Adhesion molecule expression on skin endothelia in atopic dermatitis: Effects of TNF-α and IL-4

I. Jolanda M. de Vries, PhD,[a] Elisabeth G. Langeveld-Wildschut, MD, PhD,[a] Frank C. van Reijsen, PhD,[a] Gerald R. Dubois, PhD,[a] J. Annet van den Hoek, MSc,[a] Ilse C. Bihari, MSc,[a] Dick van Wichen, MSc,[b] Roel A. de Weger, PhD,[b] Edward F. Knol, PhD,[a] Theo Thepen, PhD,[a] and Carla A. F. M. Bruijnzeel-Koomen, MD, PhD[a] *Utrecht, The Netherlands*

Background: Atopic dermatitis (AD) is characterized by skin infiltrates of leukocytes, such as lymphocytes and eosinophils.
Objective: To describe the mechanisms determining this inflammatory process, we have analyzed expression of adhesion molecules and their regulation on skin endothelial cells (ECs).
Methods: Expression of adhesion molecules on ECs was analyzed by immunohistochemistry by using *Ulex europaeus* agglutin 1 as a pan-endothelial marker.
Results: Vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin were not found in skin of nonatopic individuals, whereas expression of these surface molecules was observed in nonlesional skin of patients with AD and was even more pronounced in lesional skin or after epicutaneous application of aeroallergen. Induction of adhesion molecule expression was examined on both macrovascular ECs from human umbilical cord vein (HUVECs) and human microvascular ECs (HMEC-1) from skin. TNF-α very potently upregulated adhesion molecule expression in vitro on both EC cell types. To verify the in vivo relevance of TNF-α, we performed TNF-α staining in the skin. TNF-α was observed in the dermis of nonatopic skin, both in chymase-containing mast cells and CD68$^+$ macrophages. The increase in the number of TNF-α–containing cells was concomitant with the increase in adhesion molecule expression in the skin of patients with AD. IL-4 is supposed to be important in atopic diseases because of its IgE- and VCAM-1–inducing properties. However, IL-4 addition failed to induce VCAM-1 expression on HMEC-1, although in the same set of experiments, a clear induction of VCAM-1 expression by IL-4 on HUVECs was demonstrated. Flow cytometry revealed the absence of IL-4 receptor α-chains on HMEC-1 and their presence on HUVECs. Immunohistochemistry examination on skin sections showed no binding of the IL-4R α-chain antibodies to ECs.
Conclusion: We conclude that adhesion molecule expression is increased in the skin of patients with AD. Most probably, this increased expression is not a (direct) effect of IL-4 on skin endothelium, but other cytokines, such as TNF-α, might be responsible for this increased adhesion molecule expression. Continuous adhesion molecule expression may facilitate T-cell extravasation in a nonantigen–specific manner, thus explaining the presence of increased T-cell numbers in nonlesional skin of patients with AD. (J Allergy Clin Immunol 1998;102:461-8.)

Key words: *Adhesion molecules, atopic dermatitis, allergic inflammation, atopy patch test, microvascular endothelium, IL-4 receptor, TNF-α, endothelium, skin microvasculature*

The mechanisms involved in extravasation of T cells are not fully understood. During immune surveillance under normal circumstances, this is considered to be a highly specific mechanism involving homing receptors, which direct T cells to their original site of antigenic stimulation or associated lymphoid tissues.[1] Cutaneous lymphocyte-associated antigen (eg, the skin homing receptor) binds its ligand E-selectin on endothelial cells (ECs) during the initial step of the adhesion cascade.[2] This cascade is a multistep process in which the first step is mediated by adhesion molecules of the selectin family. Members of this family are L-selectin, present on almost all circulating leukocytes, and E- and P-selectin, which are expressed on ECs on stimulation. Selectins interact with carbohydrate ligands and mediate rolling and tethering of leukocytes on ECs. In the second step additional signals result in firm attachment and subsequent transendothelial migration involving immunoglobulin gene superfamily molecules and their β2-integrin ligands. Immunoglobulin gene superfamily adhesion receptors on ECs, such as intercellular cell adhesion molecule (ICAM)-1, ICAM-2, or ICAM-3 and vascular cell adhesion molecule (VCAM)-1, bind their integrin ligands Mac-1, lymphocyte function–associated antigen-1, and very late antigen-4. Integrin-mediated adhesion can be rapidly upregulated by activation of cells on which they are expressed.[1] In the third step, when cells are actually attracted to the site of inflammation, the presence of chemoattractants is required.[1]

Atopic dermatitis (AD), a chronic inflammatory skin disorder, is characterized by a large influx of cells, consisting mainly of activated cells, memory cells, helper T cells, dendritic cells, and macrophages.[3] The atopy patch test (APT) provides a model to study the inflammatory response in patients with AD.[4] APT-induced eczematous skin lesions are characterized by a dermal influx of T cells, eosinophils, dendritic cells, and macrophages.[5]

From the Departments of [a]Dermatology-Allergology and [b]Pathology, University Hospital Utrecht, Utrecht.
Supported by the Dutch Asthma Foundation, grant 32.91.55.
Received for publication Apr 8, 1997; revised May 8, 1998; accepted for publication May 12, 1998.
Reprint requests: Edward F. Knol, PhD, Department of Dermatology-Allergology G02.124, University Hospital Utrecht, P.O. Box 85500, NL-3508 GA Utrecht, The Netherlands.

0091-6749/98 $5.00 + 0 **1/1/91754**

Abbreviations used

AD: Atopic dermatitis
APT: Atopy patch test
EC: Endothelial cell
HMEC-1: Human microvascular endothelial cell-1
HUVEC: Human umbilical cord vein endothelial cell
ICAM: Intercellular adhesion molecule
IL-4R: Interleukin-4 receptor
NHuS: Normal human serum
PECAM-1: Platelet endothelial cell adhesion molecule-1
TRITC: Tetraethyl-rhodamine-isothiocyanate
VCAM-1: Vascular cell adhesion molecule-1

TABLE I. Characteristics of mAbs used

CD designation	Specificity	Source	Dilution
CD31	PECAM-1	Immunotech	1:25
CD54	ICAM-1	Immunotech	1:100
CD58	LFA-3	Immunotech	1:25
CD62E	E-selectin	Genzyme	1:100
CD62P	P-selectin	Immunotech	1:25
CD106	VCAM-1	Genzyme	1:100
	HLA-DR/DQ/DP	Serotech	1:100
CD124	IL-4R	Genzyme	1:25

LFA, Lymphocyte function–associated antigen.

