Human Cutaneous Dendritic Cells Migrate Through Dermal Lymphatic Vessels in a Skin Organ Culture Model

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The capacity to migrate from peripheral tissues, where antigen is encountered, to lymphoid organs, where the primary immune response is initiated, is crucial to the immunogenic function of dendritic cells (DC). The skin is a suitable tissue to study migration. DC were observed to gather in distinct nonrandom arrays ("cords") in the dermis upon culture of murine whole skin explants. It is assumed that cords represent lymphatic vessels. Using a similar organ culture model with human split-thickness skin explants, we investigated migration pathways in human skin.

We made the following observations. 1) Spontaneous emigration of Langerhans cells took place in skin cultured for 1–3 d. Nonrandom distribution patterns of strongly major histocompatibility complex class II-expressing DC (cords) occurred in cultured dermis. A variable, yet high (>50%) percentage of these DC coexpressed the Birbeck granule-associated antigen "Lag." Ultrastructurally, the cells corresponded to mature DC. 2) Electron microscopy proved that the dermal structures harboring the accumulations of DC (i.e., cords) were typical lymph vessels. Moreover, markers for blood endothelia (monoclonal antibody PAL-E, Factor VIII-related antigen) and markers for cords (strong major histocompatibility complex class II expression on nonrandomly arranged, hairy-appearing cells) were expressed in a mutually exclusive pattern. 3) On epidermal sheets we failed to detect gross changes in the levels of expression of adhesion molecules (CD44, CD54/ICAM-1, E-cadherin) on keratinocytes in the course of the culture period.

The reactivity of a part of the DC in the dermal cords with Birbeck granule-specific monoclonal antibody "Lag" suggests that the migratory population is composed of both epidermal Langerhans cells and dermal DC. We conclude that this organ culture model may prove helpful in resolving pathways and mechanisms of DC migration. *Key words: Langerhans cells/dermal dendritic cells/migration. J Invest Dermatol 106: 1293–1299, 1996*

Dendritic cells (DC) of the skin (epidermal Langerhans cells (LC) and dermal DC) are the best-studied representatives of immature "tissue DC." They act as sentinels specialized to generate immunogenic complexes of major histocompatibility complex (MHC) molecules and antigenic peptides. Mature "lymphoid DC" (i.e., short term cultured DC) are superior to other cell types in presenting these complexes together with an array of adhesion and costimulator molecules to resting T cells. These events, which take place in lymphoid organs, lead to the efficient activation of antigen-specific T cells and thus to a primary immune response (Steinman, 1991). The initiation of a successful primary T-cell response is crucially dependent on a third group of accessory functions, namely the *migratory* properties of DC (Steinman *et al*, 1995). Immunogenic MHC/peptide complexes must be transported from tissues like the skin to lymph nodes or spleen. Also, these functions are expressed in DC to a higher degree than in other cell types.

Upon application of contact sensitizers, it was noted that the numbers of LC in human epidermis decrease; CD1a-expressing cells (i.e., DC) in the dermis increase in numbers (Sterry *et al*, 1991; Groves *et al*, 1995). Occasional DC have repeatedly been found in afferent dermal lymphatic vessels (Schuler *et al*, 1991). Silberberg-Sinakin *et al* (1976) were the first to describe LC in dermal lymphatics draining a site of exposure to contact allergen. DC that display the morphology of "veiled cells" were collected after epicutaneous or parenteral antigen administration from cannulated lymph vessels in sheep (Bujdoso *et al*, 1989), rat (Liu and MacPherson, 1993), and humans (Brand *et al*, 1993). Little is known, however, about the regulation of DC/LC migration. In experimental animals, the contact hypersensitivity model is a useful approach to address such questions. For obvious reasons this model is less useful in humans. Therefore, skin organ cultures are preferred. It has been shown that DC emigrate out of cultured explants of human whole skin (Morelli *et al*, 1995; Pope *et al*, 1995; Rambukkana *et al*, 1995; Richters *et al*, 1995) or dermis (Lenz *et al*,

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Abbreviations: DC, dendritic cell(s); LC, Langerhans cell(s); MHC, major histocompatibility complex.

