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The ICAM-3/LFA-1 interaction is critical for epidermal Langerhans cell alloantigen presentation to CD4$^+$ T cells

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Summary Intercellular adhesion molecule (ICAM)-3 is a recently described member of the immunoglobulin superfamily and, as such, is closely related to ICAM-1 and ICAM-2. All three ICAMS are cognate for the counter-receptor lymphocyte function associated antigen-1 (LFA-1, CD11a/CD18). Unlike ICAM-1 and ICAM-2, ICAM-3 is constitutively expressed at high levels on resting leucocytes. We investigated the expression and function of ICAM-3 in normal skin ($n=5$), as well as its expression in psoriasis ($n=4$), atopic eczema ($n=4$), allergic (rhus) contact dermatitis ($n=3$), and cutaneous T-cell lymphoma (CTCL, $n=2$).

Five-micrometre cryostat sections of skin were stained using monoclonal antibodies to ICAM-3 and a well characterized immunoperoxidase technique. In normal skin, ICAM-3 was expressed by all cutaneous leucocytes but most striking was the strong expression of ICAM-3 by Langerhans cells within both epidermis and dermis. This observation was confirmed by double-labelling with CD1a and negative staining with an IgG1 isotype control. In psoriasis, atopic eczema, allergic contact dermatitis, and CTCL, ICAM-3 was co-expressed on all CD1a$^+$ cells, although, in psoriasis, the intensity of ICAM-3 expression was reduced. Functional blocking experiments were performed to determine whether the observed ICAM-3 expression on Langerhans cells was functionally important in antigen presentation. CD4$^+$ T cells were prepared from peripheral blood and 10^5 CD4$^+$ T cells combined with 10^5 epidermal cells harvested from keratome biopsies of normal skin of an individual allogeneic to the T-cell donor. Addition of 50 μg anti-ICAM-3 to the co-culture resulted in a consistent (50%) reduction in degree of alloantigen presentation by Langerhans cells to T cells. Inhibition was 77% of that produced by the addition of anti-LFA-1.

These data indicate that ICAM-3 is constitutively expressed by Langerhans cells and is a major ligand for LFA-1 on CD4$^+$ T cells during their response to Langerhans cells. Because fresh Langerhans cells constitutively express little ICAM-1, whereas ICAM-3 is constitutively expressed at high levels, it would appear that ICAM-3 is the dominant functional ICAM on *in situ* Langerhans cells in the normal epidermis.

Three immunoglobulin superfamily members, intercellular adhesion molecules (ICAM)-1, ICAM-2 and ICAM-3, in conjunction with their ligand lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) are important facilitators of adhesion events in inflammation.[1–4] ICAM-1, ICAM-2 and ICAM-3 are membrane glycoproteins whose structure and immunobiology are well characterized.[2–7] ICAM-1 has five immunoglobulin-like domains[8] whereas ICAM-2 contains only two such domains; however, these have 36% sequence homology to the first two domains of ICAM-1.[3] The most recently identified member of the ICAM family is a 124,000 molecular weight glycosylated glycoprotein, ICAM-3,[4,9–11] that contains five immunoglobulin-like domains — the first two of which share 37% sequence homology with those of ICAM-2[6,9,12] Although ICAM-3 is structurally similar to the other two ICAMs it differs from them in that it is constitutively expressed on resting lymphocytes and monocytes thereby implying potential importance in lymphocyte activation.[4,13]

The distribution of ICAM-1 in normal and diseased skin is well characterized.[14–16] ICAM-1 is constitutively expressed by dermal endothelial cells and is inducible on keratinocytes by gamma interferon,[14] tumour necrosis

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factor-α[14] and by a variety of non-cytokines including phorbol ester,[17] urushiol,[17] and all-*trans* retinoic acid[18,19]. ICAM-1/LFA-1 interactions are instrumental in cutaneous leucocyte trafficking, keratinocyte/leucocyte adhesion, and antigen presentation by Langerhans cells. In contrast, in normal skin, ICAM-2 is probably expressed solely by endothelium and appears not to be inducible on other cellular components of the epidermis or dermis. The distribution and function of ICAM-3 in normal and inflamed skin is poorly elucidated at present.[20] Using anti-ICAM-3 monoclonal antibodies we have investigated: (i) the expression of ICAM-3 in normal skin and inflammatory dermatoses, and (ii) the functional role of ICAM-3 as a costimulatory molecule in Langerhans cell/T-cell interactions.

