British Journal of Dermatology (1990) **122**, 333–342.

Differential modulation of keratinocyte intercellular adhesion molecule-1 expression by gamma interferon and phorbol ester: evidence for involvement of protein kinase C signal transduction

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Accepted for publication 20 September 1989

SUMMARY

There is growing evidence that keratinocyte (KC) intercellular adhesion molecule-1 (ICAM-1) expression is involved in the epidermal trafficking of T lymphocytes. To further characterize the molecular basis of KC ICAM-1 expression, the detailed kinetics of induction by gamma interferon (IFN-γ), as well as the phorbol ester, 12-O tetradecanoylphorbol-13-acetate (TPA), were studied. This study reports that KCs express both the class II major histocompatibility antigen (HLA-DR) and ICAM-1 in response to IFN-γ, although the response is distinctive for each molecule. Also, TPA induces ICAM-1, but not HLA-DR expression, whilst the protein kinase inhibitor, H7, blocks the TPA, but not the IFN-γ-mediated response. The results provide a molecular basis whereby non-cytokine-mediated stimuli (e.g. TPA) alter KC signal transduction events involving protein kinase-C (PK-C) and thereby generate such immunologically relevant events as ICAM-1 expression. Thus, KCs may be targets for both T-cell derived cytokines (e.g. IFN-γ), and non-cytokine TPA-like molecules which stimulate PK-C. Induction of ICAM-1 by either mechanism would be capable of instigating intraepidermal T-cell trafficking.

There is evidence implicating keratinocyte (KC) intercellular adhesion molecule-1 (ICAM-1) expression in the epidermal trafficking of T lymphocytes.[1–6] Recently, we have reported on the ability of gamma interferon (IFN-γ) to induce different patterns of KC ICAM-1 and HLA-DR expression.[2] T cells which express lymphocyte-function-associated antigen-1 (LFA-1) bind to

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Presented in part at the Central Region Society for Investigative Dermatology 11 November 1988; at the 47th Annual Meeting of the American Academy of Dermatology, 5 December 1988; and the Tricontinental Meeting of the Society for Investigative Dermatology, 29 April 1989.

ICAM-1-expressing KCs *in vitro*,[7-9] and the *in vivo* appearance and localization of intraepidermal T cells associated with KC ICAM-1 expression has been correlated to the induction, maintenance, and resolution phases of a wide variety of skin diseases.[3-6,10,11] Thus, the regulation of KC ICAM-1 expression is likely to be important in our understanding of the mechanism of cutaneous inflammatory reactions. In psoriasis, for example, KC ICAM-1 expression[3-6] is accompanied by increased levels of diacylglycerol (DAG) with activation of its target molecule, PK-C.[12-13] Furthermore, topical application to mice of TPA and DAG causes marked inflammation and increased epidermal proliferation which simulates the hyperplastic response as seen in psoriasis.[14] To determine whether altered PK-C signal transduction could directly influence ICAM-1 expression, cultured KCs were exposed to both TPA, a potent PK-C agonist, and a known ICAM-1 inducer (IFN-γ).[2,5,6,9] The subsequent expression of ICAM-1 was measured with the use of immunoperoxidase staining techniques and fluorescence-activated cell sorting (FACS).

METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Serum free, KC growth medium containing low calcium (KGM) was obtained from Clonetics Co., (San Diego, CA, U.S.A.). Recombinant IFN-γ (specific activity = $1{\cdot}7 \times 10^7$U/mg) was provided by Dr M. Shepard, Genentech, Inc., (South San Francisco, CA, U.S.A.). Calcium ionophore (A23187), digitonin, TPA, EDTA, EGTA, Tris, gum tragacanth, and mercaptoethanol (β-ME) were purchased from Sigma Chemical Co., (St Louis, MO, U.S.A.). (^{32}P) deoxycytidine triphosphate was from ICN Radiochemicals (Irvine, CA, U.S.A.) DEAE cellulose (DE52) was obtained from Whatman (Hilsboro, OR, U.S.A.), protein kinase-C inhibitor H7[15] was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). All other chemicals were of at least reagent grade.