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Table I. Antibodies Used for Immunohistochemistry

Specificity	Clone/Name	Immunoglobulin Class	Source	Reference
HLA-DR/DQ	9.3F10/HB180	Mouse IgG2a	ATCC[a]	
HLA-DR	L243/HB55	Mouse IgG2a	ATCC	
HLA-DR-FITC	L243	Mouse IgG2a	BDIS[b]	
CD1a	OKT-6/CRL8020	Mouse IgG1	ATCC	
CD1a-FITC	OKT-6	Mouse IgG1	Ortho[c]	
Birbeck granules	Lag	Mouse IgG1	M. Kashihara	(Kashihara *et al.*, 1986)
CD54/ICAM-1	R6.1	Mouse IgG2a	Bender[d]	
CD44-standard	SFF-2	Mouse IgG1	Bender	
CD44-variant 5	FFV8	Mouse IgG1	Bender	
CD44-variant 6	FFV18	Mouse IgG1	Bender	
CD44-variant 9	FFV16	Mouse IgG1	Bender	
E-cadherin	HECD-1	Mouse IgG1	Takara[e]	
ELAM-1	2G7	Mouse IgG1	Otsuka[f]	
Collagen type IV	polyclonal	Rabbit Ig	DAKO[g]	
Factor VIIIr-Ag	polyclonal	Rabbit Ig	DAKO	
Blood endothelia	PAL-E	Mouse Ig2a	Seralab[h]	(Schlingemann *et al.*, 1985)

[a] American Type Culture Collection, Rockville, MD.
[b] Becton-Dickinson Immunocytometry Systems, Mountain View, CA.
[c] Ortho, Raritan, NJ.
[d] Bender Immunsysteme, Vienna, Austria.
[e] Takara Inc., Otsu, Japan.
[f] Otsuka America Pharmaceuticals, Rockville, MD.
[g] DAKO, Roskilde, Denmark.
[h] Seralab, Crawley Down, UK.

1993; Nestle *et al*, 1993). The intradermal pathways of DC migration have not been defined, however.

In the murine system Larsen *et al* (1990) have shown that, in skin transplants and skin explant cultures DC line up in nonrandom arrays, termed "cords," in the dermis. It has been strongly assumed that dermal cords represent lymphatic vessels. To our knowledge, however, this has not yet been shown directly. Moreover, unequivocal cord formation has hitherto been described only in the murine system (Larsen *et al*, 1990, 1994). The goal of our study was therefore to extend the murine work to the human system. We intended to investigate the pathways of migration of human cutaneous DC in skin organ culture using an immunohistochemical and electron microscopical approach.

MATERIALS AND METHODS

Medium Culture medium used throughout was RPMI 1640 (supplemented with 10% fetal calf serum) and gentamycin (all from Biological Industries, Kibbutz Beit Haemek, Israel) and 2-mercaptoethanol (Sigma, St. Louis, MO).

Skin Organ Culture The human skin organ culture system was based on recently described methods (Lenz *et al*, 1993; Pope *et al*, 1995). Split-thickness skin (0.15-0.2 mm) from corrective plastic surgery of mammae and abdomina was trimmed to pieces of approximately 1 × 1 cm. These pieces were placed on the bottom membrane of the inner chamber of a 6-well, double-chamber tissue culture plate (Transwell™ 3414; Costar, Cambridge, MA). The outer chamber was filled with culture medium so that it could only get to the skin from underneath. The epidermal part of the skin was thus exposed to the air. These cultures were incubated at 37°C for 1 to 4 (mostly 2) d. In some experiments, skin was separated into epidermis and dermis by means of the protease "dispase" as described (Lenz *et al*, 1993; Kitano and Okado, 1983) before the onset of culture.