Methods

Patients

Four mm skin biopsies were procured under 1% plain lignocaine anaesthesia from five normal volunteers and from the untreated involved skin of chronic plaque psoriasis ($n = 4$), atopic eczema ($n = 4$), allergic (rhus) contact dermatitis: a 48-h patch test to poison ivy/poison oak mix 1:50 (w/v) in alcohol (Hollister Stier, Elkhart, IN, U.S.A.; $n = 3$) and plaque stage cutaneous T-cell lymphoma (CTCL, $n = 2$). No patients were receiving systemic therapy for their disease at time of biopsy. All biopsies were orientated in optimal cutting temperature embedding medium (OCT, Miles Laboratories, Elkhart, IN, U.S.A.), snap frozen in liquid nitrogen, and stored at -70°C until use.

For proliferation assay studies, keratome biopsies were taken from normal buttock skin under local 1% plain lignocaine anaesthesia. Biopsies were taken using a Castro Viejo keratome set at 0·2 mm so that the majority of the biopsy was composed of epidermis.

The procurement of biopsies was performed under approval of the University of Michigan Medical Center Institutional Review Board.

Immunohistology

Five-micrometre cryostat sections were stained using monoclonal antibodies to ICAM-3 (ICR1 and ICR8; ICOS Corporation, Bothell, WA, U.S.A.) and a well-characterized immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.); 3-amino-9-ethyl carbazole was used as the chromogen and the sections were counterstained with 1% haematoxylin. Substitution of the primary antibody with an IgG1 isotype control, and omission of the primary antibody, served as negative controls.

To ascertain whether ICAM-3 expression was localized to Langerhans cells, a double-labelling technique was used. Antibody ICR1 was used to identify ICAM-3 and Leu 6 (Becton Dickinson, Burlingame, California, U.S.A.) labelled $CD1a^+$ Langerhans cells. The first antibody was visualized with the avidin–biotin immunoperoxidase technique using 3,3′-diaminobenzidine as the chromogen and the second antibody was visualized using an alkaline phosphatase/anti-alkaline phosphatase technique, with fast blue as chromogen, as previously described.[21]

Epidermal proliferation assay

Epidermal cell preparation. Keratome biopsies were trypsinized overnight at 4°C in 10 ml of Dulbecco's phosphate-buffered saline (PBS) containing dispase (Collaborative Research, Becton Dickinson Labware, Bedford, MA, U.S.A.). The epidermis was then separated from the dermis and transferred to 0·25% trypsin for 20 min at 37°C. Fetal bovine serum (FBS) containing 0·01% DNAse was added to stop trypsinization and the epidermis was dispersed into a single cell suspension before filtering through a 112 μm nylon mesh. Epidermal cells were adjusted to a concentration of 1×10^6 cells/ml of RPMI (Whittaker Bioproducts Inc, Walkersville, MA, U.S.A.) and 10% FBS.

Mononuclear cell preparation. Heparinized peripheral blood was obtained from normal volunteers and mononuclear cells (MNC) isolated. Blood was diluted 1:1 with PBS and layered on to Ficoll-Hypaque (Sigma Chemical Co, St Louis, MO, U.S.A.) and centrifuged at 1500 r.p.m. for 35 min at 20°C without braking. The MNC were harvested, washed three times in PBS plus 10% FBS, and adhered to plastic flasks for 1 h at 37°C. All non-adherent cells were collected as peripheral blood lymphocytes (PBL).