Allergen-specific $CD4^+$ T-cell clones have been cultured from APT sites and lesional skin.[6,7] Furthermore, the cytokine pattern of 24-hour APT lesions is similar to that of acute AD lesions (increase in IL-4–producing T cells), whereas 48-hour APT lesions resemble chronic AD lesions (increase in predominantly IFN-γ–producing cells).[8,9] Therefore the APT appears to be an appropriate model to study the role of adhesion molecules in cutaneous T-cell infiltration.

In this article the expression of adhesion molecules on ECs, identified by double staining with Ulex europaeus,[10] was studied. Therefore biopsy specimens were taken from both nonlesional and lesional skin of patients with AD, skin from APT sites, and skin of nonatopic control subjects. This in vivo expression was compared with cytokine-induced expression of adhesion molecules on cultured EC lines. Isolation, purification, and propagation of human dermal microvascular ECs have proven difficult. An immortalized human microvascular EC line (HMEC-1) was established by Ades et al.[11] HMEC-1 has morphologic similarities with human dermal microvascular ECs and expresses characteristic microvascular EC markers, such as EN4, PAL-E, H3/5-47, and CD34.[11] In this study we compared HMEC-1 with macrovascular ECs (human umbilical cord vein ECs [HUVECs]) regarding cell adhesion molecule expression after stimulation with cytokines. Moreover, expression of the α-chain of the IL-4 receptor was determined. Combined with microscopic localization of cytokine protein expression in skin sections of patients with AD and nonatopic control subjects, the data may provide a better understanding of the relationship between cytokine production and adhesion molecule expression.

METHODS

Isolation and culture of HUVECs

HUVECs[12] were seeded in tissue culture flasks coated with human fibronectin (4 μg/cm^2; Biomedical Technologies Inc, Stoughton, Mass). HUVECs were grown in Medium 199 enriched with sodium heparin (90 μg/mL; Leo Pharmaceutical Products, Ballerup, Denmark), basic fibroblast growth factor (0.5 ng/mL; Sigma, St Louis, Mo), and 20% normal human serum (NHuS) (Red Cross blood laboratory, Utrecht, The Netherlands). Confluent primary cultures were trypsinized and seeded in a 1:3 split ratio, and further propagation was performed as described by Moser et al.[13] Confluent monolayers were used in their second to fourth passage.

Culture of the EC line HMEC-1

HMEC-1 (a generous gift of Dr Edwin W. Ades and Dr Thomas Y. Lawley, Emory University, Atlanta, Ga) is an immortalized human microvascular EC line derived from foreskin that retains morphologic, phenotypic, and functional characteristics of normal HMECs.[11] HMEC-1 cells were grown at 37°C in 5% CO_2 in a growth medium consisting of the following components: endothelial basal medium (MCDB 131; Clonetics, San Diego, Calif), 10 ng/mL epidermal growth factor (Sigma), 1 μg/mL hydrocortisone (Sigma), and 10% FCS (Gibco, Grand Island, NY).[11]

Stimulation of HMEC-1 cells and HUVECs with cytokines

EC monolayers were grown in 6-well plates precoated with fibronectin and stimulated with cytokines for 4, 24, 48, and 72 hours. Cytokines used for stimulation were IFN-γ (50 ng/mL; a kind gift of Dr Peter H. van der Meijde, BPRC, Rijswijk, The Netherlands), TNF-α (10 ng/mL; Pepro Tech Inc, Rocky Hill, NJ), IL-4 (10 U/mL; Novartis, Vienna, Austria), and LPS (1 μg/mL; Sigma). Optimum doses of cytokines were determined by titration.

Surface marker analysis HUVEC/HMEC-1

Surface marker analyses were performed by indirect immunofluorescence with flow cytometric assessment. To recover EC lines for flow cytometry, cells were washed twice with PBS and incubated for 30 minutes with PBS, 5 mmol/L EDTA, and 1% BSA (Boehringer Mannheim, Germany) at 37°C. Cells were recovered from plates and washed once with PBS. Cells (50 μL at 0.5 to 1 × 10^6/mL) were incubated for 30 minutes at 4°C with either unconjugated specific first mAb (Table I) diluted in PBS supplemented with FCS (2% vol/vol) and sodium azide (5 mmol/L) (buffer A) or control mAb of the appropriate isotype class but of irrelevant antigen specificity. Cells were then washed twice with buffer A before incubation with a secondary FITC-conjugated rabbit anti-mouse immunoglobulin (1:40 Dako; Dako A/S, Glostrup, Denmark) in buffer A for 30 minutes at 4°C. After immunofluorescence labeling, cells were washed twice in buffer A and then fixed with 2% paraformaldehyde. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif).

Patients and test procedures

AD. We studied 16 patients with AD diagnosed according to the criteria of Hanifin and Rajka,[14] who were selected on the demonstration of a positive APT response at 24 hours, a positive skin prick test response (defined as >3 mm induration 20 minutes after intradermal allergen challenge), and the presence of serum IgE specific

for house dust mite allergen. APTs were performed as described previously.[4] Punch biopsy specimens (3 mm) were taken under local anaesthesia (1% lidocaine) from nonlesional skin (n = 8), lesional skin (n = 5), and skin from APT sites at 24 hours (n = 4).

Nonatopic control subjects. Control skin biopsy specimens were taken from healthy nonatopic volunteers (n = 3) with negative skin prick test responses for series of inhalant allergens.

All biopsy specimens were snap frozen in liquid nitrogen and stored at –70°C before use. Six-micrometer sequential sections were cut on a freezing microtome and mounted on 3-aminopropyl tri-ethoxy silane (Sigma)–coated slides. All participants in this study gave their informed consent.

