Entry Into Afferent Lymphatics and Maturation *In Situ* of Migrating Murine Cutaneous Dendritic Cells

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An important property of dendritic cells (DC), which contributes crucially to their strong immunogenic function, is their capacity to migrate from sites of antigen capture to the draining lymphoid organs. Here we studied in detail the migratory pathway and the differentiation of DC during migration in a skin organ culture model and, for comparison, in the conventional contact hypersensitivity system. We report several observations on the capacity of cutaneous DC to migrate in mouse ear skin. (i) Upon application of contact allergens *in vivo* the density of Langerhans cells in epidermal sheets decreased, as determined by immunostaining for major histocompatibility complex class II, ADPase, F4/80, CD11b, CD32, NLDC-145/DEC-205, and the cytoskeleton protein vimentin. Evaluation was performed by computer assisted morphometry. (ii) Chemically related nonsensitizing or tolerizing compounds left the density of Langerhans cells unchanged. (iii) Immunohistochemical double-staining of dermal sheets from skin organ cultures for major histocompatibility complex class II and CD54 excluded blood vessels as a cutaneous pathway of DC migration. (iv) Electron microscopy of organ cultures revealed dermal accumulations of DC (including Birbeck granule containing Langerhans cells) within typical lymphatic vessels. (v) Populations of migrating DC in organ cultures upregulated markers of maturity (the antigen recognized by monoclonal antibody 2A1, CD86), but retained indicators of immaturity (invariant chain, residual antigen processing function). These data provide additional evidence that during both the induction of contact hypersensitivity and in skin organ culture, Langerhans cells physically leave the epidermis. Both Langerhans cells and dermal DC enter lymphatic vessels. DC mature while they migrate through the skin. ***Key words: contact hypersensitivity/Langerhans cells/migration/organ culture. J Invest Dermatol 110:441–448, 1998***

T cell responsiveness requires the involvement of accessory cells that fulfil three broad functions. First, they have to be able to capture antigens and generate immunogenic major histocompatibility complex (MHC)/peptide complexes. Second, they have to migrate from the sites of antigen capture to draining lymphoid organs in order to find antigen specific T lymphocytes. Third, they must be efficient in presenting the MHC/peptide complexes to the T cells together with the necessary adhesion molecules and costimulatory signals. Dendritic cells (DC) are highly specialized to perform this task (Steinman, 1991; Schuler *et al*, 1997).

A precondition for the generation of primary immune responses is that antigens be brought into lymphoid organs by blood, lymph, or mobile antigen-presenting cells (Zinkernagel, 1996). It follows that motility and mobility must be key properties of DC. It is essential that they be able to carry immunogenic MHC/peptide complexes from the places of antigen capture, which are often tissues bordering to the outside world, to those locales where a primary immune response takes place, i.e., the draining lymphoid organs. Classical experiments by Austyn *et al* have established that DC can migrate and home to the T areas of lymphoid organs (reviewed in Austyn, 1989, 1996). Evidence for migration of DC upon antigenic challenge comes mainly from studies in the murine contact hypersensitivity model. Upon epicutaneous application of contact sensitizers, the numbers of immunohistochemically detectable MHC class II expressing cells in the epidermis (i.e., Langerhans cells) decreases (Bergstresser *et al*, 1980; Botham *et al*, 1987; Steinbrink *et al*, 1996), occasional veiled cells (i.e., DC) can be found in lymphatic vessels (Silberberg-Sinakin *et al*, 1976; Schuler *et al*, 1991), and hapten-loaded, functional DC can be detected in and isolated from the draining lymph nodes (Macatonia *et al*, 1987). Larsen *et al* (1990) established a murine organ culture model where they could directly show migration of epidermal Langerhans cells from the epidermis through the dermis into the culture medium. They noted that strongly MHC class II expressing cells, i.e., DC, accumulated in the dermis in a characteristic string-like pattern that they termed "cords." Dermal "cords" were eventually also found *in vivo* in mice injected with lipopolysaccharide (Roake *et al*, 1995). In human skin organ cultures we could show that "cords" are lymphatic vessels (Lukas *et al*, 1996). For the murine skin culture model this has not yet been shown. Moreover, the relative contributions of epidermal Langerhans cells and dermal DC to the "cord" populations are unclear. Therefore, we attempted to study in more detail the pathway of DC migration in murine skin organ cultures. Moreover, we extended earlier investigations on the issue of Langerhans cell emigration from the epidermis in response to haptens *in vivo*. We used antibodies to the cytoskeletal protein vimentin and electron microscopy as a means to monitor immunohistochemically and ultrastructurally the disappearance of Langerhans cells from the epidermis.