Immunohistochemical Techniques Pieces of cultured skin were removed from the wells and epidermis was separated from dermis by means of ammoniumthiocyanate as described (Juhlin and Shelley, 1977). Epidermal and dermal sheets were trimmed to approximately 3 × 3 mm, fixed in acetone for 5 min at room temperature, and immunostained in Eppendorf tubes applying the following sequence of steps: primary mouse monoclonal antibodies (mAbs) (24 h at 4°C; see **Table I**), biotinylated anti-mouse immunoglobulin (Amersham International, Amersham, UK; 90 min at 37°C), streptavidin-peroxidase complex or streptavidin Texas Red (both from Amersham; 90 min at 37°C). The immunoperoxidase protocol was continued by visualizing peroxidase activity with 3′,3′-diaminobenzidine-H_2O_2. For immunofluorescence, the protocol was extended by double-labeling for MHC class II in order to identify dendritic cells. After a blocking step with mouse immunoglobulin (100 μg/ml), sheets were incubated in a final step with fluorescein isothiocyanate-conjugated anti-MHC class II/HLA-DR for 90 min at 37°C. Fluorescent sheets were mounted onto microscopic slides in Vectashield (Vector, Burlingame, CA) mounting medium. Alternatively, unseparated cultured human skin was snap-frozen in liquid nitrogen and sectioned on a cryostat (Frigocut, Reichert-Leitz, Vienna, Austria) in a plane tangential to the surface of the skin. Thick sections (60-100 μm) were collected in phosphate-buffered saline, fixed in acetone for 5 min at room temperature and immunostained like sheets in Eppendorf tubes. Isotype-matched nonrelevant immunoglobulins were used as specificity controls.

Evaluation of Immunohistochemistry The density of LC in epidermal sheets was counted under the microscope using 40 × objective lenses and a calibrated grid. At least 20 randomly chosen fields were counted. Fields containing hair follicles were excluded from analyses. Mean values and standard deviations were determined.

Electron Microscopy Skin organ cultures were fixed with Karnovsky's half-strength formaldehyde-glutaraldehyde reagent and further processed as described (Stössel *et al*, 1990). Ultrathin sections were evaluated on a Philips EM 400 (Philips, Eindhoven, The Netherlands) electron microscope.

RESULTS

Epidermal Langerhans Cells Leave the Epidermis During the Culture Period In epidermal sheets from cultured skin, LC density dropped in a similar fashion as described for murine skin (**Fig 1** and **Table II**). The decrease was observed with antibodies directed to three different molecules (HLA-DR, CD1a, Birbeck granule-associated). It was best appreciated using mAb Lag, directed against a Birbeck granule-associated protein. It is noteworthy that the reduction of LC in human skin seemed to happen in a less regular manner than in murine skin. We often encountered parts of a sheet with reduced LC density adjacent to parts with unchanged density even as late as day 3 of culture. The decrease of LC became also apparent in standard cryostat sections of cultured skin, although less clearly. In double-labeling experiments, we could occasionally observe LC, identified by anti-MHC class II staining, that seemed to penetrate the basement membrane, identified by anti-collagen type IV staining **(Fig 2)**. Like in the murine system (Larsen *et al*, 1990), we observed that LC remaining in the epidermal sheets after 2–3 d of culture were bigger, more dendritic in shape, and expressed higher levels of MHC class II molecules.

Dendritic Cells Accumulate in Dermal Cords In initial experiments, we failed to detect cords in cultured human skin. Only after we decided to use 60-100-μm tangentially cut cryostat