Immunohistochemistry

To determine adhesion molecule expression on ECs in vivo, sections were double-stained with biotinylated *Ulex Europaeus* agglutinin 1 (1:200; Sigma) as a pan-endothelial cell marker[10] and mAbs against adhesion molecules listed in Table I, as described.[15]

To determine TNF-α expression in vivo, sections were single-stained with anti-TNF-α (Medgenix, Fleuris, Belgium) according to the method of Thepen et al.[9] Absorption of anti-TNF-α to recombinant TNF-α coupled to magnetic beads, omission of the primary antibody, and replacement of the primary antibody by isotype-matched irrelevant antibodies served as negative controls.

Chymase, an enzyme present in 80% to 90% of mast cells in skin,[16] was stained enzymatically. Chymase is a mast cell–specific serine protease with chymotrypsin-like activity. After staining of skin sections with anti-TNF-α as described above, chymotrypsin-like activity was developed by using the following reaction mixture: 0.53 mmol/L naphthol-AS-D-chloroacetate (Fluka Chemie AG, Buchs, Switzerland), 0.3 mmol/L fast blue BB salt (Sigma), 40 mmol/L NaF (BDH Chemicals Ltd, Poole, England), and 1% Triton X-100 (Fluka Chemie AG) dissolved in 50 mmol/L Tris-HCl (pH 6.8). Sections were incubated for 30 minutes at 37°C with this reaction mixture, resulting in blue staining. Sections were washed in aqua dest and embedded in gelatin. With this method, TNF-α–positive cells stain pink/red, and chymase-containing cells stain blue. TNF-α–positive mast cells appear as purple cells.

The phenotype of TNF-α dimly positive cells in the skin sections was analyzed with the T-cell marker CD3 (Leu-4, 1:50; Becton Dickinson Immunocytometry Systems, San Jose, Calif) and the macrophage marker CD68 (KP1, 1:10; Dako). Frozen sections were fixed in 100% acetone for 10 minutes at room temperature. Sections were rinsed in PBS supplemented with Tween (0.05% vol/vol) for 10 minutes followed by overnight incubation (4°C) with anti-TNF-α (1:160; Medgenix) diluted in PBS supplemented with human serum albumin (1% vol/vol; Behring Werke, Marburg, Germany). Sections were washed 3 times for 5 minutes in TNT buffer (0.1 mol/L Tris, 0.15 mol/L NaCl, and 0.05% Tween) and incubated with a PO-labeled rabbit anti-mouse (1:200; Dako) diluted in TNT buffer supplemented with NHuS (10% vol/vol) for 30 minutes. After washing in TNT buffer (3 × 5 minutes), an amplification with biotinyl-Tyramide (NEL 700, indirect Tyramide kit; Dupont NENTM Lifescience Product, Boston, Mass) diluted 1:50 in amplification buffer (1:1 diluted in H_2O) was performed for 8 minutes and the sections were again washed in TNT buffer (3 × 5 minutes). The sections were then incubated with Streptavidin Texas Red (1:250 in TNT buffer; Dupont, NEL 721) for 30 minutes followed by washing in TNT buffer (3 × 5 minutes). PO reactivity was inactivated by 1% H_2O_2 in PBS (25 minutes). After washing in TNT buffer (3 × 5 minutes), sections were then incubated (20 minutes) with 10% normal mouse serum (Dako) in PBS to block final reactivity of the second-step anti-mouse antibody.

Finally, FITC-labeled mouse CD3 or CD68 was applied to the sections and diluted (CD3 1:100; CD68 1:600) in PBS/1% human serum albumin for 1 hour. After washing in TNT buffer, sections were incubated with PO-labeled rabbit-anti-FITC for 30 minutes and again washed with TNT buffer. An amplification with Tyramide-FITC (NEL 701, direct Tyramide kit, Dupont) was performed, and sections were diluted 1:50 in amplification buffer (1:1 diluted in H_2O) for 8 minutes and again washed in TNT buffer (3 × 5 minutes). After embedding with Vectashield (Brunschwig H1000, Burlingame, Calif), sections were analyzed with an MRC 1000 confocal laser scanning microscope (BioRad, Hemel Hempstead, UK).

To determine IL-4R α-chain expression on ECs in vivo, sections were double-stained with tetraethyl-rhodamine-isothiocyanate (TRITC)-labeled Ulex europaeus agglutinin 1[10] (1:200; Sigma) and anti-IL-4R α-chain mAb (1:25; Genzyme Diagnostics, Cambridge, Mass). After fixation with dry acetone with H_2O_2 (30%, 100 μL/100 mL, 7 min), sections were air-dried and preincubated for 20 minutes in 10% NHuS, after which they were incubated with anti-IL-4R α-chain (1:25) overnight at 4°C in 1% NHuS. Sections were incubated with Alkaline phoshatase-conjugated rabbit-anti-mouse (1:50) (Dako) in 1% NHuS. In between incubations, sections were washed with PBS/Tween (3 × 5 minutes). AP activity was demonstrated by using naphthol AS-BI phosphate (sodium salt, 50 mg/100 mL; Sigma) as substrate and new fuchsin (10 mg/100 mL; Merck & Co, Inc, Whitehouse Station, NJ) as chromogen dissolved in 0.1 mol/L Tris-HCl (pH 8.5), which resulted in pink/red staining. Endogenous AP activity was inhibited by addition of levamisole (35 mg/100 mL, Sigma) to the reaction mixture. After washing with PBS, sections were incubated with Ulex TRITC for 45 minutes and were again washed in PBS (3 × 5 minutes). After embedding with Vectashield, sections were analyzed with a Zeiss (Germany) microscope. With this method, IL-4R α-chain positive cells stain pink/red as analyzed by light microscopy, and Ulex-positive cells are red as analyzed by immunofluorescence (546 nm).

In subsequent experiments IL-4R α-chain expression on ECs in vivo was also determined by double-staining with biotinylated Ulex Europaeus agglutin and anti-IL-4R α-chain as described above. Similar results on IL-4R α-chain expression as those obtained with TRITC-labeled Ulex were found.