KC culture

Samples of normal, human skin obtained from cosmetic surgery were processed to single cell suspensions and viable KCs were then seeded on to, and grown in either 35-mm diameter, plastic Petri dishes (Lux. Flow Laboratories Inc., Naperville, IL, U.S.A.) or Lab-Tek chamber slides (Miles LAB, Naperville, IL, U.S.A.) in KGM as previously described.[2] Cells were maintained in a humidified incubator in 5% CO_2/95% air at 37°C and used after passage 3.

TPA induction of KC expression of ICAM-1 and HLA-DR

TPA, A23187 and H-7 were added to subconfluent KCs growing in KGM in the indicated combinations, concentrations and time courses. KCs grown in Petri dishes were then trypsinized, stained, and examined by FACS analysis. KCs grown in Lab-Tek chamber slides were stained *in situ* using an immunoperoxidase technique.

Staining of KCs for ICAM-1 and HLA-DR

KCs grown in 35-mm dishes were trypsinized using 0·03% trypsin/0·01% EDTA and an aliquot of approximately 10^6 KCs incubated for 40 min on ice with 10 μg/ml of the primary monoclonal antibodies, anti-ICAM-1 (RR 1/1; Dr T. Springer, Boston, MA, U.S.A.) and anti-HLA-DR (TS 1·16; Dr C. Clayberger, Stanford, CA, U.S.A.). Anti-Leu 2a (CD8, Becton-Dickinson, CA, U.S.A.) was used as an isotype control antibody. The cells were washed and

then stained with 1 μg of fluoroscein isothiocyanate-conjugated (FITC) goat anti-mouse IgG (Tago, Burlingame, CA, U.S.A.) for 30 min. After washing, the stained cells were fixed in 2% paraformaldehyde and staining intensity and numbers of cells stained, determined by FACS (EPICS 541, Coulter Corp.) as previously described.[2] KCs grown in Lab-Tek chamber slides were stained *in situ* with the aforementioned monoclonal antibodies using an immunoperoxidase technique (Vectastain ABC Kit, Vector laboratories, Burlingame, CA, U.S.A.) with 3-amino-9-ethylcarbazole as the chromogen and counterstained with 1% haematoxylin.[3]

Protein kinase-C activity

Gamma interferon, at 10^3 U/ml, was added to 100-mm diameter Petri dishes containing subconfluent KCs for either 15, 30 or 60 min. TPA 100 nM was added to similar dishes for 10 min to serve as a control. At the indicated times, the treated dishes were washed with cold, phosphate-buffered saline (PBS) (calcium and magnesium free) and then incubated on ice for 5 min in the presence of 3 ml of permeabilizing buffer (20 mM Tris, 5 mM EGTA, 2 mM EDTA, 10 mM β-ME, Leupeptin and Digitonin).[16] The permeabilizing buffer was collected (soluble fraction) and kept on ice whilst the KCs were harvested in 3 ml of homogenizing buffer (20 mM Tris, 5 mM EGTA, 2 mM EDTA, 10 mM βME, Leupeptin and 0·5% Triton X-100) and centrifuged at 10 000 ***g*** for 40 min, the resulting supernatant being the membrane fraction. Both soluble and membrane-associated PK-C were partially purified by DEAE cellulose chromatography and the fractions assayed in duplicate for PK-C activity by measuring the incorporation of ^{32}P from ^{32}P-ATP into histone as described.[17] Protein estimation was performed using the Bio-Rad assay.[18]

Time course and dose-dependence of IFN-γ induced KC expression of ICAM-1 and HLA-DR

IFN-γ was added to subconfluent, KC monolayers in 35-mm dishes at the indicated concentrations and incubated with the KCs for the various time courses. The KCs were then trypsinized, stained and quantified by FACS analysis, or the IFN-γ was washed from the dishes and the KCs incubated further in KGM alone for the indicated periods of time until trypsinization and staining.