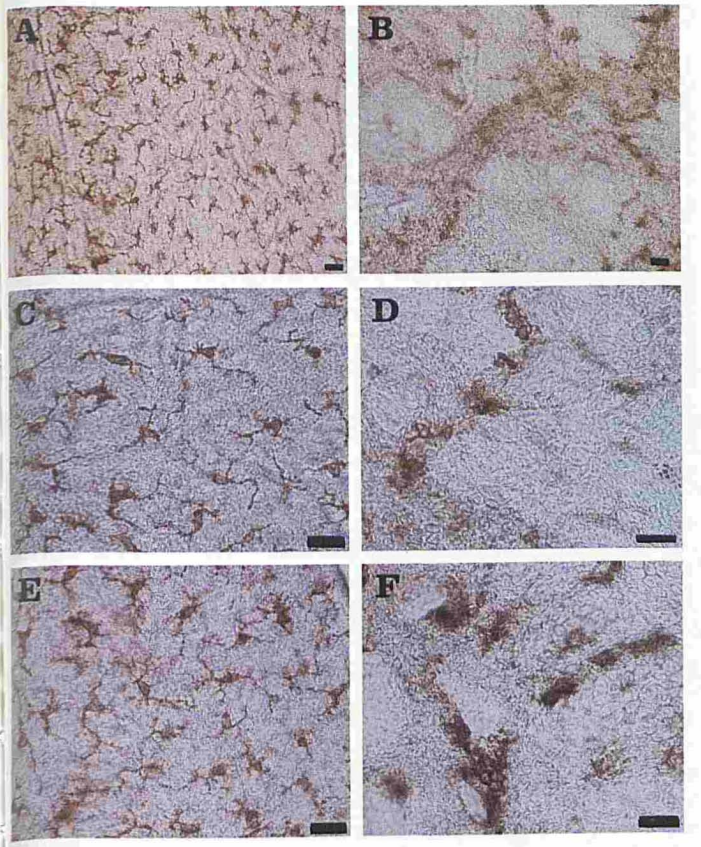

Figure 1. Epidermal Langerhans cells leave the epidermis upon organ culture. Epidermal sheets obtained from human skin cultured for 2 d were immunostained with mAbs against Birbeck granule-associated antigen Lag (*B* and *D*) and HLA-DR (*F*). Sheets from the same skin prepared before the onset of organ culture serve as control (*A*, *C*, and *E*). Note that LC remaining in the epidermis tend to aggregate and express higher levels of MHC class II (*F* vs. *E*). *Scale bars*, 100 μm.

sections rather than standard cryostat sections or the much thicker dermal sheets, we reproducibly (five different experiments) discovered the structures **(Fig 3)**. Of crucial help was also mAb Lag, which stains only very few cells in the normal, uncultured dermis (Kashihara *et al*, 1986) and thus makes it easier to make out nonrandom arrangements of cells such as cords. Double-labeling experiments disclosed that a surprisingly high proportion of class II-positive cells within the cords expressed the Birbeck granule-associated Lag antigen **(Fig 3)**. It ranged between 50% and 80%. CD1a, another marker for epidermal dendritic cells (LC) was expressed at high levels (i.e., at levels detectable with a directly fluoresceinated mAb) on only a small percentage of DR-positive cells (<20%).

Table II. Reduction of the Density of Human Epidermal Langerhans Cells With Culture as Determined With mAb Lag

Day of study	Experiment 1	Experiment 2	Experiment 3
0	800 ± 124[a]	1025 ± 159	966 ± 138
1	464 ± 125	562 ± 153	672 ± 139
2	280 ± 128	355 ± 124	521 ± 140
Reduction[b]	65%	66%	46%

[a] Numbers of LC/mm^2 ± standard deviations.
[b] Reduction after 2 d of culture in percent.

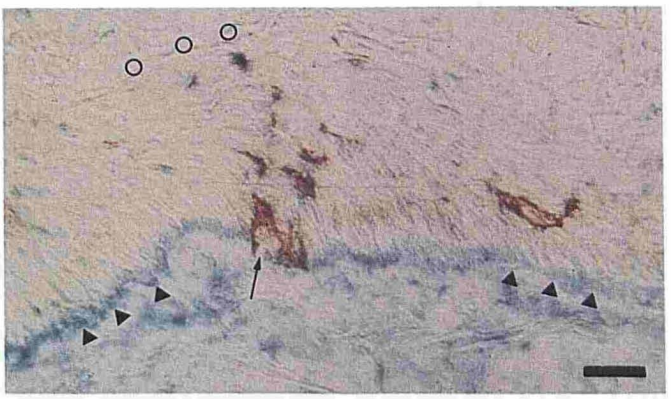

Figure 2. Epidermal Langerhans in transit through the basement membrane (▲). Double labeling for collagen type IV (blue alkaline phosphatase reaction product) and MHC class II (red peroxidase/AEC reaction product) shows a rare event where the basement membrane is interrupted (→) and a class II-positive LC seems to penetrate through it. ○, level of stratum corneum. *Scale bar*, 100 μm.

Dermal Cords Are Lymphatic Vessels On tangential cryostat sections of organ cultures, we used polyclonal antibodies against Factor VIII-related antigen (Van Willebrand factor) and mAb PAL-E (Schlingemann *et al*, 1985; Erhard *et al*, 1996) as a marker for blood endothelia. Double-labeling with MHC class II gave the following picture: Cords filled with HLA-DR–positive cells were not lined by Factor VIII- or PAL-E–expressing structures, and, vice versa, Factor VIII- or PAL-E–positive blood vessels did not contain accumulations of HLA-DR–positive dendritic cells **(Fig 4)**. These immunohistochemical data formally exclude the possibility that dermal cords might be blood vessels.

