ADHESION OF T LYMPHOBLASTS TO EPIDERMAL KERATINOCYTES IS REGULATED BY INTERFERON γ AND IS MEDIATED BY INTERCELLULAR ADHESION MOLECULE 1 (ICAM-1)

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The skin is an important site for immunological reactions (1–3). Cutaneous infiltration by T lymphocytes is characteristic of a number of disease states and T lymphocytes have been observed within the epidermal compartment in graft-vs.-host disease (4), lichen planus (5), and delayed-type hypersensitivity (6). In addition, a heterogeneous group of T lymphomas, collectively termed cutaneous T cell lymphomas, show directed migration to the skin (7, 8). In contrast, cutaneous infiltration by B lymphocytes is rare.

Epidermal keratinocytes (EK)[1] do not typically express MHC class II antigens, however, in the presence of a cutaneous T cell infiltrate, EK frequently express high levels of MHC class II HLA-DR antigens (9–15). It is likely that the enhanced expression of HLA-DR is a result of T lymphocyte–derived cytokines including IFNγ. In vitro experiments have documented that IFN-γ induces the expression of HLA-DR on EK (16); however the functional significance of HLA-DR expression by EK is not known. EK produce the important immunoregulatory cytokine IL-1 (17, 18). In addition to its role in enhancing T cell proliferation, IL-1 has been reported to be chemotactic for T cells (19). Currently, very little is known about the molecular basis of epidermal T cell interactions and the consequences of those interactions.

Lymphocyte adhesion to a wide array of cells of hematopoietic and nonhematopoietic origin is required for the induction and maintenance of immune responses and for effector functions of lymphocytes (20). Interactions of lymphocytes with many cell types are inhibited by lymphocyte function–associated antigen 1 (LFA-1) (CD11a,CD18) mAb (21–25), and consistent with the importance of adhesion for immune function, several in vitro assays of immune function are blocked by LFA-1 mAb (26). In the epidermis, T lymphocytes are

known to be closely apposed to basal EK and Langerhans' cells (4–6, 27), and intercellular adhesion may be important in maintaining this proximity.

We have described a cell surface glycoprotein, intercellular adhesion molecule 1 (ICAM-1), that is involved in LFA-1-dependent adhesion of lymphocytes (28). ICAM-1 expression on dermal fibroblasts and umbilical vein endothelial cells is increased by IFN-γ, IL-1, TNF, lymphotoxin, and LPS (24, 29). Staining of tissue sections has shown that the expression of ICAM-1 on epithelial cells is restricted to thymic epithelial cells and some kidney tubule epithelial cells. Additionally, staining of mucosal epithelial cells and EK was seen at sites of immune responses (24). This is very similar to the staining pattern of HLA-DR on epithelial cells, suggesting that ICAM-1 and HLA-DR expression on EK and other epithelial cells might be under common regulation. Since ICAM-1 is involved in adhesive interactions of T lymphocytes, the coexpression of ICAM-1 and MHC class II antigens on EK could be important for cutaneous immune responses. Therefore, we have examined the interaction of allogeneic and autologous T lymphoblasts with cultured EK treated with a variety of cytokines and the relationship between ICAM-1 expression and lymphocyte adhesion. The role of ICAM-1 in adhesion has been studied with mAb blockade and reconstitution of purified epithelioid cell ICAM-1 in planar membranes. Our findings suggest that ICAM-1 is the primary EK surface molecule that regulates adhesion to T lymphoblasts.

Materials and Methods

Materials.   rIFN-γ expressed in CHO cells with a sp act of 10^8 U/mg was a gift of Drs. Daniella Novick or Dina Fisher (Weizmann Institute of Science, Rehovot, Israel); rIFN-γ expressed in *Escherichia coli* with a sp act of 5×10^7 U/mg was purchased from Collaborative Research, Lexington, MA). Human rTNF with a specific activity of 10^8 U/mg was from Genentech (South San Francisco, CA). Human rIL-1-β with a sp act of 2×10^7 U/mg was a gift of Dr. Charles A. Dinarello (Tufts University, Boston, MA). Cell culture reagents and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), with the exception of epidermal growth factor from Collaborative Research, cholera toxin from Schwarz-Mann/ICN (Irvine, CA) and hydrocortisone from Calbiochem-Behring Corp. (San Diego, CA). Protein A–Sepharose CL-4B and Sepharose CL-4B were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL). FITC–goat anti–mouse IgG was purchased from Zymed Laboratories (San Francisco, CA). Carrier-free Na^{125}I and Na$_2$^{51}CrO$_4$ were purchased from New England Nuclear (Boston, MA).

Monoclonal Antibodies.   RR1/1 (anti-ICAM-1 IgG1) (28), TS1/22 (anti-LFA-1α IgG1), TS1/18 (anti-LFA-1, Mac-1, p150,95 β IgG1), TS1/16 and TS1/2 (anti-HLA DR framework IgG1), TS2/18 (anti-CD2 IgG1), TS2/9 (anti-LFA-3 IgG1) (30), LM2/1 (anti-Mac-1 α IgG1), SHCL3 (anti-p150,95α IgG1) (31), P3X63Ag8 (nonbinding myeloma IgG1) (32), and W6/32 (anti-HLA-A,B IgG2a) (33) were used as protein A affinity-purified IgG or as dilutions of culture supernatants. Anti-Leu-2a(anti-CD8 IgG1), anti-Leu-3a(anti-CD4 IgG1), and L243 (anti-HLA-DR IgG2a) ascites were obtained from Becton Dickinson & Co. (Mountain View, CA). OKT3 (anti-CD3 IgG2a) was obtained from Ortho Pharmaceutical (Raritan, NJ). A polyclonal rabbit antisera reacting primarily with the VLA-β subunit was a gift of Dr. Martin Hemler, Dana Farber Cancer Institute, Boston, MA (34).

Cell Culture.   Con A T lymphoblasts were prepared from PBMC by culturing mononuclear cells in RPMI 1640, 20% FCS, 2 μg/ml Con A for 3 d followed by washing once and culturing with 1 ng/ml IL-2 for 4–10 d before use. The B lymphoblastoid cell line JY was maintained in RPMI 1640, 10% FCS (complete medium). EK were isolated from

neonatal foreskins or adult skin biopsies and cultured with mouse 3T3 feeder cells in supplemented DME as previously described (35).