Microscopic evaluation

Skin sections were examined by light microscopy at 400× magnification. Before examination of the double-staining, sections were compared with their control-stained countersections. For quantification of cell numbers and homing receptor expression, all double- and single-stained cells between the dermal-epidermal junction to the base of the hair follicles were counted independently by 2 observers. The mean interobserver coefficient of variation was 10%. In fields containing sweat ducts and hair shafts, only intervening dermal regions were counted. Of all biopsy specimens, two sections (1 to 1.5 mm^2 per section), containing at least 82 endothelial cells, were stained and counted. Results are expressed as percentages of double-stained ECs. Percentages were calculated per patient after which the mean was taken.

Statistical analysis

Statistical analysis was performed by using the Wilcoxon rank-sum test for nonparametric distributions. Probability *(P)* values of less than .05 were considered significant.

RESULTS

Adhesion molecules

Adhesion molecule expression was studied in vivo and in vitro by immunohistochemistry on skin sections (Fig 1) and indirect immunofluorescence detected by flow cytometry on an EC line from different origin, respectively.

In vivo adhesion molecule expression. Expression of E-selectin, P-selectin or VCAM-1 was not observed on

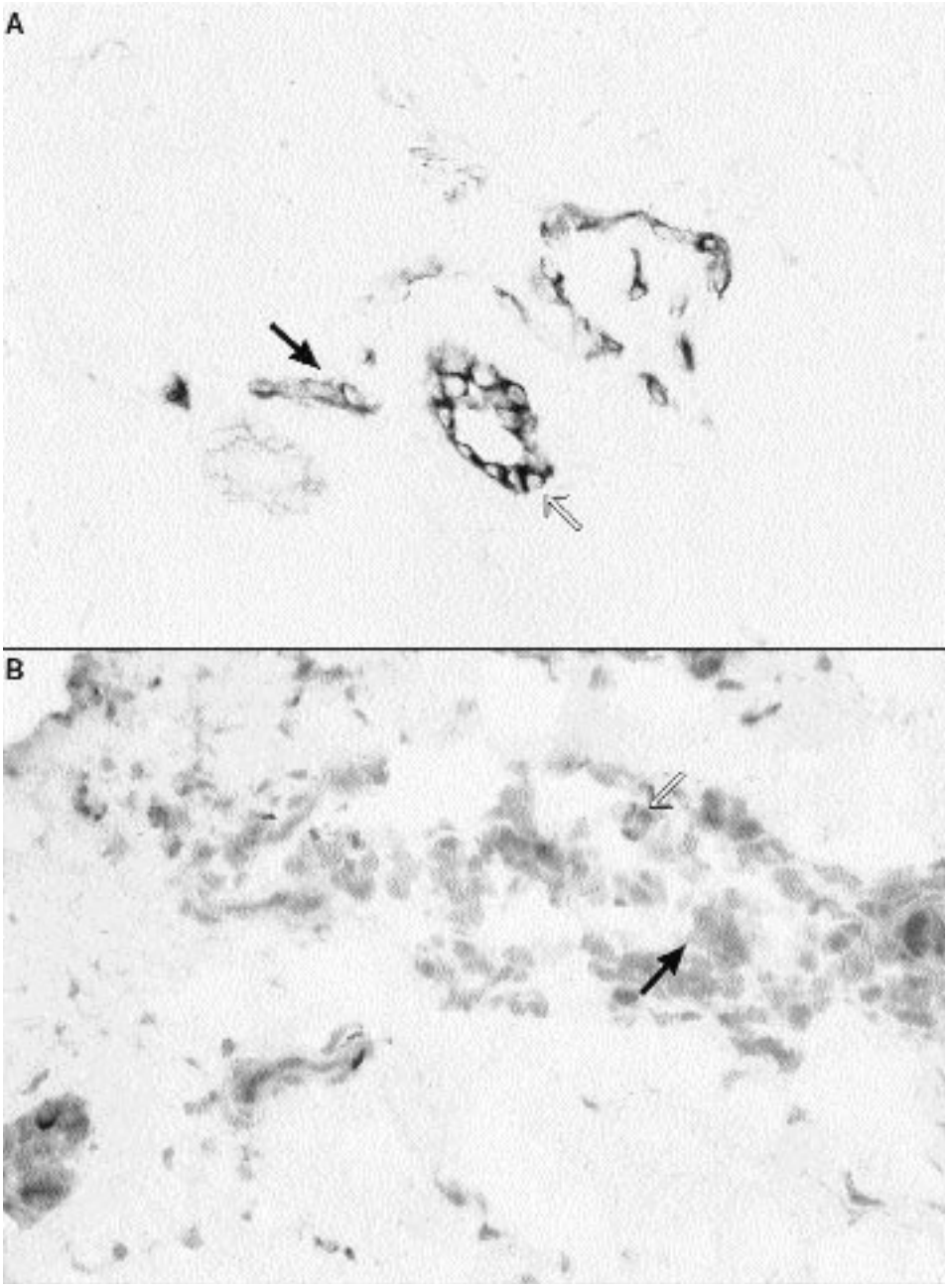

FIG 1. Immunohistochemical stainings on lesional skin of patients with AD. **A,** Double-staining with Ulex (red) and anti-P-selectin (blue). P-selectin–expressing ECs are indicated by purple staining. *Double arrows* indicate double-positive cell, and *single arrows* indicate single red positive cell. No single blue staining cells are seen. **B,** Single-staining with anti-TNF-α (pink/red). *Double arrows* indicate strong positive cell, and *single arrows* indicate dimly stained cell. Magnification 40×.

blood vessels in skin of nonatopic individuals. However, in nonlesional skin of patients with AD, expression of these adhesion molecules was noted (Table II). This expression was even further increased in lesional skin or after epicutaneous application of aeroallergen in the APTs. Although ICAM-1 was expressed in nonatopic skin, its expression further increased in the skin of patients with AD. Platelet endothelial adhesion molecule (PECAM)-1 expression was similar in all skin types.

