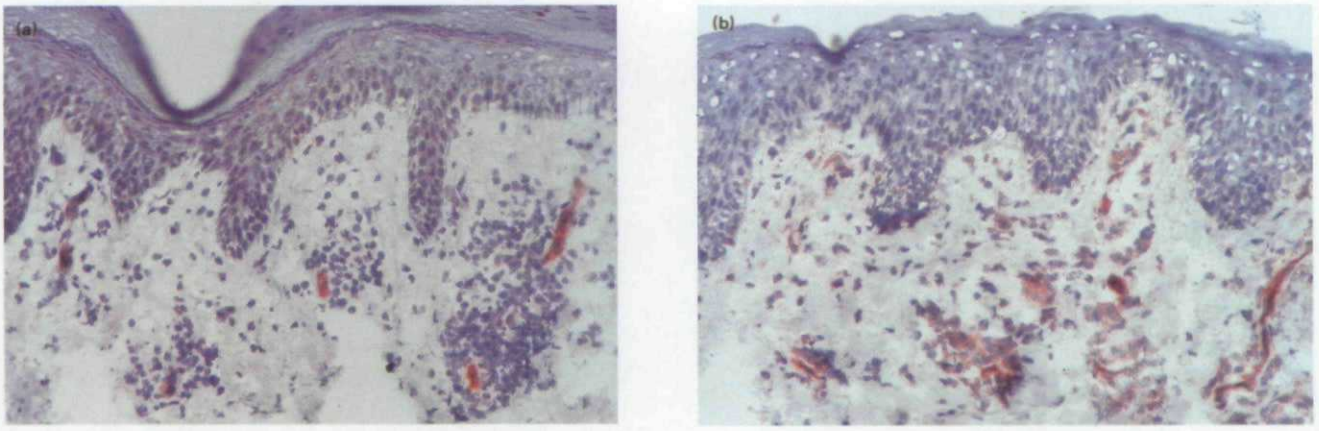

Figure 7. (a) ELAM-1 expression persisting on endothelial cells 3 weeks after treatment. (b) VCAM-1 expression persisting on endothelial cells 3 weeks after treatment.

VCAM-1 (Fig. 7b). The epidermal keratinocyte TNF-α and IL-8 staining pattern had largely reverted back to baseline levels.

Discussion

This experimental model was designed to detect *in vivo* the molecular mediators of adhesive interactions between leucocytes and cultured cytokine-activated endothelial cells with neutrophils, lymphocytes and determined where and when these molecules were expressed by indigenous and migrating cells that participate in adhesive interactions during the course of cutaneous inflammation. From tissue culture experiments it is known that at least three distinct mechanisms are involved for lymphocyte adhesion to endothelial cells.[15] The studies on the combination of human endothelial cells, with neutrophils, lymphocytes and monocytes have been reviewed.[16] In this present study we have assessed three distinct adhesion molecules, a T-cell chemotaxin (IL-8) and a modulatory cytokine (TNF-α), together with the associated accumulation of leucocytes in an *in-vivo* human system.[4,17]

Endothelial cell ELAM-1 was induced within 4–8 h following topical application of the poison ivy/oak mixture, and this induction preceded migration of lymphocytes into the dermis. ELAM-1 expression was not associated with an influx of neutrophils as predicted by *in vitro* experiments,[18] or as observed in baboon skin following intradermal injections of TNF-α and IFN-γ.[19] Moreover, *in vivo* ELAM-1 expression was more persistent (> 3 weeks) than previously observed for cultured endothelial cells[13] and endothelium in short-term skin organ cultures incubated with various cytokines,[20] in which ELAM-1 expression was essentially absent after only 24 h. The differences in results between cultured endothelial cells[13] and the current study may be partly explained by the experimental methods used. The study of cultured endothelial cells emphasized cell-surface immunoreactivity, whereas in frozen sections of intact skin specimens fixed with acetone, the resulting membrane permeabilization does not allow easy distinction between cytoplasmic and cell surface staining. It should be noted that different ELAM-1 antibodies have been used, and it is possible that the H4/18 antibody recognizes a different epitope on endothelial cells than the currently used BB11 antibody. However, it is also possible that the regulation of ELAM-1 expression and functional binding interactions between neutrophils and cultured endothelial cells is quite different from regulatory pathways *in vivo*. There may be pulses of endothelial cell activation *in vivo*, operating on a large reservoir of vascular-lining cells, which could give the appearance of persistence by individual cells, of ELAM-1 expression and a cell-to-cell contiguity.