RIA for ICAM-1 Expression. Purified RR1/1 was iodinated using iodogen to a specific activity of either 10 or 1 μCi/μg (24, 36). EK were seeded into 96-well plates at high density. The cells were allowed to grow to between 60 and 90% confluence. Cells were then treated as indicated and washed with HBSS 1 mM EDTA to remove dead cells and any residual feeder cells (RR1/1 does not react with 3T3 cells) and once with complete medium with 25 mM Hepes at 4°C. An amount of iodinated RR1/1 + cold RR1/1 was added to each well such that a linear signal was obtained over a range of three orders of magnitude including the range of ICAM-1 expression obtained on EK (sp act, $\leq$ 2 μCi/ μg, with 20 ng/well). Nonspecific binding was determined in the presence of a 100-fold excess of cold RR1/1. After 30 min at 4°C, the wells were washed three times and the bound radioactivity was measured after solubilization with 0.1 N NaOH. Sites/cell were calculated using a mol wt of 150,000 for IgG.

Immunofluorescence Flow Cytometry. T lymphoblasts were washed three times with HBSS/10% FCS, 1mM EDTA, 0.025% NaN$_3$. This buffer was used in all subsequent steps. 10^6 cells were incubated with specific mAb or P3X63.Ag8 IgG1 at a 1:1 dilution of hybridoma culture supernatant for 30 min at 4°C and then washed three times. The cells were then incubated with FITC–goat anti–mouse IgG at 100 μg/ml (50 μl) for 30 min and washed three times. The stained cells were fixed with 1% paraformaldehyde and stored up to 1 wk at 4°C before analysis on a flow cytometer (Epics V; Coulter Electronics Inc., Hialeah, Fl or Ortho Diagnostic Systems Inc., Westmead, MA).

Cell Binding Assay. EK were grown in 96-well plates exactly as for the RIA. T lymphoblasts or other cells were labeled with Na^{51}CrO$_4$ by incubating 4 $\times$ 10^6 cells with 200 μCi of Na^{51}CrO$_4$ in 1.2 ml of complete medium/isotonic saline (4:1) for 1.5 h at 37°C. Cells were then washed four times with complete medium. T lymphoblasts were also washed twice with 5 mM methyl-α-D-mannopyranoside after labeling to remove any residual surface Con A. Cells (0.5–1 $\times$ 10^5) were added and the plate was incubated at 37°C for 1 h. The wells were washed four times with 37°C medium (23, 24). All points were determined in quadruplicate. mAbs were added just before the labeled cells, or cells were pretreated with the relevant mAb and then washed three times before labeled cells were added.

Immunoprecipitation and SDS-PAGE Analysis. Purified RR1/1 mAb was coupled to CNBr-activated Sepharose CL-4B using the procedure of March et al. (37). EK incubated for 24 h without or with 100 U/ml IFN-γ or JY cells were lysed in 25 mM Tris, pH 8.0, 150 mM NaCl, 0.025% NaN$_3$ (TSA), 1% Triton X-100, 1 mM PMSF, 5 mM iodoacetamide, 200 mTIU (Trypsin inhibitor units)/ml aprotinin (lysis buffer) for 30 min at 4°C at 5 $\times$ 10^7 cells/ml. Nuclei and other insoluble material were removed by centrifugation at 12,000 g for 15 min. The lysate was incubated overnight, with 20 μl/ml mouse IgG Sepharose (prepared as for RR1/1), centrifuged to remove nonspecifically bound components, and then ICAM-1 was immunoprecipitated with 20 μl/ml RR1/1 Sepharose for 6 h at 4°C. The precipitate was washed thrice with TSA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS (38), and finally with TSA and then 50 mM Tris, pH 6.8. The Sepharose was boiled with Laemmli sample buffer containing 50 mM iodoacetamine (nonreducing) and subjected to 8% SDS-PAGE (39). Proteins were visualized using the silver staining (40).

Purification of ICAM-1 from HeLa Cells. ICAM-1 was purified from 2–3 g of HeLa cells (2–3 $\times$ 10^9) after solubilization in lysis buffer at 5 $\times$ 10^7 cells/ml using the procedure of Marlin and Springer (41). The lysate was passed over a 1-ml RR1/1-Sepharose column (1.5 mg/ml) at a flow rate of 1 ml/min. The column was washed with 10 vol of TSA, 1% Triton X-100, with 5 vol of 25 mM triethylamine, pH 11, 0.15 M NaCl, 0.1% Triton X-100, and finally with 5 vol of 20 mM sodium phosphate, pH 7.2, 0.15 M NaCl (PBS), 1% octyl-β-D-glucopyranoside (OG) (41). ICAM-1 was eluted in 50 mM triethylamine, pH 12.4, 0.15 M NaCl, 1% OG and collected into tubes containing 0.1 vol of 1 M Tris, pH 7.5, 1% OG.

Reconstitution of ICAM-1 into Liposomes and Preparation of Planar Mem-

branes. Liposomes were prepared by addition of phospholipid and cholesterol (7:2 molar ratio) and removal of detergent by dialysis (42, 43). The final protein/lipid ratio was between 1:500 and 1:5,000, and the lipid concentration was 0.1 mM. Planar membranes were prepared as described by Brian and McConnell (44) with several modifications. Cleaned coverslips (5 mm, Bellco Glass, Inc., Vineland, NJ) were placed in the bottom of 96-well plate wells, and 30 μl of liposome suspension was added to each well. The plate was covered with a damp paper towel to retard evaporation and left for 1 h at room temperature. The wells were washed five times with HBSS and twice with complete medium, always leaving ∼40 μl in the well to keep the membrane submerged; exposure to air results in instantaneous destruction of the membrane. Cell binding in this system was done exactly as described for binding of ^{51}Cr-labeled T lymphoblasts to cell layers except that the planar membrane was never exposed to an air/water interface.