RESULTS

Induction of KC ICAM-1 expression by TPA

By FACS analysis, TPA induced KC expression of ICAM-1 but no HLA-DR and the maximal induction occurred at 1 day with no subsequent increase or decrease in intensity of expression with incubation continued for as long as 3 days. Incubation beyond 3 days resulted in TPA-induced KC toxicity. The maximum dose response was reached at 10–20 nM TPA (Fig. 1). Neither A23187 nor the protein kinase inhibitor, H-7 alone induced ICAM-1 or HLA-DR expression, but A23187 at 1 μM enhanced TPA-induced expression of ICAM-1. H-7 at 40 μM inhibited TPA induction of ICAM-1 by 50% (higher H-7 concentrations could not be used due to cytotoxicity), and TPA + A23187 induction of ICAM-1 by 60% (Fig. 2), but did not inhibit IFN-γ-induced ICAM-1 expression (data not shown).

Immunoperoxidase staining of untreated KC monolayers showed few KCs ($< 1\%$) positive for ICAM-1. However ICAM-1, but not HLA-DR expression, occurred after incubation for 48 h with TPA alone, or with TPA and A23187, although the intensity and extent of ICAM-1 expression was less than that induced by IFN-γ (Fig. 3). Light microscopy showed that those

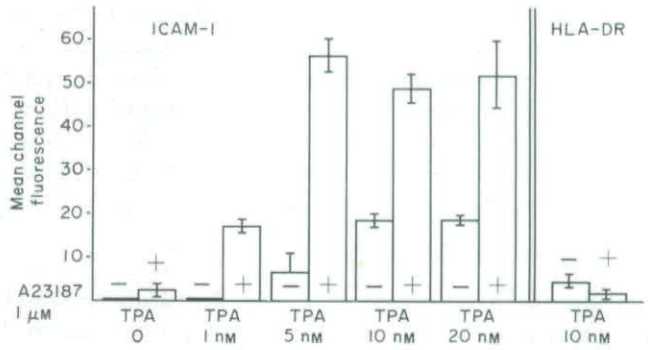

FIGURE 1. KC expression of ICAM-1 and HLA-DR following incubation for 48 h with TPA 0–20 nM ± calcium ionophore, A23187, 1 μM, as determined by FACS analysis. A23187 enhances the induction of ICAM-1 by TPA, and there is only minimal HLA-DR expression in response to TPA with no enhancement by A23187. ($n = 3$–7 for all experiments, values are mean ± SEM.)

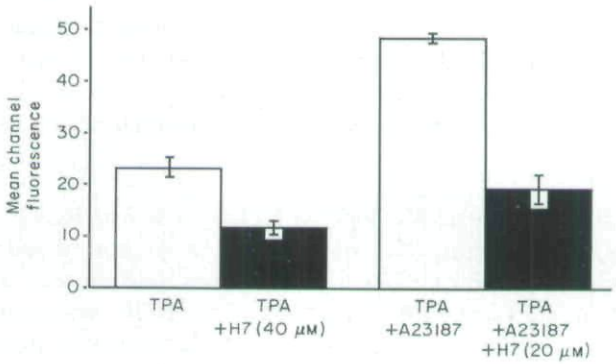

FIGURE 2. KC expression of ICAM-1 after 48 h incubation with either TPA 10 nM or TPA 10 nM + calcium ionophore, A23187, 1 μM and subsequent inhibition of this effect by co-incubation with PK-C inhibitor H7 at either 40 or 20 μM. FACS analysis determination. ($n = 4$, values are mean ± SEM.)

KCs expressing ICAM-1 in response to TPA differed from surrounding non-ICAM-1-expressing cells in that they were slightly elongated and had migrated above the monolayer.

PK-C modulation

TPA produced a rapid (10 min) reduction in soluble fraction PK-C activity (Table 1) with a concomitant rise in membrane-associated PK-C activity, as previously reported.[19,20] IFN-γ had induced no change in membrane-associated PK-C activity by 15 min. However, extension of the IFN-γ co-incubation time to 30 min produced a rise in the PK-C activity of both the membrane-associated and soluble fractions with the membrane-associated PK-C activity returning to the original value at 60 min.