Preparation of $CD4^+$ T cells. Purified $CD4^+$ T cells were obtained by treating PBL with the following monoclonal antibody cocktail: anti-CD8 (OKT8, ATCC, Rockville, MD, U.S.A.), anti-HLA-DR (HB55, ATCC), and anti-CD11b (OKM1, ATCC). Antibodies were diluted 1:50 using RPMI and 1% FBS. One ml of the antibody cocktail was added to 20×10^6 PBL in a 15 ml round-bottom, polypropylene tube (Falcon, Lincoln Park, NJ, U.S.A.) and incubated at 4°C for 45 min with agitation

every 15 min, and then washed three times with PBS and 1% FBS.

Goat antimouse IgG-conjugated magnetic beads (Dynabeads, M-450, Dynal, Great Neck, NY, U.S.A.) were washed twice with PBS and 10% FBS, resuspended in RPMI 1640 plus 1% FBS, and added at a concentration of 3 (beads)/1 PBL. The PBL–bead mixture was incubated for 45 min at 4°C with regular (5 min) agitation. The solution was then diluted to 10 ml with PBS plus 10% FBS, and incubated for a further 15 min at 4°C, again with 5 min agitation. B cells, monocytes, natural killer cells, and $CD8^+$ T cells were removed from the PBL suspension by applying the tube containing them to a magnet for 3–5 min, drawing the bead-attached cells to the tube wall, and thus allowing the unbound cells to be removed in the supernatant. The removed supernatant was centrifuged at 1400 r.p.m. at 4°C for 8 min. The viability of $CD4^+$-enriched cells present in the supernatant was assessed using trypan blue.

T-cell proliferation assay. To each well of a 96-well, round-bottom microfilter plate (Costar, Cambridge, MA, U.S.A.) was added 50 μl of purified $CD4^+$ T cells (10^5 cells) and10^5 epidermal cells. Various experiments were performed in triplicate with addition of either 50 μg anti-ICAM-3 (ICR8), 50 μg IgG1 isotype control, or 50 μg anti LFA-1 (CD11a/18). The cultures were incubated at 37°C for 6 days before pulsing with 0·037 MBq per well of triated thymidine, harvested 18 h later, and counted in a liquid scintillation counter. The mean ± standard error of the mean (SEM) count per minute (c.p.m.) was calculated for each triplicate of wells.

Results

Immunohistology

Both antibodies to ICAM-3 (ICR1 and ICR8) showed equivalent staining in normal and diseased skin. In normal skin, the predominant, constitutive staining was of epidermal Langerhans cells (Fig. 1) as confirmed by double-labelling with CD1a (Fig. 2). Staining with IgG1 isotype control was negative. Other cell types that stained were predominantly of leucocyte lineage but neither keratinocytes, fibroblasts nor endothelial cells expressed ICAM-3.

Psoriasis, atopic eczema, allergic contact dermatitis, and CTCL also demonstrated positive leucocyte staining for ICAM-3 (Figs 3–6), again with expression by Langerhans cells, confirmed by double-labelling (Fig. 4) in both epidermis and dermis. ICAM-3 expression by psoriatic epidermal Langerhans cells was fainter than that observed in either normal skin or the other dermatoses studied (Fig. 3). In none of the four inflammatory dermatoses studied was ICAM-3 expressed on keratinocytes, fibroblasts or endothelial cells (Figs 3–6).

Proliferation assay

Combining allogeneic epidermal cells to antigen-presenting cell-depleted $CD4^+$ T cells resulted in T-cell proliferation that was not significantly inhibited by the addition of IgG1, isotype control immunoglobulin (Fig. 7). Addition of ICR8 (IgG1 antibody to ICAM-3) to the epidermal cell + $CD4^+$ T-cell culture resulted in a 50 ± 14% reduction in alloantigen presentation, as