Results

ICAM-1 Expression on EK. ICAM-1 expression on cultured EK under basal conditions or after incubation for 24 h with rIFN-γ, rIFN-β, rTNF, and rIL-1-β alone or in combinations was quantified using saturating concentrations of ^{125}I-ICAM-1 mAb (Table I). The basal ICAM-1 expression varied between experiments even on the same line from 0 to 50,000 ICAM-1 mAb binding sites/cell. The basis of the variation has not been systematically studied but small variations are also observed on fibroblasts (24). Both rIFN-γ and rTNF greatly increased ICAM-1 expression at 24 h. rIFN-γ at 1,000 U/ml increased ICAM-1 25-fold (range, 12.7–33). rTNF had a moderate effect, increasing ICAM-1 expression about eightfold (range, 2–10). rIFN-β (1,000 U/ml), rIL-1-β (10 ng/ml), and LPS (10 μg/ml) (not shown) had no effect on ICAM-1 expression on EK. In combinations, 1,000 U/ml rIFN-γ and 10 ng/ml rTNF were greater than additive in effect, increasing ICAM-1 expression 49-fold (range, 20–64). Although rIL-1-β had little effect by itself, it was weakly synergistic with IFN-γ, increasing ICAM-1 expression 32-fold. Cross titration between rIFN-γ and rTNF showed

TABLE I
ICAM-1 Site Number Analysis on EK

Treatment (24 h)	Sites/cell	Fold increase
	$\times 10^{-3}$	
None	35	—
rIFN-γ (1,000 U/ml)	940	25
rTNF (10 ng/ml)	296	8
rIL-1-β (10 ng/ml)	50	1.3
rIFN-β (10,000 U/ml)	25	0.7
TNF + IFN-γ	1,800	49
TNF + IL-1-β	281	7.5
TNF + IFN-β	254	6.9
IFN-γ + IL-1-β	1,200	32
IFN-γ + IFN-β	940	25
IL-1-β + IFN-β	86	2.3

EK in 96-well plates were treated for 24 h with the indicated mediators and sites/cell was determined with ICAM-1 mAb labeled with ^{125}I to low specific activity. All points were determined in triplicate and cells/well were determined after removal by trypsinization.
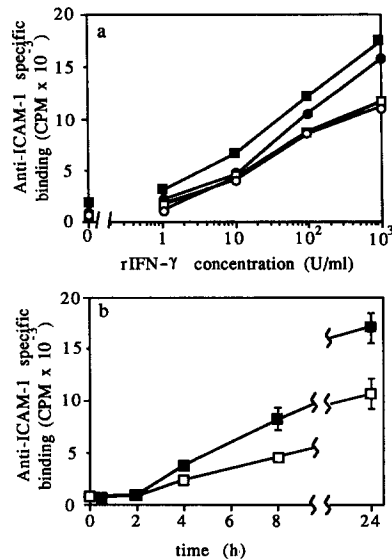
FIGURE 1. (a) Synergism between rIFN-γ and rTNF for upregulation of ICAM-1. EK were grown to 80% confluence in 96-well plates and treated with the indicated combination rIFN-γ and/or rTNF for 24 h. An RIA was then done on washed monolayers as in methods. TNF concentrations: 0 (O), 0.1 (□), 1.0 (●), and 10 ng/ml (■). (b) Time course of rIFN-γ and rIFN-γ/rTNF effect on ICAM-1 expression. EK were treated for indicated time with 500 U/ml rIFN-γ (□)) or 500 U/ml rIFN-γ + 5 ng/ml rTNF (■). The monolayers were washed and an RIA done as in Materials and Methods.

moderate synergism at high TNF concentrations (Fig. 1 a). ICAM-1 was significantly increased by rIFN-γ at 1 U/ml with a half-maximal increase occurring at ~20 U/ml. Marked synergism between rIFN-γ and rTNF was only seen at 1 and 10 ng/ml rTNF; 10 ng/ml was the optimal rTNF concentration for increasing ICAM-1 on dermal fibroblasts and endothelial cells (44a).

The time-course of the rIFN-γ effect on EK ICAM-1 expression was determined out to 24 h (Fig. 1 b). An increase in ICAM-1 expression was not detectable at 2 h, but became apparent at 4 h for EK treated with rIFN-γ or rIFN-γ and rTNF. This is similar to the time course of the rIFN-γ effect on dermal fibroblast ICAM-1 expression (24).

Adhesion of T Lymphoblasts to Epidermal Keratinocytes. Adhesion of T lymphoblasts to epidermal cell monolayers was assayed using ^{51}Cr-labeled lymphocytes (Table II). T lymphoblasts were used as a model for large activated lymphocytes that are known to extravasate most readily during local immune responses, especially early on (45), and may be more likely to invade the epidermis. When previously examined, intraepidermal T lymphocytes were found to express HLA-DR, suggesting that they were activated (5, 14). Adhesion of T lymphoblasts to untreated epidermal monolayers was minimal and was not affected by mAbs specific for ICAM-1, LFA-1, CD2, or LFA-3. When epidermal cells were treated for 24 h with 500 U/ml IFN-γ, the adhesion of T lymphoblasts was dramatically increased from 2.4 to 42%. Adhesion of T lymphoblasts to IFN-γ-treated EK was inhibited to the level seen for untreated EK monolayers by mAb to ICAM-1 or LFA-1 (Table II). Other mAbs were tested that were previously shown to block adhesion of HLA-DR-specific cytolytic T lymphocytes to lymphoid target cells; mAb to HLA-DR, CD2, and LFA-3 were found to have no effect in this system. The experiments shown were done after a 24-h treatment of EK with IFN-γ, when ICAM-1 is strongly induced but HLA-DR is only weakly or not expressed (Singer, K., and D. Tuck, unpublished observation). Identical results