We could detect accumulations of DC in the dermis on *semithin sections* (1 μm) of specimens embedded for electron microscopy. This was not as frequent, though, as in murine organ cultures (Larsen *et al*, 1990). Substantial numbers of cells displaying DC morphology ("veils," lobulated nuclei, light cytoplasm) were found in wide clefts **(Fig 5)**. Most likely they correspond to the cords that were detected by immunostaining. Characteristic nuclei that bulged into the lumen of the clefts indicated the presence of an endothelial lining. Such structures had never been observed in normal, uncultured skin. Cords were detected both in the papillary dermis close to the epidermis and deeper in the reticular dermis. It was not possible, however, to determine a preferred site for the occurrence of human cords.

Ultrastructural analysis definitively established that the cords were lymphatic vessels **(Fig 6)**. The dermal clefts were surrounded by a thin monolayer of endothelial cells that occasionally formed large gaps opening to the surrounding dermal matrix. These gaps were wide enough for a DC to pass through **(Fig 6)**. We could not observe this event, though. Pericytes were not found. As opposed to blood vessels, the endothelial cells of the cords were *not* supported by a continuous basement membrane. Only short fragments of poorly developed lamina densa were found. The cells also lacked Weibel-Palade bodies, which are characteristic for blood endothelial cells (Ryan, 1989). The cells that had accumulated in the lymph vessels qualified unequivocally as mature DC by ultrastructural criteria. They possessed thin cytoplasmic, organelle-free sheets ("veils") that extended in all directions. The nuclei were strongly indented. The electron-light cytoplasm contained many profiles of smooth endoplasmic reticulum, multivesicular bodies, and few electron-dense lysosomes. As expected from the immunohistochemical staining with mAb Lag, we found Birbeck granules in a substantial number of DC. We counted three out of 15 profiles of DC containing Birbeck granules. Most of the granules were small and rod-shaped; few showed the typical tennis racket morphology.

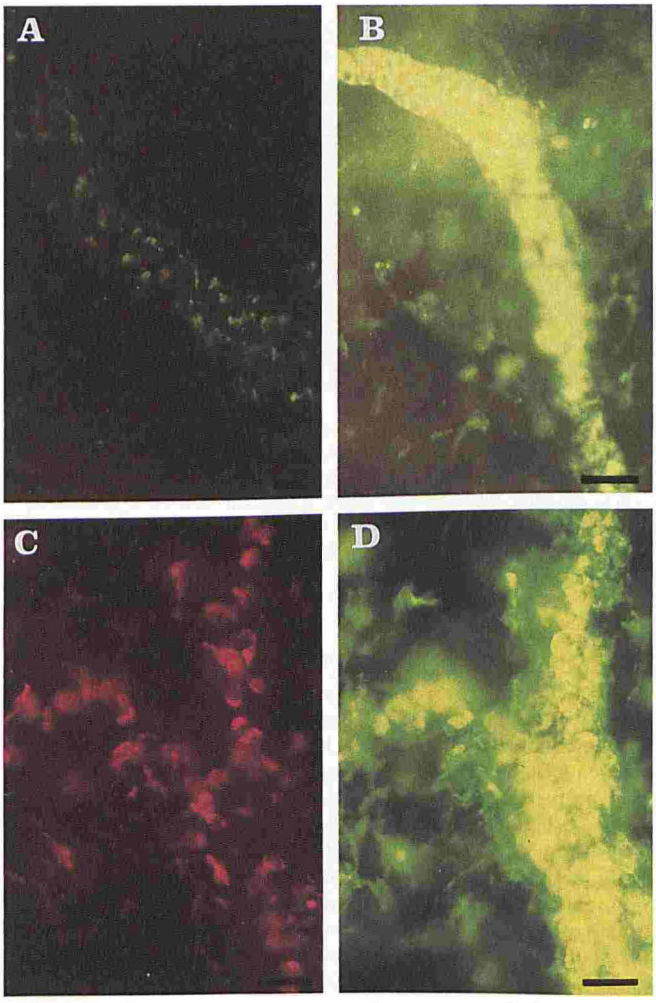

Figure 3. Dermal cords in tangential cryostat sections of skin cultured for 2 d. Note the characteristic nonrandom, string-like distribution of labeled cells and the difference in staining pattern between Lag (*A*) and HLA-DR (*B*): In Lag-stained sections the DC in the cords are virtually the only cells that stain (*A*), whereas many more dermal elements are recognized by anti–HLA-DR mAb (*B*). Double labeling (*C* and *D*) shows that most HLA-DR–positive cells (*green fluorescence*) also express Lag antigen (*red fluorescence*). *Scale bars*, 100 μm.