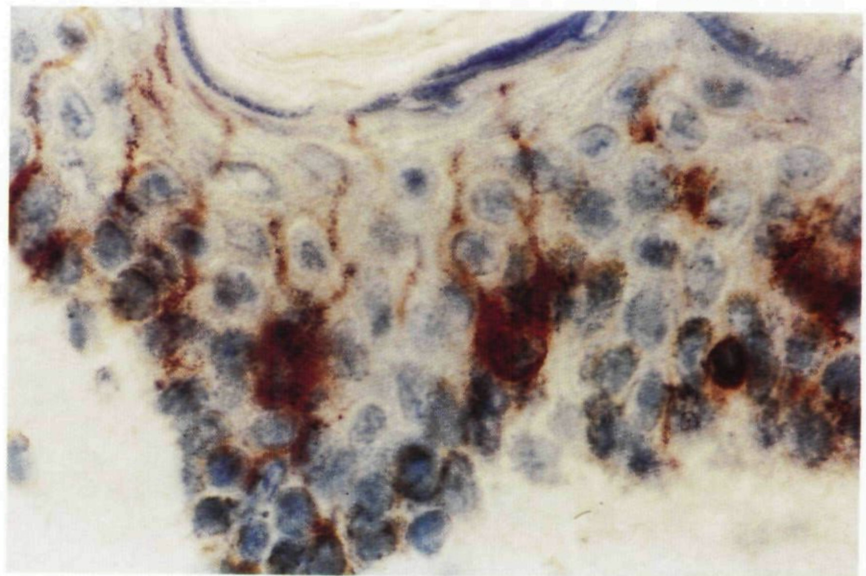

Figure 1. Immunoperoxidase staining of normal skin with anti-ICAM-3. Dendritic epidermal Langerhans cells are strongly positive for ICAM-3 (×250).

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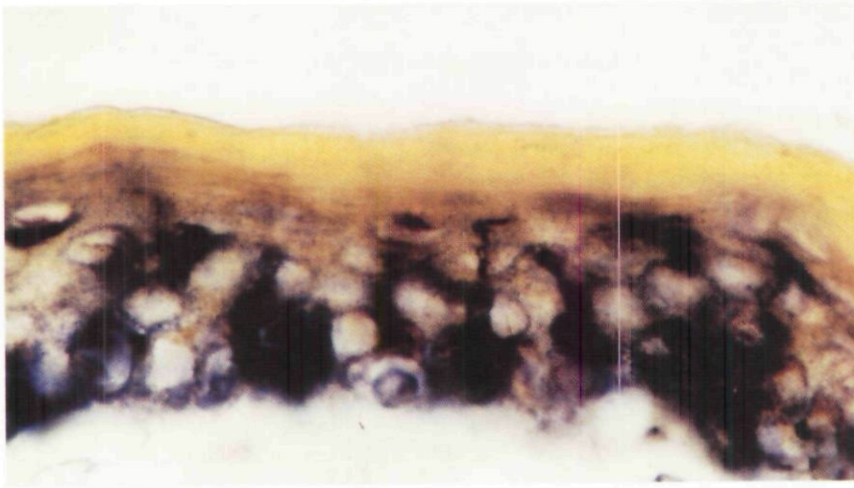

Figure 2. Double-labelling of normal epidermis with anti-ICAM-3 (brown) and anti CD1a (blue). Note concurrence of ICAM-3 and CD1a (black staining) on epidermal Langerhans cells indicating constitutive expression of ICAM-3 (×100).

measured by T-cell proliferation. Inhibition of T-cell proliferation by addition of anti-LFA-1 was $65 \pm 5\%$. Thus, anti-ICAM-3 inhibited T-cell proliferation to about 77% of that observed with anti-LFA-1, $n = 3$ (Fig. 7).

Discussion

In this study we have demonstrated that ICAM-3 is constitutively and strongly expressed by Langerhans cells *in situ* in the epidermis of normal skin. ICAM-3 expression is not limited to normal skin as it is also observed on dermal and epidermal Langerhans cells and cutaneous leucocytes in psoriasis, atopic eczema, allergic contact dermatitis and CTCL. The reduced ICAM-3 expression on epidermal Langerhans cells in psoriatic skin is not readily explicable, although it is in agreement with observations that similar reductions in CD1a and PKCβ occur in psoriasis.[22] Furthermore, the presence of ICAM-3 on epidermal Langerhans cells appears to be integral for alloantigen presentation to $CD4^+$ T cells and their subsequent proliferative response.