TABLE II

LFA-1⁺ and LFA-1⁻ T Lymphoblast Binding to EK

mAb	T cells bound			
	Normal donor		LAD donor	
	EK: untreated	IFN-γ	Untreated	IFN-γ
	% ± SD			
IgG1 control	2.4 ± 0.5	42 ± 1.8	1.4 ± 0.7	1.8 ± 0.9
Anti-ICAM-1	2.3 ± 0.7	2.4 ± 1.0*	0.8 ± 0.7	1.7 ± 1.0
Anti-LFA-1	2.1 ± 0.8	2.1 ± 4.1*	—	2.0 ± 0.4
Anti-Mac-1	—	40 ± 3.0	—	—
Anti-p150,95	—	43 ± 2.1	—	—
Anti-HLA-DR	—	44 ± 1.2	0.9 ± 0.3	1.9 ± 0.6
Anti-CD2	—	40 ± 1.8	1.5 ± 0.9	2.0 ± 0.8
Anti-LFA-3	—	41 ± 2.0	2.1 ± 0.4	1.4 ± 1.0

EK (80% confluent) were treated with medium or with 500 U/ml IFN-γ for 24 h. T lymphoblasts from a normal donor and from a severely affected LAD patient were ⁵¹Cr labeled and monolayer binding was measured. mAbs were added at between 5 and 25 μg/ml. Results are percent binding ± SD of quadruplicates.
* Significantly different than IgG control for same treatment category.

were obtained after 48- and 72-h treatment of EK with IFN-γ when both ICAM-1 and HLA-DR are strongly expressed (not shown). T lymphoblasts prepared from PBMC of a patient with a severe deficiency in the LFA-1, Mac-1, p150,95 family of adhesion glycoproteins (46) were also tested for their ability to bind to EK (Table II). LFA-1⁻ T lymphoblasts were deficient in binding to IFN-γ treated EK, confirming the mAb-blocking results. Since the population of normal T lymphoblasts prepared by stimulating with Con A contains cells weakly positive for Mac-1 and p150,95 by immunofluorescence flow cytometry, the ability of anti–α chain mAbs specific for these antigens was tested. Neither of these mAbs inhibited adhesion of normal T lymphoblasts to treated EK (Table II).

In some adhesion experiments, subconfluent monolayers were used to avoid differentiation of EK, which occurs to a greater extent in confluent cultures. Adhesion to subconfluent EK monolayers was increased by IFN-γ treatment of EK (Fig. 2, compare *b* and *a*) and this adhesion to EK was blocked by anti-ICAM-1 and anti-LFA-1 mAb (Fig. 2, *c* and *d*). However, with subconfluent EK we observed adhesion of T lymphoblasts to the spaces between EK colonies (Fig. 2 *d*). This substratum adhesion was not inhibited by anti-ICAM-1 (not shown) or anti-LFA-1 mAb (Fig. 2 *d*). This binding was responsible for the background of 1–2% anti-ICAM-1–resistant adhesion seen in more confluent untreated and IFN-γ-treated EK (Table II), as shown by inspection of washed monolayers. Adhesion of T lymphoblasts to plastic was not seen in binding to subconfluent dermal fibroblasts (24), suggesting that the plastic binding was dependent on the presence of EK and possibly the result of binding to an extracellular matrix component secreted by EK. A polyclonal rabbit antiserum to the VLA family of surface proteins, which includes the human fibronectin receptor and two poten-
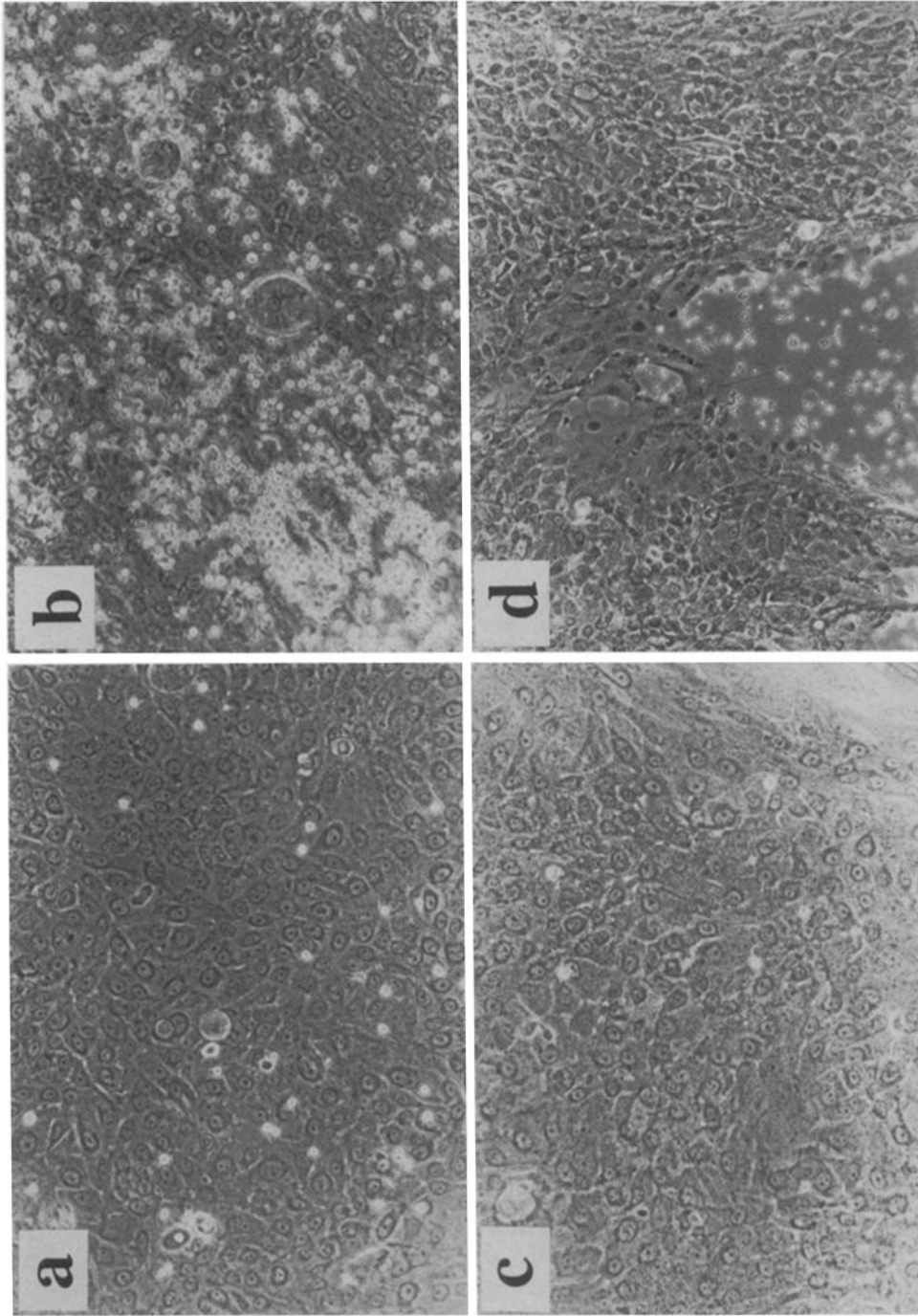
FIGURE 2. Adhesion of T lymphoblasts to EK. EK at 70% confluence were treated with media (*a*) or with 200 U/ml rIFN-γ (*b–d*) for 24 h. Normal T lymphoblasts were allowed to settle onto the monolayer and bind for 1 h in the presence of nonbinding control mAb (*a* and *b*), ICAM-1 mAb (*c*) or LFA-1 mAb (*d*) after which the wells were extensively washed and photomicrographs were taken on a Nikon Diaphot microscope. Scale bar, 50 μm.
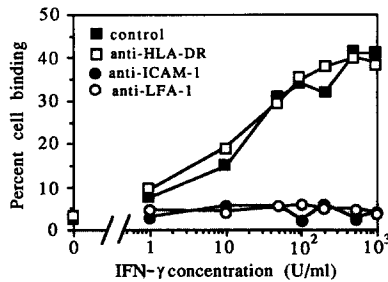
FIGURE 3. Dose-response for IFN-γ effect on T lymphoblast adhesion to EK. EK were grown to 90% confluence in 96-well plates and treated with the indicated concentration of rIFN-γ for 24 h. The monolayers were extensively washed and ^{51}Cr-labeled T lymphoblasts were added and incubated with cells for 1 h at 37°C in the presence of the indicated mAb. Unbound cells were washed out and the percent bound cells determined.