Clearly, the numbers of Birbeck granules in these DC were less than in resident LC in uncultured epidermis.

Expression of Molecules Involved in Migration in Cultured Human Skin We searched for differential expression of such molecules in the epidermis during organ culture based on data that suggest the involvement of adhesion molecules (Jalkanen *et al*, 1990; Tang *et al*, 1993) in migratory processes. Staining of epidermal sheets before and after 2 d of culture with mAbs against E-cadherin, CD44-standard, and CD44-splice variants 5v, 6v, and 9v did not show any gross differences: Keratinocytes *in situ* of both fresh and cultured epidermis expressed these molecules abundantly (not shown). Expression on LC could therefore not be evaluated with this immunostaining approach. CD54 was not expressed by keratinocytes and LC *in situ* before and after 2 d of culture. mAb against ELAM-1 (CD62) failed to stain blood vessels in skin cultures.

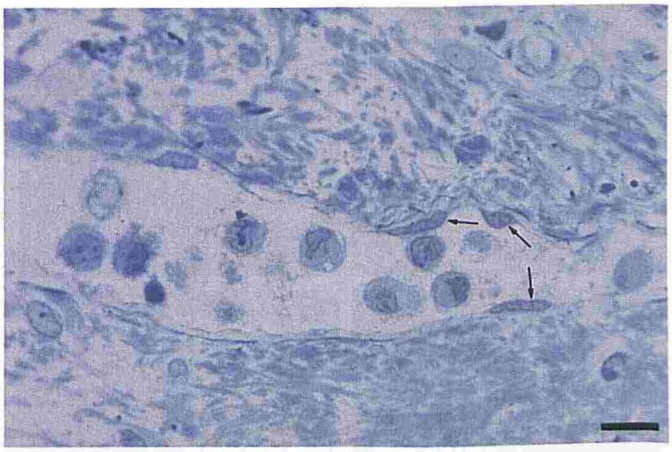

Figure 5. Semithin section (1 μm) of skin cultured for 2 d. Cells with the morphology of dendritic cells (lobulated and eccentric nuclei, light cytoplasm) are located in wide clefts. Of the thin endothelial cell lining, only the characteristic nuclei (→) that bulge into the lumen can be appreciated. *Scale bar*, 20 μm.

DISCUSSION

We show here that *human* cutaneous DC migrate along the same routes that have previously been defined in a *murine* skin organ culture model. There, DC accumulate in the dermis in immunohistochemically appreciable structures termed "cords." Such cords could hitherto not unequivocally be demonstrated in human skin. Here we reproducibly detected cords in human skin organ cultures and proved by immunohistochemistry and electron microscopy that they represent afferent lymphatic vessels.

Cords have not been found previously in human skin organ cultures for two major reasons. 1) Because human cords were not as frequently encountered as murine cords, it was mandatory to screen relatively large areas of skin. Therefore, standard cryostat sections were not suitable. Dermal sheets were often too thick to allow clear interpretation of immunostaining results. This problem

Figure 4. Double labeling of tangential sections of skin cultured for 2 d with markers for dendritic cells (MHC class II; *green fluorescence* in *A* and *C*) and blood endothelia (mAb PAL-E; *red fluorescence* in *B* and *C*). *C* represents a different section from that shown in *A* and *B*. Slides were double-exposed in *C*. Note that there is no overlap between the two markers. *Scale bars*, 50 μm.