Previous investigations of ICAM-1 and ICAM-2 expression in normal skin,[14–16] have demonstrated constitutive expression limited to endothelium. Langerhans cells do not express ICAM-2 and their constitutive epidermal and dermal expression of ICAM-1 is minimal,[14] demonstrable only by highly sensitive immunoelectron microscopy techniques.[23] Prior studies have demonstrated strong expression of ICAM-3 on epidermal Langerhans cells in normal skin,[20] although ICAM-3 distribution in inflammatory

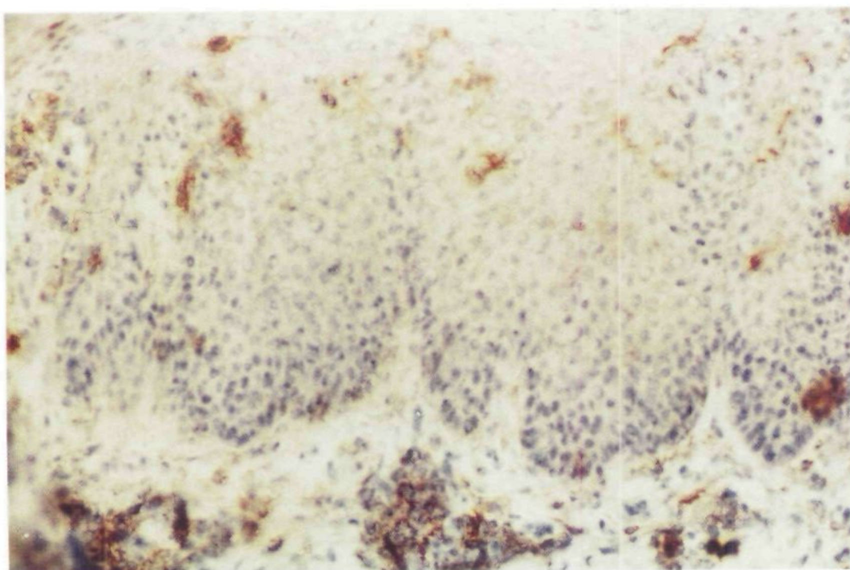

Figure 3. Immunoperoxidase staining of involved psoriatic skin with anti-ICAM-3. Epidermal Langerhans cells and dermal leucocytes are positive for ICAM-3 (×100).

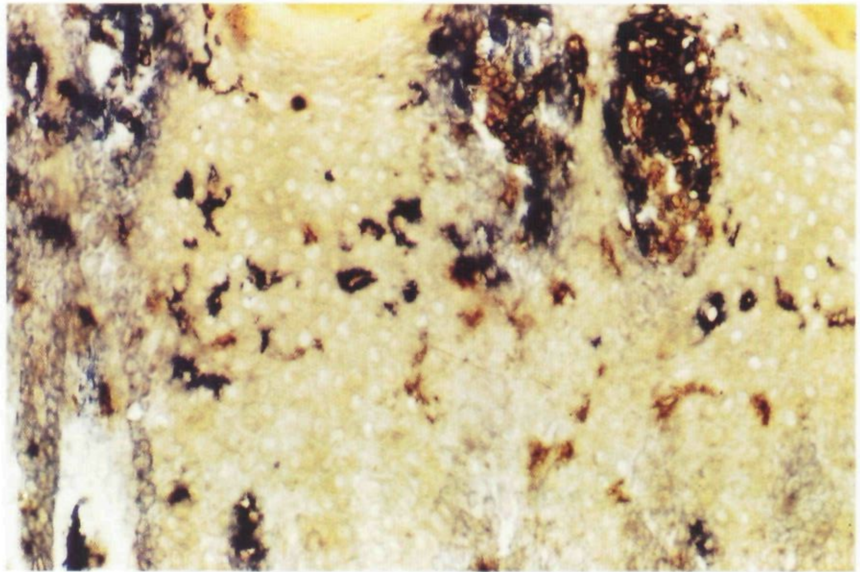

Figure 4. Double-labelling of involved psoriatic skin with anti-ICAM-3 (brown) and anti-CD1a (blue). Note concurrence of ICAM-3 and CD1a (black staining) on Langerhans cells. ICAM-3 positive leucocytes within the epidermis stain brown (×100).