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Abbreviations: DC, dendritic cells; DCNB, 2,4-dichloro-1-nitrobenzene; DNTB, 2,4-dinitrothiocyanatebenzene.

MATERIALS AND METHODS

Mice and media Mice of inbred strains C57BL/6, BALB/C, and C3H/He were purchased from Charles River (Sulzfeld, Germany) and used at 6–12 wk of age. Each control or experimental panel consisted of three to five mice. The culture medium used throughout was RPMI 1640 supplemented with 10% fetal calf serum, gentamycin (all from Biological Industries, Kibbutz Beit Haemek, Israel), and 2-mercaptoethanol (Sigma, St. Louis, MO).

Induction of contact sensitivity The contact sensitizers 2,4-dinitro-1-chloro-benzene (DNCB) and 2,4-dinitro-1-fluorobenzene (DNFB) were obtained from Sigma, and 2,4,6-trinitro-1-chlorobenzene (picryl-chloride, TNCB) was from Kodak Eastman (Rochester, NY). The tolerogen 2,4-dinitrothiocyanatebenzene (DNTB) was purchased from ICN Biochemicals (Plainview, NY), and the nonsensitizer 2,4-dichloro-1-nitrobenzene (DCNB) was from Aldrich (Gillingham, U.K.) (Botham *et al*, 1987). All substances were applied onto the left ears of nonsensitized animals (10 μl per dorsal ear-half) at the subtoxic concentration of 1% in acetone-olive oil (4:1). A toxic effect was excluded by histologic examination of paraffin sections. The sensitizing potency was verified by elicitation experiments.

Elicitation of contact sensitivity Five days after sensitization, the right dorsal ear-halves were challenged with 5 μl of 1% hapten in acetone-olive oil. Twenty-four and 48 h after antigen challenge ear thickness was measured and compared with that of unsensitized control animals that had received only the challenge dose (Phanuphak *et al*, 1974). Ear thickness was quantitated using an engineer's micrometer (Peacock, Ozaki, Japan).

Skin organ culture Dorsal (i.e., cartilage-free) ear halves were rinsed in 70% ethanol, air-dried for ≈10 min, and cultured in 24 well tissue culture plates (Costar, Cambridge, MA) in 1.5 ml of culture medium at 37°C (Larsen *et al*, 1990; Ortner *et al*, 1996; Romani *et al*, 1997). One ear-half was placed into one well. Cultures were fed after 2 d by carefully aspirating 800 μl of spent medium and adding back the same volume of fresh medium. In some experiments skin was separated into epidermis and dermis by means of protease dispase before the onset of culture, using a previously described method (Kitano and Okado, 1983; Lenz *et al*, 1993).

Immunohistochemical techniques The epidermis was separated from the dermis by means of ammoniumthiocyanate as previously described (Juhlin and Shelley, 1977). Acetone-fixed (5 min at room temperature) epidermal and dermal sheets were immunostained applying the following sequence of steps: primary rat or mouse monoclonal antibodies (MoAb) (24 h at +4°C), biotinylated anti-rat or mouse Ig (Amersham International, Amersham, U.K.; 90 min at 37°C), streptavidin-peroxidase complex or streptavidin Texas Red (both from Amersham; 90 min at 37°C). Alternatively, rat MoAb were visualized by a previously described sensitive three-step method (Romani *et al*, 1985) using a mouse polyclonal anti-rat Ig (a kind gift of Dr. R. North, Trudeau Institute, Saranac Lake, NY) followed by tetramethylrhodamine isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse IgG + IgM (90 min at 37°C, Tago, Burlingame, CA). The immunoperoxidase protocol was continued by visualizing peroxidase acitivity with 3′3′-diaminobenzidine-H_2O_2. Immunofluorescence was carried on by double-labeling for MHC class II in order to identify DC. After a blocking step with rat Ig (100 μg per ml), sheets were incubated in a final step with fluorescein isothiocyanate-conjugated anti-MHC class II [clone 2G9; Pharmingen (San Diego, CA) or MoAb B21–2/anti-I-$A^{b,d}$ conjugated with fluorescein according to standard methods; 4°C/overnight]. Fluorescent sheets were mounted onto microscopic slides in Vectashield (Vector, Burlingame, CA) mounting medium. To visualize vimentin intermediate filaments it was advantageous to add 0.5% saponin (Sigma) to all washing solutions and antibody dilutions. This treatment enhanced antibody penetration into the cytoplasm of the cells. It did not change the staining pattern of the other antibodies used in this study.