In vitro adhesion molecule modulation. P-selectin was not demonstrated on cultured HUVECs and HMEC-1 under unstimulated or stimulated (LPS, TNF-α, IL-4, IFN-γ, or IL-1 for 20 minutes, 1 hour, and 2 hours) conditions (data not shown). Although PECAM-1 was demonstrated on both EC lines, there was no change in expression after in vitro stimulation with the above-mentioned stimuli (not shown). ICAM-1 expression was upregulated on the 2 EC lines by both TNF-α and IFN-γ

TABLE II. Percentages of adhesion molecule expressing ECs

	Nonatopic skin (n = 4)	Nonlesional skin (n = 8)	Lesional skin (n = 4)	24-hour APT sites (n = 4)
VCAM-1	0 ± 0*,a	8 ± 3a,b,c	24 ± 2c	26 ± 6b
E-selectin	0 ± 0d	21 ± 3d,e	49 ± 3e	35 ± 13
P-selectin	0 ± 0f	14 ± 2f,g	34 ± 4g	24 ± 5
PECAM-1	51 ± 3	45 ± 7	59 ± 6	44 ± 8
HLA-DR	38 ± 11	42 ± 5h,i	70 ± 2i,j	85 ± 1h,j
ICAM-1	16 ± 7k	47 ± 4k	62 ± 5	64 ± 5

Statistical analyses of corresponding letters (a, b, c, d, e, f, g, h, and i) resulted in significant differences ($P < .05$).
*Data represent mean of percentage per patient ± SEM.

TABLE III. TNF-α–expressing cells in skin

	TNF-α+/chymase+	TNF-αdim
Nonatopic skin (n = 4)	4 ± 2*†	18 ± 4
Nonlesional AD skin (n = 8)	16 ± 7†	49 ± 9
24-hour APT sites (n = 7)	7 ± 6	31 ± 6
Lesional skin (n = 7)	10 ± 3	42 ± 13

*Numbers represent the number of TNF-α–positive cells per mm^2 (mean ± SEM).
†Significant difference ($P < .05$).

(not shown). Although E-selectin was not expressed on both EC lines, it was induced on both lines with TNF-α or LPS. Increased E-selectin already occurred after 4 hours and lasted until 24 hours, after which it declined. After TNF-α stimulation, E-selectin was expressed on 98% of the HUVECs, but only on 26% of the HMEC-1. LPS was a more potent stimulus for the HMEC-1; 54% of the cells expressed E-selectin after LPS (1 μg/mL) stimulation for 4 hours. Neither IFN-γ nor IL-4 induced E-selectin expression on either EC line. Expression of VCAM-1 was induced on HUVECs by IL-4 and TNF-α (74% ± 7% and 94% ± 4%, respectively) (Fig 2). In contrast, only TNF-α induced VCAM-1 expression on HMEC-1 (67% ± 6%). Even very high concentrations of IL-4 (1000 U/mL) failed to induce VCAM-1 on HMEC-1 (data not shown). On both EC lines, VCAM-1 was found after 4 hours of TNF-α stimulation and reached peak levels at 24 hours. IFN-γ did not induce VCAM-1 on either EC line (not shown).

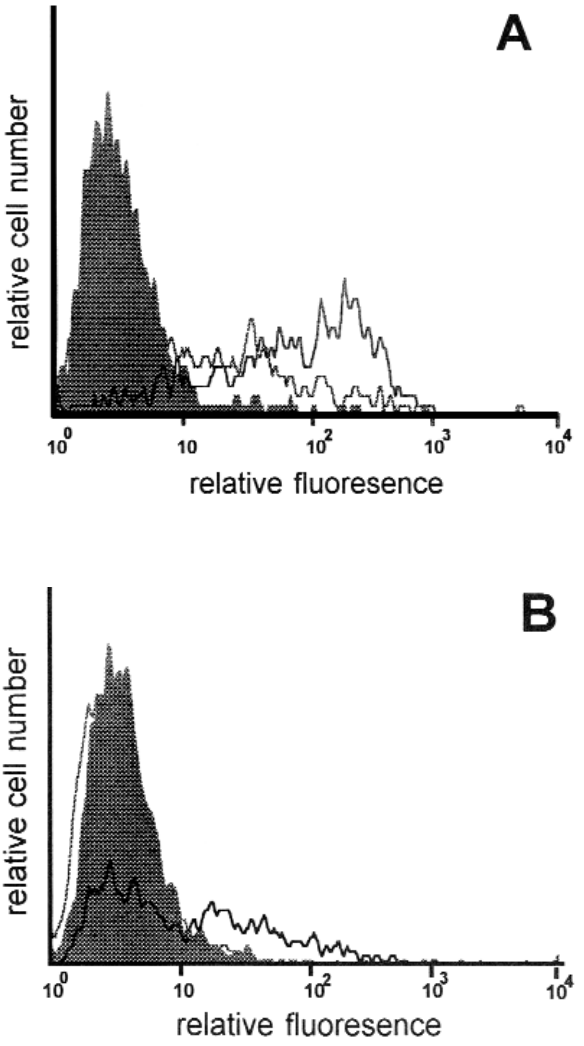

FIG 2. Induction of VCAM-1 on EC lines as examined by indirect immunofluorescence and flow cytometry. Expression, MFI, on HUVEC (**A**) and HMEC-1 (**B**) either on nonstimulated (basal level) or on cytokine-stimulated EC lines. Cytokines were used at optimal concentrations (IL-4, 10 U/mL and TNF-α, 10 ng/mL) for 24 hours. *Filled histogram,* Unstimulated endothelial cells; *grey line,* IL-4; *black line,* TNF-α. HUVECs demonstrated 95% ± 3% and 74% ± 7% positive cells after TNF-α or IL-4 addition, respectively, whereas HMECs demonstrated only positive cells after TNF-α addition (67% ± 6%) (n = 3 to 5 [mean ± SD]).

Presence of TNF-α in skin

TNF-α, a potent cytokine for adhesion molecule upregulation in vitro, was immunohistochemically stained in sections of nonatopic skin, nonlesional AD skin, 24-hour APT sites, and lesional AD skin. The staining was not uniform; all sections contained brightly stained cells and dimly stained cells (Fig 1, Table III). Of the brightly stained cells, 80% were also positive for chymase, indicating that chymase-containing mast cells in skin are a major source of TNF-α. To identify the dimly stained cells, immunofluorescent double-labeling with antibodies to cell membrane markers and TNF-α was performed. The majority of the TNF-α dimly stained cells were macrophages, as was seen by colocalization of TNF-α with CD68; less than 5% of the dimly stained cells expressed the T-cell marker CD3.