Endothelial cell VCAM-1 was induced within 4–8 h following topical application of the poison ivy/oak mixture, and this induction preceded migration of lymphocytes into the dermis. This VCAM-1 expression occurred much earlier than that observed at 72 h in a delayed-type, cutaneous inflammatory reaction.[7] However, the rapid induction and prolonged expression of endothelial VCAM-1 is very similar to the results obtained by treating cultured endothelial cells with TNF-α.[14] The perivascular dermal dendritic cells were also VCAM-1 positive both in untreated control skin, and

in inflamed skin which agrees with a previous study using a different anti-VCAM-1 antibody (INCAM-110).[7]

The induction of keratinocyte ICAM-1 occurred very rapidly before the development of a dermal T-lymphocytic infiltrate and an ICAM-1 network was formed in the upper portion of the skin involving endothelial cells, dermal dendritic cells, and basal keratinocytes.[2] At 2–3 weeks post-treatment when the cutaneous inflammation was resolving and there was no further trafficking of mononuclear cells into the epidermis, the keratinocytes became ICAM-1 negative. This temporal and spatial co-localization of keratinocyte ICAM-1 with intraepidermal lymphocyte trafficking[17] is found in several skin diseases (psoriasis, lichen planus, alopecia areata) before and during treatment with cyclosporin A.[21–23] We previously observed the ability of urushiol to activate directly keratinocyte ICAM-1 expression,[2] and noted that urushiol strongly induced TNF-α expression on cultured keratinocytes.[4] We therefore examined whether TNF-α protein expression could be detected early in our *in vivo* model, as this is the only known cytokine capable of inducing endothelial cell ELAM-1, VCAM-1 and keratinocyte ICAM-1 expression.

Keratinocyte TNF-α expression was induced within 4–8 h following topical application of the poison ivy/oak mixture, and the keratinocyte was the principal cutaneous cell type which was TNF-α positive. The ability of the keratinocytes to rapidly respond to urushiol by producing TNF-α, which could autoinduce keratinocyte ICAM-1 and then activate the underlying dermal endothelial cells, supports our earlier contention that the keratinocyte regulates the movement of dermal cells.[4,17,24] While the notion that the 'activated' endothelial cell is pivotal in T-cell-mediated inflammatory reactions has gained support in recent years,[1,11,25] the current results suggest that the epithelium, and perhaps perivascular dendritic macrophages, are very important in the endothelial cell–T-cell interactions, and may even initiate them.[4]

Keratinocyte IL-8 expression occurred somewhat later than the appearance of TNF-α, but correlated well with the migration of T lymphocytes towards the epidermis. This *in vivo* correlation involving keratinocyte TNF-α expression with the co-ordinated induction of ICAM-1 and IL-8, resembles that found *in vitro* and supports the notion that keratinocyte-derived IL-8 is an important T-cell chemotaxin both *in vitro*, and *in vivo*.[26]

In conclusion, these results indicate that the molecular basis for the precise cellular movement of leucocytes during the genesis, evolution, and termination of cutaneous inflammation induced by poison ivy/oak involves the production of TNF-α by epidermal keratinocytes, with the subsequent induction and diminution of multiple adhesion molecules on endothelial cells (ELAM-1, VCAM-1) and keratinocytes (ICAM-1), as well as the production of chemotactic factors such as IL-8. While there are almost certainly other components of this molecular and cellular network beyond those studied here, this experimental system can further our understanding of normal immunohomeostasis and the pathophysiology of acute and chronic cutaneous inflammation.

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