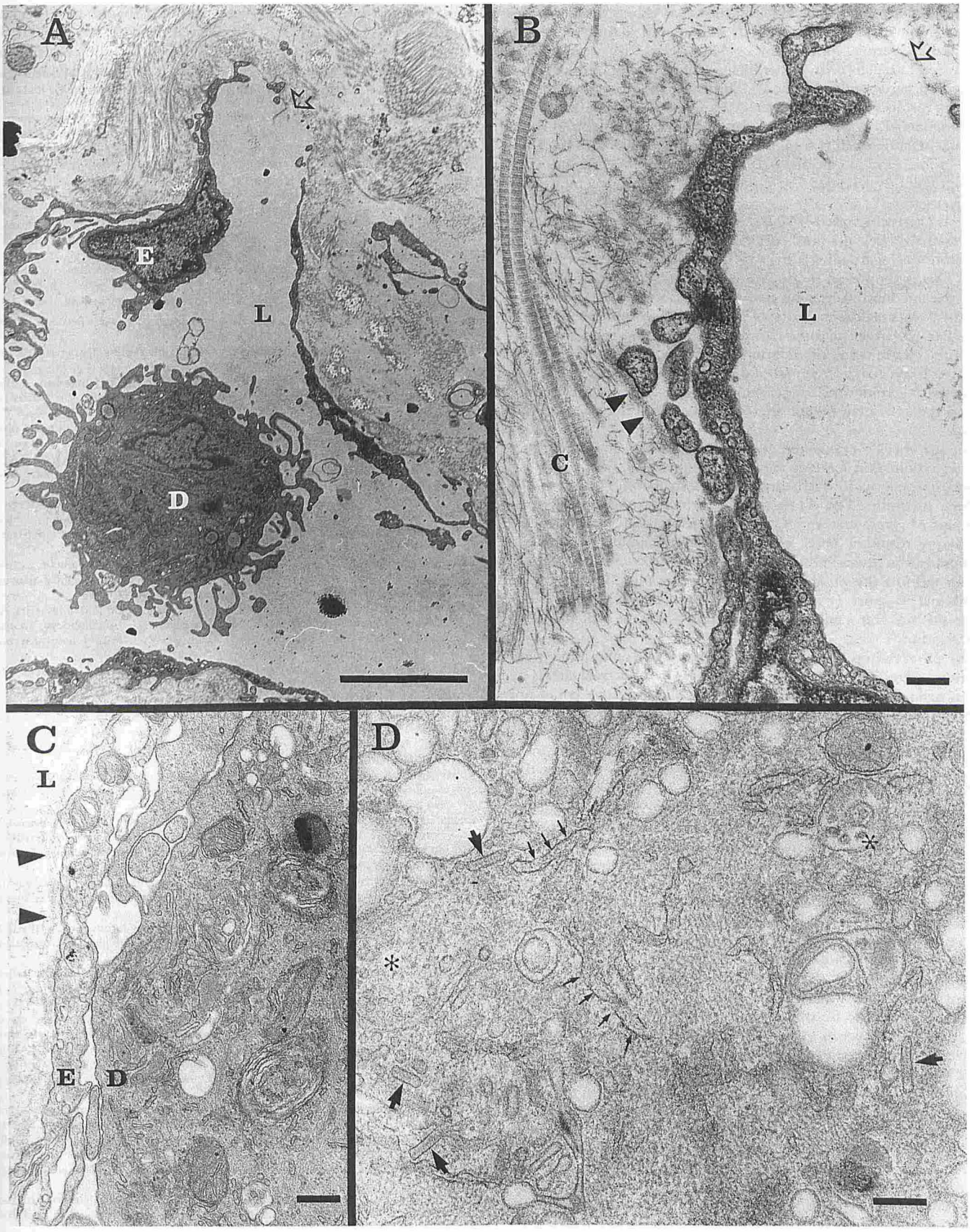

Figure 6. Ultrastructural appearance of a cord after 2 d of culture. Note thin cytoplasmic processes ("veils") of a dendritic cell (*D*) in a lymph vessel. The thin endothelial lining has wide gaps (⇐ in *panels A* and *B*). Only fragments of lamina densa (▶) can be found at the ablumenal side of the endothelial cells (*E*); a continuous basement membrane is not present (*panels B* and *C*). In *panel D* the high magnification of the cytoplasm of a DC contained within the lymph vessel shows small Birbeck granules (➡), stretches of smooth endoplasmic reticulum (→), and multivesicular bodies (*). *E*, endothelial cell; *D*, dendritic cell; *L*, lumen; *C*, collagen fibrils. *A*: *Scale bar*, 5 μm; *B,C*: *Scale bar*, 1 μm; *D*: *Scale bar*, 0.2 μm.