dermatoses has not been examined. ICAM-3 is not, however, unique to cutaneous cells as it has been described on cells of leucocyte lineage in a variety of organs including lymph node, spleen, liver, kidney, lung and rheumatoid synovium.[24] Furthermore, ICAM-3 has rarely been observed on endothelium, although there is one report of its presence on endothelial cells in lymphomas.[25]

The observation that ICAM-3 is strongly and constitutively expressed by Langerhans cells *in situ* in normal epidermis indicates that this adhesion molecule is important in initial antigen presentation to T cells. Both ICAM-1 and ICAM-2 are costimulatory when ligated to LFA-1,[26,27] and ICAM-1 is costimulatory for activated T cells.[13,28] By contrast ICAM-3 is costimulatory for both resting as well as activated T cells[13] and this may be a consequence of its high constitutive expression. *In vivo*, Langerhans cell expression of ICAM-1 is very low, although *in vitro* culture conditions significantly enhance ICAM-1 expression and such enhancement is concurrent with increased potency of antigen presentation.[29,30] Blocking antibody to ICAM-3 significantly abrogates alloantigen presentation and subsequent T-cell proliferation and this observation underscores the importance of ICAM-3 to Langerhans cell function. ICAM-3 should be added

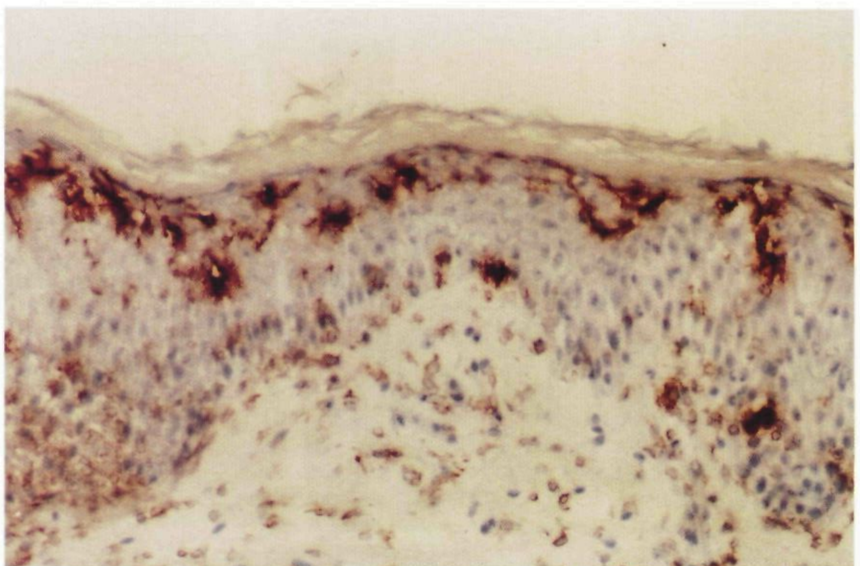

Figure 5. Immunoperoxidase staining of rhus allergic contact dermatitis with anti-ICAM-3. Epidermal Langerhans cells are strongly positive for ICAM-3 whereas infiltrating mononuclear leucocytes are less positive for ICAM-3 (×50).