tial receptors found on T lymphoblasts (34), did not block this adhesion suggesting that this adhesion is not mediated by a member of this family (not shown).

Dose-dependent Effect of IFN-γ on Lymphocyte Adhesion. The relationship between adhesion and ICAM-1 density was further examined by measuring adhesion of T lymphoblasts to EK monolayers treated with increasing concentrations of IFN-γ (Fig. 3). The IFN-γ dose responses for ICAM-1 upregulation and T lymphoblast adhesion were very similar (compare Figs. 1 *a* and 3). The mAb blocking pattern was the same over the entire range of IFN-γ concentrations; anti-ICAM-1 and anti-LFA-1 inhibited strongly and anti-HLA-DR had no effect.

Binding of T Lymphocytes to Autologous and Allogeneic EK. While antibodies to HLA-DR and HLA-A,B (not shown) did not block the adhesion of T lymphoblasts to IFN-γ-stimulated EK, it remained a possibility that the adhesion might be enhanced by alloreactivity of T lymphoblasts toward the EK. Therefore, binding experiments were done using syngeneic combinations of T lymphoblasts and EK (forearm biopsy) in parallel with allogeneic combinations (Table III). No significant difference was seen between the binding of T lymphoblast line A to EK line A or B, and this binding was similar to the binding of T lymphoblast line C to either EK A or B in both efficiency and inhibition by anti-ICAM-1 and anti-LFA-1 mAb (Table III). No inhibition of syngeneic adhesion was seen with anti-HLA-DR.

Biochemical Characterization of IFN-γ-Induced ICAM-1 from EK. ICAM-1 immunoprecipitated from IFN-γ-treated EK had a mean M_r of 100,000 with a range from 88,000 to 113,000. ICAM-1 immunoprecipitated from the same number of untreated EK could not be detected (Fig. 4, lanes *2* and *4*). A higher quantity of HLA-A,B was also seen with IFN-γ treatment (Fig. 4, lanes *3* and *5*). ICAM-1 immunoprecipitated from JY cells in parallel has a slightly lower M_r of 92,000 (Fig. 4, lane *6*).

Binding of T Lymphoblasts to ICAM-1 in Planar Membranes. It has been demonstrated (25) that ICAM-1 from a B lymphoblastoid cell line has ligand activity for the LFA-1-dependent lymphocyte adhesion pathway. To extend this result to ICAM-1 isolated from an epithelial source, ICAM-1 was immunoaffinity purified from HeLa cells (Fig. 4, lane *1*) and reconstituted into glass-supported planar lipid membranes. As a control, human glycophorin was also reconstituted into planar membranes. T lymphoblasts efficiently bound to ICAM-1-bearing

TABLE III

Allogeneic and Syngeneic T Lymphoblast/EK Adhesion

T cell type	mAb	T cells bound			
		EK type A		EK type B	
		Untreated	IFN-γ	Untreated	IFN-γ
		% ± SD			
A	IgG control	4.6 ± 1.0	34 ±1.8	8.4 ± 2.0	35 ±2.9
	Anti-ICAM-1	3.3 ± 0.8	4.5 ±1.2*	8.1 ± 1.9	9.8 ±1.1*
	Anti-LFA-1	1.9 ± 0.7	5.7 ±0.7*	7.6 ± 1.7	12 ±2.2*
	Anti-HLA-DR	2.4 ± 0.5	31 ±3.8	9.0 ± 3.1	35 ±3.6
C	IgG control	4.8 ± 0.6	29 ±1.4	7.1 ± 2.5	31 ±2.5
	Anti-ICAM-1	4.1 ± 0.8	5.0 ±0.3*	6.8 ± 1.6	10 ±1.3*
	Anti-LFA-1	4.1 ± 1.5	6.1 ±1.4*	7.0 ± 2.3	11 ±1.7*
	Anti-HLA-DR	4.2 ± 1.1	38 ±2.1	8.1 ± 0.9	33 ±0.8