IL-4R α-chain expression on endothelium

The IL-4 receptor (IL-4R) consists of an IL-4R-specific α-chain and a common γ-chain, which is shared with the receptors for IL-2, IL-7, IL-9, and IL-15.[17] The IL-4R was absent on ECs in lesional and nonlesional skin of patients with AD and nonatopic control subjects, as was determined with a mAb against the IL-4R α-chain.

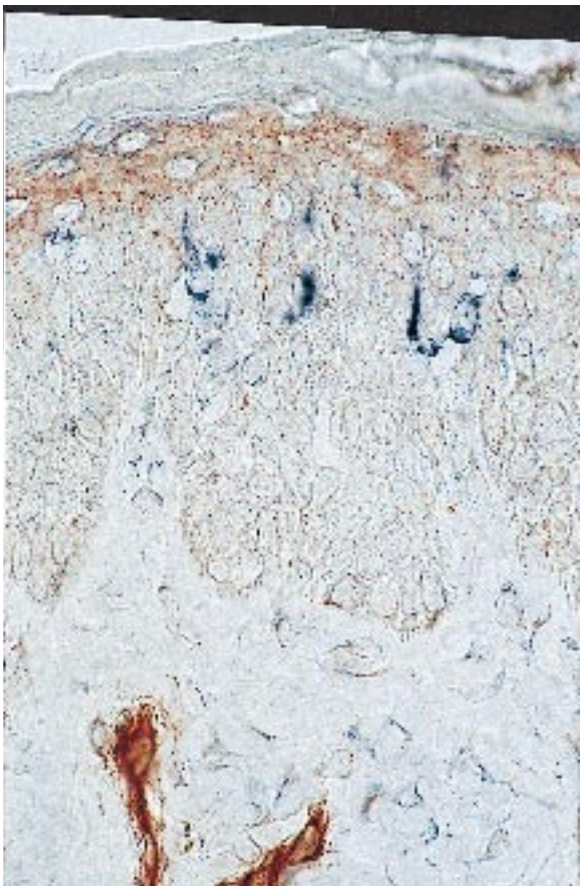

FIG. 4. Immunohistochemical staining for IL-4R α-chain expression on skin endothelia. Double-staining was performed with Ulex (red) and anti-IL-4R α-chain on lesional skin of patients with AD. Only single-positive cells are found. Magnification 100×.

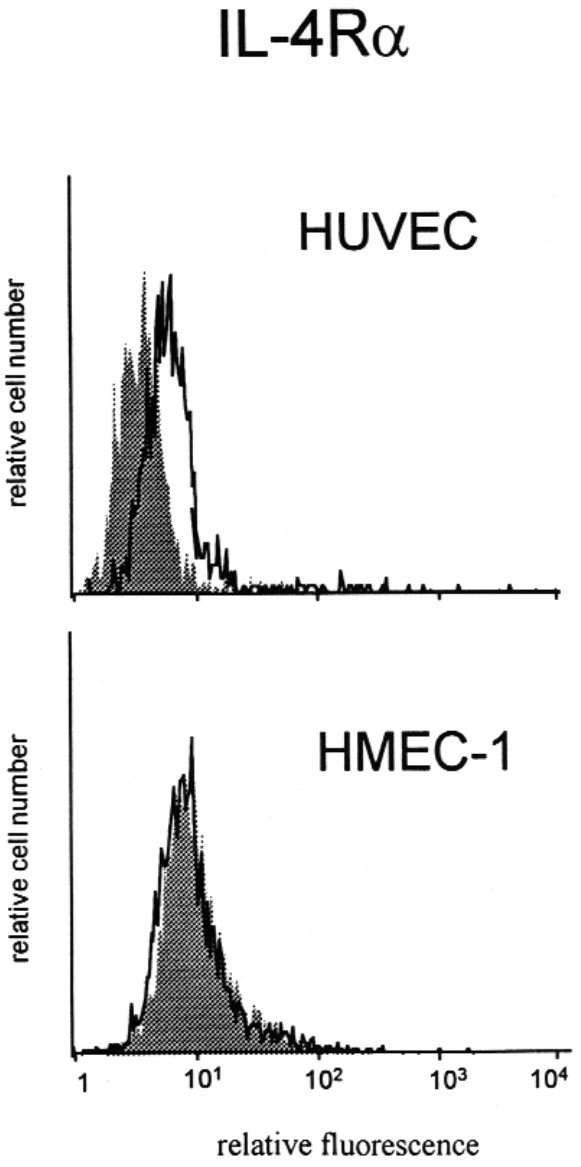

FIG 3. Surface expression of IL-4R α-chain on HUVECs and HMEC-1 cells. Histograms of flow cytometric analysis after immunofluorescent staining with anti-IL-4R α-chain *(open histogram)* as compared with an IgG1 isotype control *(closed histogram).*

Immunofluorescent staining with the IL-4R α-chain mAb demonstrated that IL-4R was present on HUVECs but absent on HMEC-1 (Fig 3).

To make sure that the lack of IL-4R α-chain detection was not limited to the HMEC-1 cell line, we also analyzed the IL-4R α-chain expression by immunohistochemistry on skin sections. Endothelial cells were marked by *Ulex europaeus* agglutinin-1 binding. No IL-4R α-chain–positive endothelial cells were detected after double staining in skin sections of nonatopic individuals and patients with AD, nonlesional skin, 24-hour APT sites, and lesional skin. Some of the cells in the epidermis demonstrated a clear IL-4R-α expression (Fig 4). Moreover, immunohistochemical analysis of HUVECs did demonstrated IL-4R α-chain expression (not shown).