Antibodies Anti-I-$A^{b,d}$ (B21–2/TIB229; rat IgG2b; R.M. Steinman, The Rockefeller University, New York, NY); anti-I-A^{d} (MK-D6/HB3; mouse IgG2a; ATCC, Rockville, MD); anti-I-$A^{k,r,f,s}$ (10–2.16/TIB93; mouse IgG2b; R.M. Steinman); anti-I-$E^{k,d}$ (14–4–4S/HB32; mouse IgG2a; ATCC); invariant chain (In1; rat IgG2b, N. Koch, University of Bonn, Germany); anti-macrophage (F4/80/HB198; rat IgG2b; ATCC); anti-CD11b/Mac-1 (M1/70/TIB128; rat IgG2b; ATCC); anti-CD32/FcγRII (2.4G2/HB197; rat IgG2b; ATCC); dendritic cells/DEC-205 (NLDC145; rat IgG2a; G. Kraal, Free University, Amsterdam, The Netherlands; Kraal *et al*, 1986); intracytoplasmic, vesicle-bound molecule (2A1; rat IgG2a; R.M. Steinman; positive in mature DC/negative in immature DC; Inaba *et al*, 1992); CD54/ICAM-1 (BE29G1; rat IgG2a; B.A. Lollo, University of California, San Diego; Kuhlman *et al*, 1991); CD86/B7–2 (GL1; rat IgG2a; Pharmingen, San Diego, CA; positive in mature DC/negative in immature DC; Inaba *et al*, 1994); anti-vimentin (mouse IgM; V. Small, Austrian Academy of Sciences, Salzburg, Austria, and P. Leoncini, Istituto Sclavo, Siena, Italy; Cintorino *et al*, 1989); and anti-vimentin (polyclonal rabbit Ig; F. Ramaekers, University of Nijmegen, The Netherlands; de Waal *et al*, 1984).

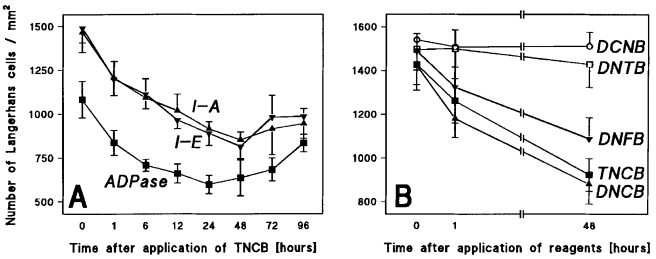

Figure 1. The density of Langerhans cells decreases specifically in response to contact allergens. One per cent solutions of different sensitizing or nonsensitizing compounds were applied epicutaneously. Epidermal sheets of BALB/C mice were evaluated by counting under the microscope. (*A*) Immunohistochemistry using MoAb against I-A (MoAb B21–2) and I-E (MoAb HB-32) as well as enzyme-histochemistry (ADPase) show a similar decrease in Langerhans cell densities in the first 2 d after application of TNCB. (*B*) Immunostaining for MHC class II (MoAb B21–2) reveals a decrease of Langerhans cells in response to three different contact sensitizers (DNFB, DNCB, TNCB). In contrast, a nonsensitizing (DCNB) and a tolerizing (DNTB) compound left the Langerhans cell numbers unchanged. *Error bars*, mean ± SD (n = 5).

ADPase-staining Epidermal sheets were prepared as described above, fixed in 5% paraformaldehyde in 0.1 M sodium cacodylate buffer, and stained for nucleoside-diphosphatase according to the method of Chaker *et al* (1984).

Evaluation of immunohistochemistry The density of Langerhans cells in epidermal sheets was counted under the microscope using ×40 objective lenses and a calibrated grid. Areas to be counted were selected for interfollicular regions of even staining and regular distribution of positive cells. There, 50 grids were randomly chosen for each antibody and experiment (150 for controls). Fields containing hair follicles were excluded from analyses. Mean values and standard deviations were determined. In addition, epidermal sheets stained with the peroxidase technique were subjected to image analysis using bright field microscopy (Axiomat; Zeiss, Oberkochen, Germany). At a magnification of ×250, at least 200 randomly selected visual fields were evaluated for each experimental condition in a fully automated system (VIDAS; Kontron, München, Germany). Langerhans cells were counted and basic statistical parameters (mean, standard deviation, Mann–Whitney U test: $p < 0.01$) were calculated.

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

Functional assays Antigen processing capacity of DC was measured by means of a hen egg lysozyme peptide specific TT hybridoma [Hd-1.AC5 (Jensen, 1988); a kind gift of Dr. S.I. Katz, Bethesda, MD] as described (Koch *et al*, 1995). The capacity to sensitize resting T cells was determined in a oxidative mitogenensis assay (Schuler and Steinman, 1985).