EK from two donors (A, adult forearm; B, foreskin) were grown to 70% confluence in 96-well plates and treated for 24 h with medium (untreated) or 500 U/ml rIFN-γ. T lymphoblasts from donor A (day 7) and an unrelated donor C (day 8) were labeled with chromium and monolayer adhesion assays were done. mAbs were present during the adhesion assay at between 5 and 25 µg/ml.
* Significantly different than IgG control for same treatment category. In the presence of anti-LFA-1 and anti-ICAM-1, all binding was to areas between cells by visual inspection.

planar membranes but not to glycophorin in planar membranes (Table IV). When ICAM-1 and glycophorin were mixed in an equimolar ratio before reconstitution, T lymphoblast adhesion was as efficient as to ICAM-1 alone. T lymphoblasts adhering to ICAM-1 in planar membranes were spread and some cells were highly elongated (not shown). This adhesion was blocked completely by anti-ICAM-1 mAb binding to the planar membrane or anti-LFA-1 mAb binding to T lymphoblasts (Table IV). Furthermore, the LFA-1-dependent adhesion was blocked by EDTA in excess of divalent cations and no adhesion occurred at 4°C. These results demonstrate that epithelial cell ICAM-1 can mediate T lymphocyte adhesion in the absence of other epithelial cell membrane components.

Isolation and Characterization of Adherent T Lymphoblasts. The highest level of T lymphoblast binding obtained for IFN-γ/TNF-treated keratinocytes or ICAM-1 in planar membranes was 50% (range, 20–50%). This percentage of binding could represent a stochastic process (i.e., equilibrium between attachment and detachment) acting on a population of cells with uniform binding capacity, or heterogeneity within the T lymphoblast population containing cells having different ligand density requirements for stable adhesion. To address this question, the surface phenotype of adherent T lymphoblasts was determined by immunofluorescence flow cytometry after panning on ICAM-1 in planar membranes supported by 12-mm glass cover slips or on IFN-γ-treated EK monolayers (Cavander, D. E., D. O. Haskard, D. Maliakkal, and M. Ziff, submitted for publication). Nonadherent cells were removed by six rinses with warm media and adherent cells were recovered by addition of HBSS containing 2.5 mM EDTA, which rapidly released the adherent blasts without damaging membranes or EK
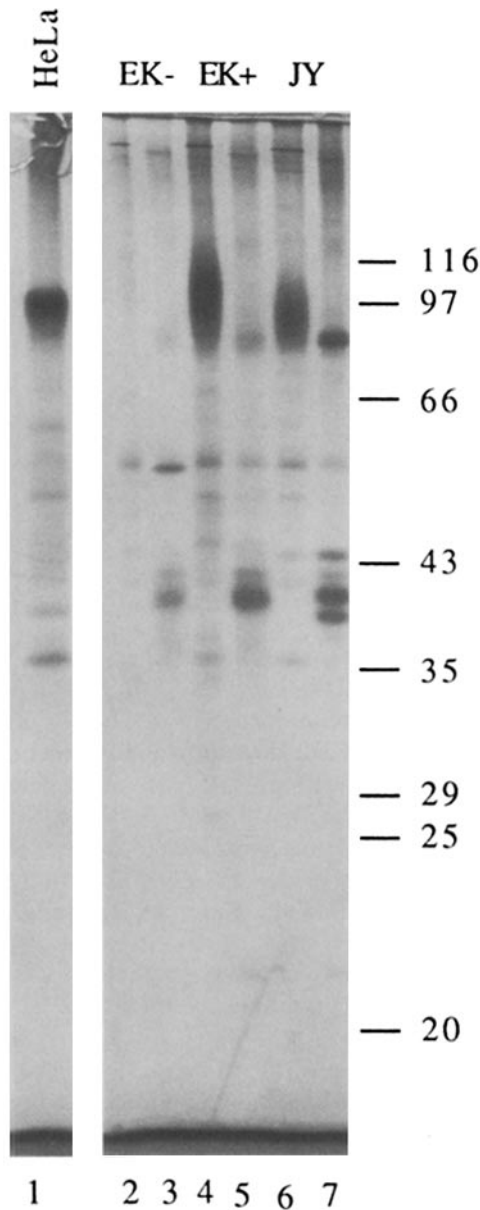
FIGURE 4. SDS-10%-PAGE of ICAM-1 from HeLa and EK. HeLa ICAM-1 was purified and 100 ng was loaded (lane *1*). Keratinocytes were grown to confluence in 75-cm^2 flasks and treated with medium (lanes *2* and *3*) or 1,000 U/ml IFN-γ (lanes *4* and *5*). Lysis buffer was added directly to the flasks and 4×10^7 JY cells were lysed in parallel. Immunoprecipitates were done using ICAM-1 mAb (lanes *2,4*, and *6*) or HLA-A,B mAb (lanes *3,5*, and *7*) on half of each lysate.

monolayers. Approximately half of the cells visibly adherent to EK monolayers were not accessible to the EDTA wash, apparently due to their intercalation between the EK. Aliquots of unseparated cells and adherent cell fractions were analyzed. Unseparated Con A blasts from several donors were similar in phenotype: 100% CD2$^+$, 100% CD3$^+$, 33% CD8$^+$, 65% CD4$^+$, and 20–30% Mac-1$^+$ (CD11c). T lymphoblasts bound to ICAM-1 planar membranes were a sub-

TABLE IV

Binding of T Lymphoblasts to HeLa ICAM-1 in Glass-Supported Planar Membranes

Pretreatments		Assay	T cells bound		
			Membrane composition		
T blasts	Membrane		ICAM-1	Glycophorin	Both
			% ± SD		
None	None	IgG1 control	26 ±3.1	0.4 ±0.2	25 ± 1.6
None	Anti-ICAM-1	IgG1 control	0.1 ±0.1*	0.3 ±0.2	—
None	Anti-LFA-1	IgG1 control	22 ±0.9	0.3 ±0.3	—
Anti-ICAM-1	None	IgG1 control	28 ±2.6	0.3 ±0.1	—
Anti-LFA-1	None	IgG1 control	0.3 ±0.02*	0.4 ±0.3	—
None	None	EDTA	0.2 ±0.3*	0.3 ±0.4	—
None	None	4°C	0.1 ±0.1*	0.05 ±0.1	—