Modulation of KC ICAM-1 by IFN-γ

FACS analysis demonstrated that in the absence of IFN-γ there was no KC expression of HLA-DR and < 1% staining for ICAM-1. After only 4 h of IFN-γ treatment, KCs strongly expressed

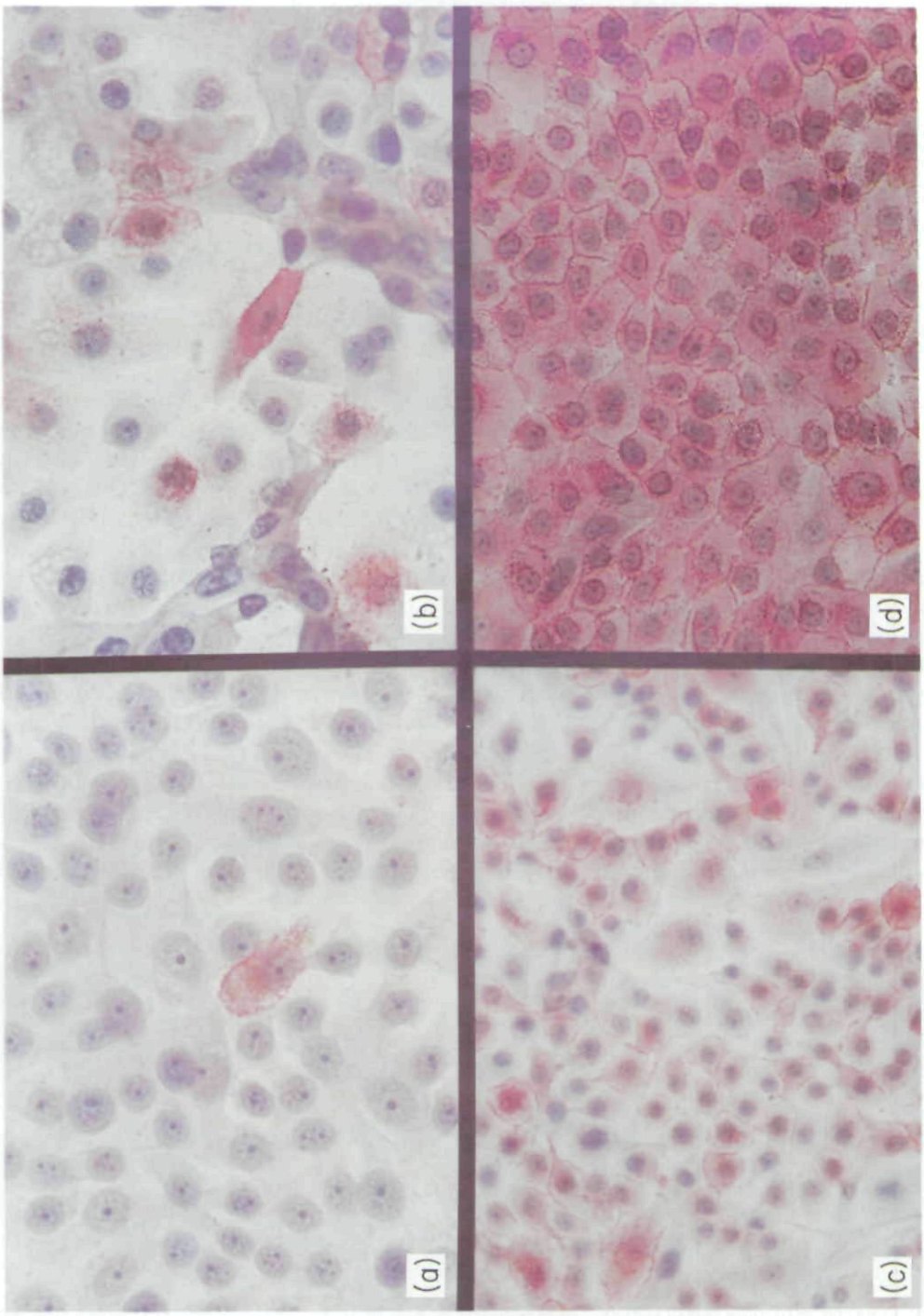

FIGURE 3. (a) Immunohistochemical ICAM-1 staining of KCs in the absence of any treatment or (b) presence of TPA alone, (c) TPA + A23187, and (d) IFN-γ after 2 days. (×75).