was solved by using tangential sections of skin that were of a constant thickness (60–100 μm). 2) As opposed to murine dermis, the human dermis contains more MHC class II-expressing cell types (endothelial cells, macrophages, infiltrating leukocytes—often in a perivascular location). This makes the interpretation of staining results obtained with anti-MHC class II mAbs difficult. We circumvented this problem by applying mAb Lag. This Birbeck granule-specific mAb (Kashihara *et al*, 1986) stained very few cells both in fresh and in cultured dermis. Therefore, the large numbers of nonrandomly arranged Lag-positive cells (i.e., cords) could easily be discovered.

mAb Lag recognizes a 42-kDa protein that is specifically associated with Birbeck granules of LC (Kashihara *et al*, 1986). It may therefore be regarded as a marker for an epidermal derivation of DC. This is supported by our observation that DC that emigrate into the medium from cultured *epidermis* were uniformly Lag-reactive, whereas the majority of DC derived from cultured *dermis* were Lag-negative. Immunocytochemistry was confirmed by ultrastructural examination of emigrated cells (Lenz *et al*, 1993). Therefore, it is likely that a large part of the DC that can be found in cords are migrating epidermal Langerhans cells. Lag-negative, MHC class II-positive cells may be considered as migratory dermal dendritic cells.

Pope *et al* (1995) collected mature DC that had emigrated from human whole skin explant cultures. Our culture system did not allow the systematic evaluation of cells that emigrated into the culture medium. Yet, in a few experiments we noted small numbers of viable mature DC that must have "squeezed" through the 3-μm–diameter pores of the supporting membrane into the medium of the lower culture chamber. This shows that the culture system permits the emigration of DC. Upon modification of the membrane support (wider pores, net insets) the numbers and properties of the emigrated DC would also be amenable to investigation.

Our observations regarding the mechanism of emigration were not conclusive. We examined keratinocytes in epidermal sheets by means of immunohistochemistry. With this method we could not detect gross changes in the expression of some molecules that are involved in migration and homing during organ culture (E-cadherin, CD44 standard and variants, CD54). Evaluation of LC was not possible with this approach and was beyond the scope of this study. Expression of E-cadherin was shown to be down-regulated on cultured epidermal LC *in vitro* (Tang *et al*, 1993; Blauvelt *et al*, 1995) and also *in vivo* after epicutaneous application of contact sensitizers, possibly mediated by the cytokines tumor necrosis factor-α and interleukin-1β. This suggests that a homotypic interaction between E-cadherin molecules on keratinocytes and LC holds the LC in place. A down-regulation of E-cadherin may lead to a weakening or disengagement of this molecular bridge which, in turn, may enable LC to leave their epidermal environment. Integrins have also be implicated in the regulation of human LC migration (Staquet *et al*, 1995). The directed migration of LC toward the dermis could occur along a chemotactic gradient. Recent data point to fibroblast products as chemotactic stimuli for human LC (Kobayashi *et al*, 1994). Chemokines like MIP-1α (macrophage inflammatory protein-1α) or RANTES can attract blood DC (Sozzani *et al*, 1995) in chemotaxis assays. In a transgenic mouse model, MCP-1 (monocyte chemotactic protein-1) appears to be a stimulus for migration of LC into the epidermis (Nakamura *et al*, 1995). The failure of anti-ELAM-1 mAb to stain endothelia in cultured skin leads us to the conclusion that inflammatory cytokines are not being produced in large amounts in the skin cultures (Bevilacqua, 1993).

In conclusion, our data suggest that the human organ culture model is representative for the migration processes occurring *in vivo*. It is worth noting that the model has an additional advantage: After separation of epidermis from dermis by means of dispase, both compartments may be studied separately. In ongoing studies we have obtained preliminary data that show a migration-enhancing effect of tumor necrosis factor–α in this organ culture system. This observation is in line with data from human (Groves *et al*, 1995) and murine (Roake *et al*, 1995; Cumberbatch *et al*, 1994; Vermeer and Streilein, 1990) experimental models. We believe, therefore, that the human skin organ culture model may help to elucidate the cytokine cascade that triggers migration and maturation of cutaneous DC.

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