Binding of normal T lymphoblasts to ICAM-1 and/or glycophorin-bearing planar membranes was measured. Data presented as percent cells bound ± SD of quadruplicate determinations.
* Significantly different than value in first row.

population enriched for larger cells by forward-angle light scatter with higher expression of LFA-1 (2.4-fold) and CD2 (2.1-fold) (Fig. 5). Less enrichment in linear fluorescence was seen for CD4 (1.4-fold), CD8 (1.2-fold), CD3 (1.1-fold) and IL-2-R (Tac) (1-fold) expression, showing the enrichment for higher surface expression was selective for some markers. The enrichment for both LFA-1 and CD2 cell adhesion glycoproteins is notable since CD2 does not appear to be directly involved in adhesion to ICAM-1 in planar membranes (not shown and Dustin, M. L. and T. A. Springer, submitted for publication). The moderate enrichment for expression of CD8 by ICAM-1 planar membrane–bound CD8+ T lymphoblasts contrasted with the substantial enrichment for CD8+ cells (73% positive) relative to CD4+ cells (27% positive). Similarly, T lymphoblasts bound to IFN-γ-treated EK monolayers were enriched for larger cells and expressed
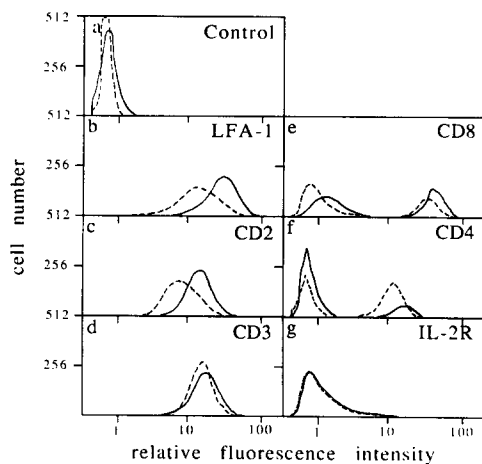

FIGURE 5. Surface phenotype of T lymphoblasts adherent to ICAM-1 in artificial membranes. Glass-supported planar membranes with ICAM-1 were made as described in Materials and Methods on 12-mm coverslips in 24-well plates. Bound T cells (10% of input) were recovered and analyzed as in Materials and Methods.

higher quantities of several surface markers (not shown). Under conditions where 25% of input T lymphocytes were bound to EK monolayers, CD2, CD8, and CD4 showed 1.1–1.2-fold higher expression, while LFA-1 showed 1.5-fold higher expression. The adherent population was enriched for CD8$^+$ cells (45 vs. 33% positive) and depleted of CD4$^+$ cells (38 vs. 66% positive). The adherent cells were also enriched for Mac-1 (40 vs. 20% positive) which is expressed on some CD8$^+$ T lymphocytes and large granular lymphocytes (not shown).

Discussion

We have studied the role of the LFA-1 ligand, ICAM-1, in lymphocyte adhesion to EK. ICAM-1 expression on cultured EK was dramatically upregulated by treatment of keratinocytes with IFN-γ and more weakly upregulated by TNF. T lymphoblasts failed to adhere to untreated EK monolayers, but were strongly adherent to IFN-γ-treated monolayers. Adherence and ICAM-1 expression showed similar dependence on IFN-γ concentration. mAb-blocking studies showed that adhesion was dependent on LFA-1 on the T lymphoblast and ICAM-1 on the EK. Studies on ICAM-1 purified from an epithelioid cell line, HeLa, and reconstituted in an artificial lipid membrane showed that it is a ligand for LFA-1.

Comparison of results on EK cells to studies on dermal fibroblasts and endothelial cells show that the effect of immunomodulatory agents on ICAM-1 expression is highly dependent on cell lineage. Human dermal fibroblast ICAM-1 is strongly upregulated not only by IFN-γ, but also by IL-1, TNF, and LPS. The latter agents have little or no effect on EK ICAM-1. On endothelial cells, the effects of these mediators is reversed with respect to EK. Endothelial cell ICAM-1 is strongly upregulated by TNF, LPS, and IL-1, and only weakly by IFN-γ (29, 44a). The polarity between endothelial and EK ICAM-1 regulation by cytokines is intriguing since these cells form the closest and farthest, respectively, tissue boundaries to lymphocytes migrating from the blood. Fibroblasts, which upregulate ICAM-1 in response to the widest range of mediators and are intermediate between endothelial cells and EK in sensitivity, are also intermediate in their distance from blood lymphocytes. The sensitivity of EK to lymphocyte-activation products, as opposed to the greater sensitivity of endothelial cells to monocyte and bacterial products, may influence the nature of the inflammatory response to these stimuli. These results are consistent with parallel observations that ICAM-1 is induced on EK in vivo in delayed-type hypersensitivity responses, but not in croton oil–induced inflammation; both cases are accompanied by mononuclear cell infiltrates, but activation of T lymphocytes would only occur in the delayed-type hypersensitivity reaction (Wantzin, G. L., E. Ralfkiaer, C. Avnstorp, M. Czajkowski, S. D. Marlin, and R. Rothlein, manuscript in preparation).

While ICAM-1 appears to be critical for lymphocyte adhesion to EK, ICAM-1 expression cannot clearly explain the initial invasion of the epidermis by T lymphocytes or T lymphoma cells since the EK ICAM-1 is separated from extravasated T lymphocytes in the dermis by a highly organized and continuous extracellular matrix, the basement membrane. It is interesting that T lymphoblasts

adhere to surfaces on which EK have been cultured. This adhesion was not blocked by anti-LFA-1 mAb. These receptors also appear to be distinct from the VLA family of heterodimers since a function blocking rabbit antisera to the common β subunit of these molecules did not inhibit adhesion to the EK matrix. These observations suggest that T lymphocytes may express uncharacterized receptors for EK basement membrane components.