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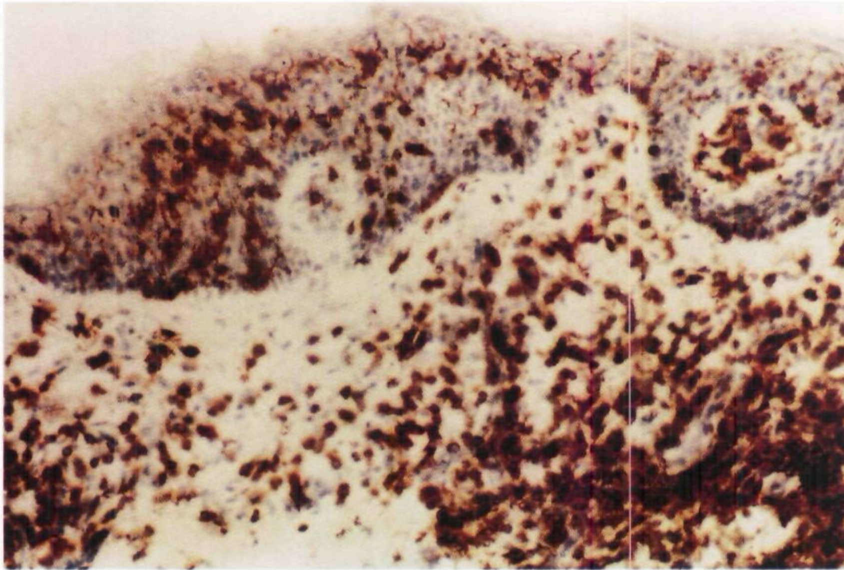

Figure 6. Immunoperoxidase staining of cutaneous T-cell lymphoma with anti-ICAM-3. Epidermal Langerhans cells and mononuclear leucocytes of the dermal and epidermal infiltrate are strongly positive for ICAM-3 (×50).

to the list of other accessory/costimulatory molecules such as B7, CTLA-4 ligand A and ICAM-1, present on cutaneous antigen-presenting cells. Most probably high, constitutive expression, of ICAM-3 is of particular value to antigen-presenting cells whose expression of ICAM-1 is low or absent, i.e. Langerhans cells. Under such circumstances of low ICAM-1 expression, a strong costimulatory signal could be generated via ICAM-3 before upregulation and subsequent activity of ICAM-1.[13] Thus, ICAM-3 may be of greater importance to T-cell activation occurring *in situ* during the effector phase of intracutaneous immune reactions than it is during primary sensitization in the draining lymph node.

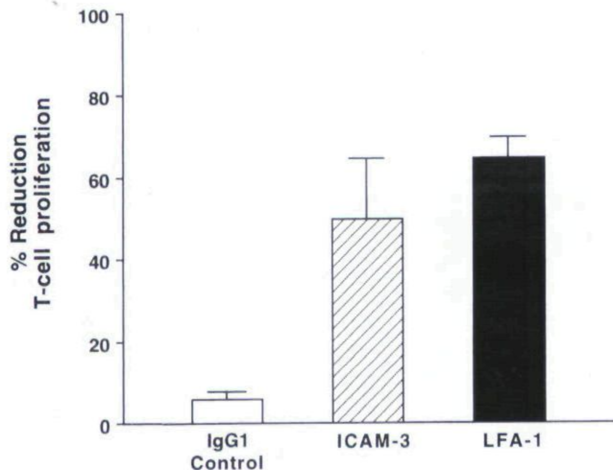

Figure 7. Percentage inhibition of $CD4^+$ T-cell proliferation by ICAM-3 and LFA-1 antibodies. Epidermal cell suspension stimulation of $CD4^+$ T-cell proliferation is inhibited by coincubation with antibody to ICAM-3 and to a slightly greater extent by antibody to LFA-1. By comparison, an IgG1 isotype control has little effect on T-cell proliferation. 10^5 T cells were incubated with 10^5 epidermal cells. Bars are means ± SEM; $n = 3$.

In conclusion, ICAM-3 is probably the dominant functional ICAM on *in situ* Langerhans cells and is likely to be an integral molecule in the initiation of antigen-driven cutaneous inflammation.

Addendum in proof

While this article was in proof two reports[31,32] were published which verify the importance of ICAM-3 as a costimulatory molecule on Langerhans cells.

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