TABLE 1. PK-C activity, soluble and membrane-associated, of normal, human cultured KC showing effect of IFN-γ (100 U/ml) and TPA (100 nM) at different time intervals. Values are mean ± SEM

	PK-C activity (pmol/mg protein per min)	
Treatment	Soluble	Membrane associated
Control	23·1 ± 7 (n=3)	43·9 ± 8 (n=4)
TPA 10 min	13·4 ± 7·6 (n=3)	138·9 ± 16·3 (n=3)
IFN-γ 15 min	42·3 ± 12 (n=3)	35·8 ± 8 (n=4)
IFN-γ 30 min	35·8 ± 16 (n=3)	71·3 ± 12 (n=3)
IFN-γ 60 min	35·5 ± 12 (n=3)	43·7 ± 12 (n=3)

ICAM-1, reaching a maximum intensity at 2 days (Fig. 4). HLA-DR was not induced until day 1, with a maximum at day 3 as previously described.[21] Continued incubation with IFN-γ over a period of 6 days resulted in a marked decline of ICAM-1 expression, but a stabilizing of HLA-DR expression with minimal loss of intensity (Fig. 4). When the KCs were washed free of IFN-γ (as detected by a sensitive radioimmunoassay; Centocour, Malvern, PA, U.S.A.) after 2 days exposure, both HLA-DR and ICAM-1 expression declined slightly over the first week with a greater decrease during week 2 (Fig. 5). The decrease in HLA-DR expression was always less than for ICAM-1 to day 11, but by day 18 there was no significant detectable KC expression of either antigen.

Minimal KC expression of ICAM-1 was induced by only 0·1 U/ml IFN-γ with a rapid increase in intensity of expression with concentrations up to 5 U/ml IFN-γ (Fig. 6). Maximum expression of ICAM-1 occurred at about 10^2 U/ml IFN-γ, with increasing concentrations up to 10^3 U/ml IFN-γ producing no further enhancement. KC expression of HLA-DR was less sensitive to IFN-γ than ICAM-1, with minimal expression first occurring at 2·5 U/ml with a maximum at 100 U/ml, equivalent to that seen for ICAM-1 (Fig. 6). Thus, ICAM-1 induction in cultured KCs is more rapid and more sensitive to lower doses of IFN-γ than is HLA-DR, as was also found with short-term, whole-skin organ cultures.[3] Whether the decreased KC

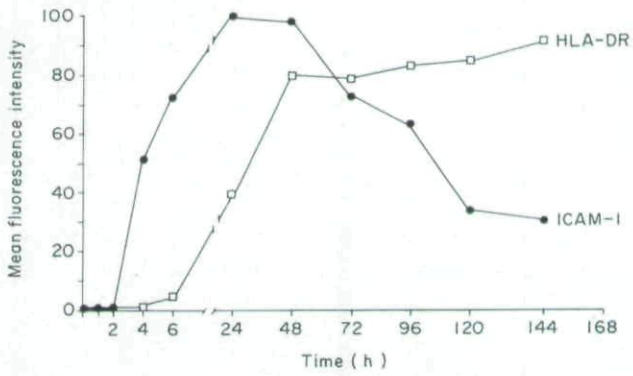

FIGURE 4. Time course of induction of ICAM-1 and HLA-DR on KCs by IFN-γ 500 U/ml for 1–144 h, as determined by FACS analysis. Note the more rapid induction and disappearance of ICAM-1 expression compared to HLA-DR.