The interaction between LFA-1 and ICAM-1 was the only detectable pathway of T lymphoblast adhesion to EK. In other experimental systems, additional LFA-1-dependent, ICAM-1-independent as well as LFA-1-independent pathways have been found. ICAM-1 mAbs do not inhibit phorbol ester–stimulated homotypic aggregation of the SKW3 T lymphoma cell line which is inhibited by LFA-1 mAbs (28), suggesting the existence of LFA-1 ligands distinct from ICAM-1. Similarly, cytolytic T lymphocyte adhesion to some target cells is inhibited by LFA-1 mAbs, but not ICAM-1 mAbs, while ICAM-1 mAb is a potent inhibitor of cytolytic T lymphocyte adhesion to and killing of, for example, the U937 histiocytic leukemia cell line (47). Adhesion of T lymphocytes to cultured endothelial cells involves at least three pathways based on functional experiments with LFA-1 and ICAM-1 mAb (44a); one is LFA-1 and ICAM-1 dependent, the second is LFA-1 dependent and ICAM-1 independent, and a third is LFA-1 independent. While endothelial cells express at least two LFA-1 ligands, only the ICAM-1 dependent pathway is upregulated (44a) by cytokines or LPS. Therefore, EK appear to express only a single pathway in contrast to dermal fibroblasts and endothelial cells with at least two and three pathways respectively.

In addition to adhesion mediated by LFA-1 and ICAM-1, another pathway for T lymphocyte adhesion is mediated by the interaction of CD2 and LFA-3 (20). This pathway was initially defined for interaction of cytolytic T lymphocytes with target cells but subsequently has been shown to mediate interaction of thymocytes and thymic epithelial cells (48). Unlike thymic epithelial cells, EK did not adhere to thymocytes by the CD2/LFA-3 pathway although EK do express LFA-3 (48). Therefore, ICAM-1 appears to be the only adhesion pathway used in lymphocyte–EK binding.

ICAM-1 purified from B lymphoblastoid cells (41), endothelial cells (44a), and epithelial cells mediates efficient, LFA-1-dependent lymphocyte adhesion when reconstituted into planar membranes. Adhesion to epithelial cell ICAM-1 has all of the properties of LFA-1-dependent adhesion to other cells including temperature sensitivity and a requirement for divalent cations. Furthermore, T lymphoblasts adhering to ICAM-1 from all three sources exhibit spreading, which is also seen when T lymphoblasts adhere to cell monolayers and is associated with a high degree of motility and crawling between cells (49). Therefore, LFA-1/ICAM-1-dependent adhesion may be important for T lymphocyte motility in the dermis (fibroblast ICAM-1) and the epidermis (EK ICAM-1) when ICAM-1 expression is increased by immunological or other stimuli.

Our results indicate that polyclonal T lymphoblasts display heterogeneity in their ability to bind IFN-γ-treated EK or ICAM-1 in planar membranes. This heterogeneity is reflected in the expression of particular antigens that are known to be involved in cell-to-cell interactions, LFA-1, CD2, CD4, CD8, Mac-1, and HLA-A,B. Adherent T lymphoblasts were enriched for cells expressing high lev-

els of these antigens, particularly LFA-1 and CD2, while expression of CD3 and CD25 epitopes was similar between the adherent and initial populations. The adherent subpopulation was enriched for cells expressing CD8, but contained a significant number of CD4$^+$ cells. An attractive hypothesis is that cells with higher LFA-1 density are more adherent to surfaces expressing LFA-1 ligands and that some other markers are coordinately expressed, although the state of lymphocyte activation may also be important (50, 51). Coordinated expression of LFA-1, CD2, and LFA-3 has been shown on resting peripheral blood T lymphocytes; and lymphocytes with immunological memory have been distinguished from naive lymphocytes by expression of LFA-3 and by higher expression of LFA-1 and CD2 (52).

It has been suggested that MHC class II antigen expression by EK may have a significant immunological role in allograft rejection (4, 10, 11). While expression of MHC class II antigens may enable EK to present antigens to helper T lymphocytes they do not appear to be required for adhesion of polyclonal T lymphoblasts to IFN-γ-stimulated EK in allogeneic or syngeneic systems. However, the cooexpression of ICAM-1 and HLA-DR could facilitate adhesion and subsequent MHC class II antigen–restricted antigen presentation to T lymphocytes. The role of LFA-1/ICAM-1 interaction in immunological function of IFN-γ-treated normal EK and other epithelial cell types requires further study in light of the finding that IFN-γ increases expression of ICAM-1 on EK. EK may require prior exposure to IFN-γ to act as efficient targets for cytolytic T lymphocytes or as APC.

Summary

The cell surface expression and function of the LFA-1 ligand, intercellular adhesion molecule 1 (ICAM-1), on epidermal keratinocytes (EK) was studied. ICAM-1 expression on the surface of cultured EK was either absent or weak, but was induced by treating EK with rIFN-γ or TNF for 4–48 h. IFN-γ and TNF were synergistic. IFN-γ treatment increased T lymphoblast adhesion from < 2% to 20–40%, with a concentration dependence similar to that seen for ICAM-1 induction. All of the adhesion to EK was inhibited by LFA-1 and ICAM-1 mAbs, but not by HLA-DR, CD2, or LFA-3 mAbs. There was no difference in the level of T lymphoblast adhesion to IFN-γ-treated allogeneic or autologous EK. ICAM-1 purified from the HeLa epithelioid cell line and reconstituted into planar membranes also supported efficient adhesion of T lymphoblasts that was blocked by LFA-1 mAb bound to the T lymphoblasts or ICAM-1 mAb bound to the planar membranes. T lymphoblasts adherent to EK or ICAM-1 planar membranes were isolated by panning, and surface markers were analyzed by immunofluorescence flow cytometry. The adherent T cells were a phenotypically skewed subpopulation. They were enriched for CD8$^+$ cells and expressed 1.5–2.5-fold higher LFA-1 and CD2 compared with the unseparated population.

Note added in proof: Nickoloff et al. (53) have recently reported that IFN-γ treatment of cultured keratinocytes enhances their binding of lymphocytes.

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