DISCUSSION

Adhesion molecules on ECs are involved in extravasation of inflammatory cells. In this article we present data on the kinetics and regulatory mechanisms of adhesion molecule expression on ECs in patients with AD. The adhesion molecules E-selectin, P-selectin, and VCAM-1 were present on ECs in nonlesional AD skin, but absent in nonatopic skin. Because these molecules are responsible for the first step in T-cell extravasation,[18,19] their very presence may lead to the increase in cutaneous T cells in nonlesional AD skin.[9] Because a proportion of these T cells are antigen specific,[20] contact with allergen through the skin may be sufficient to promote an inflammatory response. The APT is a well-studied in vivo model for allergic inflammation in patients with AD.[4,9] We applied the APT to determine the kinetics of adhesion molecule expression in vivo and found a significant upregulation of VCAM-1 and ICAM-1, whereas E-selectin and P-selectin showed only a tendency to increase. During the APT, the

upregulation of VCAM-1 and ICAM-1 squares with the presence of eosinophils and T cells,[1,21] whereas the marginal increase of E-selectin corresponds with the absence of neutrophils.[22] Thus the relative expression of the different adhesion molecules is reflected in the composition of the infiltrate.

To investigate cytokines relevant for regulation of adhesion molecules on ECs in vivo, the human dermal microvascular EC line HMEC-1 and HUVECs were stimulated with IFN-γ, IL-4, and TNF-α. IFN-γ and IL-4 play an important role in allergic disease, whereas TNF-α is known to be a potent cytokine to induce and upregulate adhesion molecule expression in vitro.[8,9,23] Most intriguing are the VCAM-inducing properties of IL-4 on HUVECs and its concomitant increase in eosinophil, but not neutrophil, adhesion.[24] Although upregulation of adhesion molecules on HMEC-1 was lower than that on HUVECs, both EC lines responded comparably to stimulation with either TNF-α or IFN-γ. A marked difference, however, was observed in response to IL-4, which upregulated VCAM-1 expression on HUVECs but not on HMEC-1. Also on freshly isolated dermal ECs, IL-4 has been shown to have no effect on VCAM-1 expression,[25] indicating that HMEC-1 is comparable to freshly isolated dermal ECs in this respect. By means of FACS analysis, we demonstrate that, in contrast to HUVECs, the IL-4R α-chain is not expressed on HMEC-1 under any condition tested (ie, addition of TNF-α, LPS, IFN-γ, or IL-4). Moreover, ECs in skin sections of patients with AD and control subjects do not express the IL-4R α-chain. This is in accordance with HMEC-1 and suggests that HMEC-1 bears greater similarity to ECs in vivo from skin microvasculature than do HUVECs. Therefore these data, together with the morphologic similarities and microvascular EC marker expression, renders HMEC-1 a better model to predict the response of microvascular ECs in skin than HUVECs.[11] Moreover, these data suggest that IL-4 has little relevance for adhesion molecule regulation on microvascular ECs in vivo, at least with regard to the skin.

Because TNF-α and IFN-γ did have an effect on adhesion molecule expression on HMEC-1, knowledge of their in situ distribution is necessary. The kinetics of IFN-γ was described previously,[9] but limited data are available on production and localization of TNF-α in the skin of patients with AD. Therefore we studied the kinetics of this cytokine at the protein level in the skin of patients with AD and nonatopic control subjects. In AD skin we observed a significant increase of TNF-α–containing cells compared with that found in nonatopic skin. A proportion of these cells, with a bright staining pattern, were chymase-positive mast cells.[16] The majority of TNF-α^+ cells showed a dim staining, which colocalized with macrophages as identified by CD68 staining.

The absence of E-selectin, P-selectin, and VCAM-1 in nonatopic skin, despite the presence of TNF-α, which upregulates these molecules on HMEC-1 in vitro, indicates that in this instance TNF-α is not present in an active form. The ability of mast cells to store preformed TNF-α has been shown previously.[26,27] Because a proportion of the TNF-α in nonatopic skin is present in mast cells, this points to storage rather than release. In nonlesional skin, the number of TNF-α^+ mast cells was higher than that found in nonatopic skin, as was the total number of TNF-α^+ cells. This is linked to an increase in adhesion molecule expression and points to a role for TNF-α released by the dimly stained cells in maintaining AD skin in a primed state.

In lesional AD skin, which is in a continuous state of inflammation, the percentage of adhesion molecule–expressing ECs was significantly higher than that found at 24-hour APT reaction sites. In vitro, however, E-selectin expression is rapidly downregulated, even after continuous stimulation by TNF-α.[28] Our in vivo observations suggest that this downregulation as such does not occur in skin. This is in accordance with previous reports that show that persistent endothelial E-selectin expression also occurs in other chronic dermatoses such as psoriasis.[29] The recent discovery of different types of E-selectin transcripts, one of which is linked to chronic expression of E-selectin in vivo, provides a plausible explanation.[30]

Previously, it has been shown that the number of eosinophils in lesional skin was relatively low,[9] although we now describe increased number of VCAM-1–expressing ECs. This implicates that adhesion molecule expression does not necessarily lead to cell extravasation because eosinophils do express very late antigen-4, a ligand for VCAM-1.[24] In cell extravasation, which is a multistep process, chemoattractants play an essential role. IL-4 can act as a chemoattractant for eosinophils from patients with allergic asthma or AD.[31] In the early (24 hour) phase of the APT, many eosinophils are present.[32] The diminished IL-4 production in lesional skin compared with the early APT reaction might therefore explain the absence of eosinophils in lesional skin.[8,9] However, the role of other chemoattractants besides IL-4 in the APT needs to be established.

In summary, our data show that HMEC-1 is a more suitable in vitro model to study the response of cutaneous microvascular ECs than HUVECs. The in vitro effect of TNF-α on HMEC-1 indicates a role for TNF-α rather than IL-4 in regulation of adhesion molecule expression in vivo. TNF-α is indeed present in AD skin, and the kinetics of TNF-α are reflected both in the expression of adhesion molecules and in the composition of the cellular infiltrate.

We thank Dr Geert Mudde (Novartis, Vienna, Austria) for the gift of IL-4 and Dr Peter H. van der Meide (BPRC, Rijswijk, The Netherlands) for the IFN-γ. Dr Edwin W. Ades from the Centers for Disease Control and Prevention and Dr Thomas Y. Lawley of Emory University (Atlanta, Ga) are acknowledged as the developers of the HMEC-1. Medgenix is acknowledged for the mAb against TNF-α.