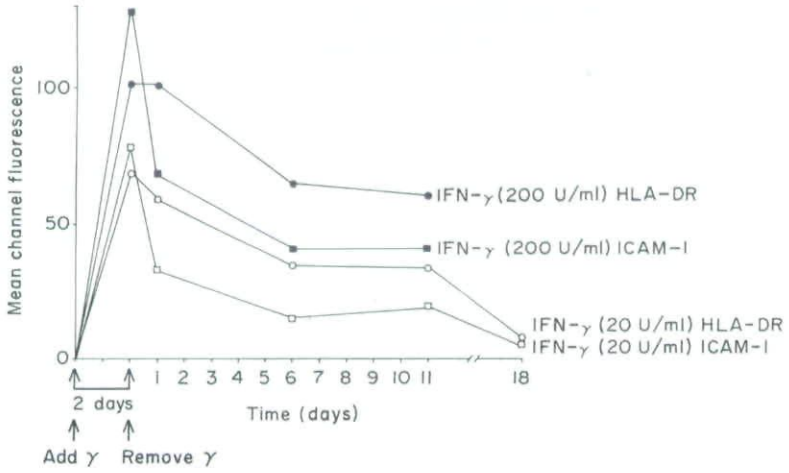

FIGURE 5. KC expression of ICAM-1 and HLA-DR after incubation with IFN-γ (20 U/ml or 200 U/ml) for 48 h, followed by washing KCs free of IFN-γ and incubation continued in KGM alone for a further 16 days by FACS analysis. Note more rapid induction of ICAM-1 expression and gradual decline in both ICAM-1 and HLA-DR expression after removal of IFN-γ.

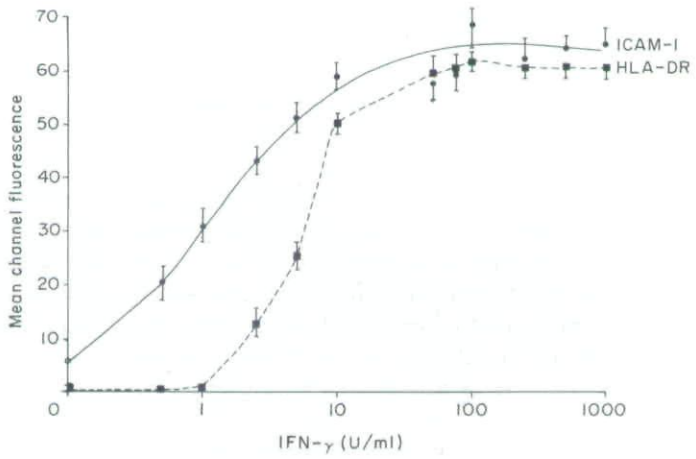

FIGURE 6. KC expression of HLA-DR and ICAM-1 in response to 48 h incubation with increasing concentrations of IFN-γ (0·5–1000 U/ml), as determined by FACS analysis. KC expression of ICAM-1 is more sensitive to lower concentrations of IFN-γ, and both KC ICAM-1 and HLA-DR expression peak at 100 U/ml of IFN-γ.

ICAM-1 expression which occurred between 48–96 h of IFN-γ exposure was due to ICAM-1 shedding, surface modification, or internalization and degradation is now being investigated.

DISCUSSION

Protein kinase C (PK-C) is the major cellular phorbol-ester receptor and it is believed that most TPA-induced events within the cell are PK-C mediated.[22] TPA induces KC ICAM-1, and this

effect is enhanced by the calcium ionophore, A23187, a carboxylic acid antibiotic which transports calcium ions across biological membranes.[23] These findings strongly indicate a role for PK-C in the secondary signalling mechanisms preceding ICAM-1 expression, which is further supported by the ability of the protein kinase inhibitor, H7, to partially block this response.[15]