REFERENCES

1. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301-14.
2. Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1

is an adhesion molecule for skin-homing T cells. Nature 1991;349:796-9.
3. Bos JD, Hagenaars C, Das PK, Krieg SR, Voorn WJ, Kapsenberg ML. Predominance of "memory" T cells (CD4+,CDw29+) over naive T cells (CD4+,CD45R+) in both normal and diseased human skin. Arch Dermatol Res 1989;281:24-30.
4. Langeveld-Wildschut EG, Van Marion AMW, Thepen T, Mudde GC, Bruijnzeel PLB, Bruijnzeel-Koomen CAFM. Evaluation of variables influencing the outcome of the atopy patch test. J Allergy Clin Immunol 1995;96:66-73.
5. Reitamo S, Visa K, Kahonen K, Kyhk K, Stubb S, Salo O. Eczematous reactions in atopic dermatitis patients caused by epicutaneous testing with inhalant allergens. Br J Dermatol 1986;114:303-9.
6. Van Reijsen FC, Bruijnzeel-Koomen CAFM, Kalthoff FS, Maggi E, Romagnani S, Mudde GC. Skin-derived aeroallergen-specific T-cell clones of Th2 phenotype in patients with atopic dermatitis. J Allergy Clin Immunol 1992;90:184-92.
7. Van der Heijden FL, Wierenga EA, Bos JD, Kapsenberg ML. High frequency of IL-4-producing CD4+ allergen-specific T lymphocytes in atopic dermatitis lesional skin. J Invest Dermatol 1991;97:389-94.
8. Grewe M, Walther S, Gyufko K, Czech W, Schöpf E, Krutmann J. Analysis of the cytokine pattern expressed in situ in inhalant allergen patch test reactions of atopic dermatitis patients. J Invest Dermatol 1995;105:407-10.
9. Thepen T, Langeveld-Wildschut EG, Bihari IC, van Wichen DF, van Reijsen FC, Mudde GC, et al. Bi-phasic response against aeroallergens in atopic dermatitis showing a switch from initial T_{H2} into T_{H1} response in situ: an immuno-cytochemical study. J Allergy Clin Immunol 1996;97:828-37.
10. Holthoefer H, Virtanen I, Kariniemi A-L, Hormia M, Linder E, Miettinen A. Ulex europaeus I lectin as a marker for vascular endothelium in human tissues. Lab Invest 1982;47:60-6.
11. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 1992;99:683-90.
12. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. J Clin Invest 1973;52:2745-56.
13. Moser R, Schleiffenbaum B, Groscurth P, Fehr J. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. J Clin Invest 1989;83:444-55.
14. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol 1980;92:44-7.
15. Langeveld-Wildschut EG, Thepen T, Bihari IC, van Reijsen FC, de Vries IJM, Bruijnzeel PLB, et al. Evaluation of the atopy patch test and the cutaneous late-phase reaction as relevant models for the study of allergic inflammation in patients with atopic eczema. J Allergy Clin Immunol 1996;98:1019-27.
16. Schwartz LB. Mast cells: function and contents. Curr Opin Immunol 1994;6:91-7.
17. Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K, et al. Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. Science 1993;262:1874-82.
18. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell 1991;65:859-73.
19. Luscinskas FW, Ding H, Lichtman AH. P-selectin and vascular cell adhesion molecule 1 mediate rolling and arrest of CD4+ T lymphocytes on tumor necrosis factor α-activated vascular endothelium under flow. J Exp Med 1995;181:1179-86.
20. Van Reijsen FC, Mudde GC, De Weger RA, Bruijnzeel-Koomen CAFM. Retention of long-lived, allergen-specific T cells in atopic dermatitis skin. J Invest Dermatol 1997;108:530.
21. Moser R, Fehr J, Olgiati L, Bruijnzeel PLB. Migration of primed human eosinophils across cytokine-activated endothelial cell monolayers. Blood 1992;79:2937-45.
22. Bevilacqua MP, Stengelin S, Gimbrone MA, Seed B. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 1989;243:1160-5.
23. Pober JS, Gimbrone MA, Lapierre LA, Mendrick DL, Fiers W, Rothlein R, et al. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J Immunol 1986;137:1893-6.
24. Schleimer RP, Sterbinsky SA, Kaiser J, Bickel CA, Klunk DA, Tomioka K, et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: association with expression of VCAM-1. J Immunol 1992;148:1086-92.
25. Swerlick RA, Lee KH, Li L, Sepp NT, Caughman SW, Lawley TJ. Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. J Immunol 1992;149:698-705.
26. Walsh LJ, Trinchieri G, Waldorf HA. Whitaker D, Murphy GF. Human dermal mast cells contain and release tumor necrosis factor α, which induces endothelial leukocyte adhesion molecule 1. Proc Natl Acad Sci USA 1991;88:4220-4.
27. Gordon JR, Galli SJ. Promotion of mouse fibroblast collagen gene expression by mast cells stimulated via the Fc epsilon RI. Role for mast cell-derived transforming growth factor beta and tumor necrosis factor alpha. J Exp Med 1994;180:2027-37.
28. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 1987;84:92-138.
29. Groves RW, Allen MH, Barker JNWN, Haskard DO, Macdonald DM. Endothelial leucocyte adhesion molecule-1 (ELAM-1) expression in cutaneous inflammation. Br J Dermatol 1991;124:117-23.
30. Chu W, Presky DH, Swerlick RA, Burns DK. Alternatively processed human E-selectin transcripts linked to chronic expression of E-selectin in vivo. J Immunol 1994;153:4179-89.
31. Dubois GR, Bruijnzeel-Koomen CAFM, Bruijnzeel PLB. IL-4 induces chemotaxis of blood eosinophils from atopic dermatitis patients, but not from normal individuals. J Invest Dermatol 1994;102:843-6.
32. Bruijnzeel-Koomen CAFM, Van Wichen DF, Spry CJF, Venge P, Bruijnzeel PLB. Active participation of eosinophils in patch test reactions to inhalant allergens in patients with atopic dermatitis. Br J Dermatol 1988;118:229-38.