The observation that 15 min incubation with IFN-γ did not increase the PK-C activity of membrane-associated fractions of human KCs is in contrast to the rapid TPA activation of PK-C by translocation from cytosol to membrane.[22] However, IFN-γ increased both membrane-associated and soluble PK-C activity within 30 min. In agreement with our results, Hamilton *et al.*[24] demonstrated a concomitant increase in both cytosolic and membrane-associated PK-C activity following treatment of murine peritoneal macrophages with IFN-γ. They concluded that this dual rise in soluble and membrane PK-C activation was not due to *de novo* synthesis of PK-C and that the molecular mechanisms were unknown. Ostrowski *et al.*[25] showed in a B-lymphoid cell line that although IFN-γ will produce transient translocation of PK-C activity from cytoplasm to membrane, the response is slower (30 min) than that reported for interleukin 3.[26]

Our study therefore agrees with previous work which demonstrated that IFN-γ does not activate PK-C in the same manner as does TPA. The inability of H7 to inhibit IFN-γ-induction of KC ICAM-1 is evidence that IFN-γ does not directly induce KC expression of ICAM-1 via a PK-C-mediated pathway. Furthermore, Koide *et al*[27] demonstrated that IFN-γ induction of HLA-DR on HL-60 cells is not inhibited by H7, but W7 (a calmodulin antagonist) will block this response, indicating that IFN-γ induction of HLA-DR may be a calcium/calmodulin and not a PK-C-mediated event. Perhaps IFN-γ induction of KC ICAM-1 expression is also via a calmodulin-dependent pathway. Previous authors have observed that certain biological effects of IFN-γ, such as induction of an anti-viral state, are PK-C dependent,[28] whilst other activation events are PK-C independent.[29]

The results suggest that KC ICAM-1 is inducible by at least two different cellular mechanisms; the first involving IFN-γ, and the second involving TPA. In the first mechanism the KCs may be viewed as passive targets for IFN-γ which can augment an ongoing immunological reaction in the dermis and which induces ICAM-1 expression. In the second mechanism, the KCs may be viewed as active initiators of epidermal T-cell trafficking in which PK-C-mediated induction of ICAM-1 leads to the activation of T cells and subsequent production of T-cell cytokines. In this model, either endogenously generated DAG within the epidermis, or exogenously applied TPA-like molecules to the skin surface, would be responsible for activation of KC PK-C and induction of KC ICAM-1 expression.

It is known that even normal appearing skin contains occasional intraepidermal T cells,[30] and accumulation of these T cells within the epidermis occurs when they bind to KCs expressing ICAM-1. These accumulated intraepidermal T cells may become activated themselves either through exposure to KC-derived cytokines such as IL-1,6, GM-CSF,[31] or through activation of their own PK-C signal transduction system by a TPA-like molecule.[32] Activated intraepidermal T cells could then produce IFN-γ to augment KC ICAM-1 expression and induce HLA-DR expression, thereby conferring antigen-presenting capacity on the KC.[33] Topical exposure to PK-C agonists such as TPA, aplysiatoxin, and urushiol which produce inflammatory psoriasiform dermatitis,[14] swimmers itch,[34] and poison ivy contact dermatitis[35] respectively, may, via the induction of KC ICAM-1, influence T cell epidermal trafficking and thereby explain the rapid appearance of intraepidermal T cells and cutaneous inflammation. In fact, we have recently observed early KC ICAM-1 expression in poison ivy contact dermatitis as described above.[36]

In summary, our results suggest a new pathophysiological mechanism for inflammatory skin diseases which emphasizes the production of cutaneous immunological events via altered KC signal transduction. Furthermore, the propensity for both cytokine (e.g. IFN-γ), and non-cytokine- (e.g. TPA) mediated mechanisms to induce ICAM-1 on KCs via PK-C-independent and dependent pathways, respectively, may aid the development of new therapeutic agents. Thus, the recently recognized ability of topically applied sphingosine, which is a protein kinase inhibitor, to block TPA-induced inflammation and KC hyperplasia,[14] may be mediated in part through modulation of adhesion molecules and subsequent T-cell trafficking in the skin.

ACKNOWLEDGMENTS

Supported in part by the Babcock Dermatological Fund, NIH Grants AR35897, AR01823, AR40065 (BJN), AR39691 (GJF), Danish Medical Research Grant 82488 (JE) and the British Association of Dermatologists' Dowling Travelling Fellowship (CEMG).

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