RESULTS

Langerhans cells leave the epidermis both after epicutaneous application of contact sensitizers and upon skin organ culture immunohistochemistry Using TNCB as contact sensitizer and anti-MHC class II MoAb, as well as ADPase reactivity, to visualize Langerhans cells, a progressive reduction in the number of reactive cells, i.e., Langerhans cells, was observed (**Fig 1**). One hour after application an insignificant decrease of 10–20% was evident. The number of reactive cells dropped in a time dependent fashion and reached the lowest levels at 48 h; 30–40% reduction as compared with the untreated control (**Fig 1**). Langerhans cell densities then slowly recovered but still remained about 20% below control levels at 96 h. It was consistently noted that the numerical reduction was more pronounced with ADPase as a Langerhans cell marker than with anti-MHC class II MoAb (**Fig 1*A***). The morphologic appearance of this reduction was a general thinning out of antibody/enzyme reactive

Table I. The density of Langerhans cells decreases specifically in response to contact allergens[a]

Treatment	0 h	1 h	24 h	48 h
Anti-MHC class II immunostaining				
None	1219 ± 189 (100%)			
Acetone/olive oil		996 ± 196 (–18%)	1070 ± 214 (–12%)	1073 ± 237 (–12%)
TNCB		994 ± 222 (–18%)	759 ± 200 (–38%)	687 ± 209 (–44%)
DNTB		nt	1037 ± 196 (–15%)	1074 ± 209 (–12%)
DCNB		nt	1091 ± 218 (–11%)	1073 ± 216 (–12%)
Anti-vimentin immunostaining				
None	1211 ± 195 (100%)			
Acetone/olive oil		nt	nt	1080 ± 165 (–11%)
TNCB		nt	nt	783 ± 153 (–36%)
DCNB		nt	nt	1054 ± 198 (–13%)

[a]Epidermal sheets from ears of BALB/C mice that had been treated epicutaneously with the indicated reagents (see *Material and Methods*) were immunostained for MHC class II (MoAb B21–2) or vimentin and evaluated by morphometry. Langerhans cell densities at 24 and 48 h after TNCB (i.e., sensitizer) treatment were significantly decreased (underlined; $p \leq 0.01$; Mann–Whitney U test) as compared with both untreated epidermis (100%) and acetone-olive oil, or nonsensitizer treated (i.e., DNTB and DCNB) epidermis. One hour values were not significantly different. One experiment of two is shown here. Numbers indicate Langerhans cells per mm^2 ± SD. Ac/ol-oil, acetone/olive oil; nt, not tested.

cells. Patches totally devoid of staining were occasionally observed. Langerhans cells in hapten treated epidermal sheets were bigger than on untreated sheets or acetone-olive oil treated sheets, and their class II expression was clearly enhanced (not shown). They did not look damaged, though. Cell counts were confirmed by computer assisted morphometrical analysis (**Table I**). An identical time dependent reduction of Langerhans cells as determined by anti-MHC class II immunolabeling was found for the other contact allergens, DNCB and DNFB (**Fig 1*B***). The application of acetone-olive oil alone resulted in a consistent reduction by about 12% in the number of cells reacting with anti-MHC class II MoAb (**Table I**) and displaying ADPase reactivity. In epidermal sheets from cultured ear-halves we noted a much more pronounced decrease in the density of class II positive epidermal cells, i.e., Langerhans cells (data not shown). The values for days 1 and 3 of culture were 50% and 75%, respectively, lower than those of untreated epidermis. This was exactly as described by Larsen *et al* (1990).

To ascertain whether the observed reduction in the numbers of antibody-reactive cells was due to the physical disappearance of the cells from the epidermis rather than to a mere loss of surface antigens, we stained epidermal sheets from TNCB treated mice with different anti-vimentin reagents. These experiments were carried out with BALB/C mice that contain only negligible numbers of vimentin expressing dendritic, γ/δ TCR bearing epidermal T cells (Bergstresser *et al*, 1983; Tschachler *et al*, 1983). Again, a concomitant reduction of vimentin and MHC class II reactive cells was observed. Forty-eight hours after application of TNCB, morphometrical analysis revealed a statistically significant difference ($p \leq 0.01$) between vimentin-positive cells in sheets from control skin *versus* sensitizer treated skin (**Table I**). Reduction of vimentin-positive cells was 36% and 28% in relation to untreated and vehicle treated epidermis, respectively (**Table I**; **Fig 2**). Likewise, in organ cultures the density of vimentin reactive cells dropped concomitantly with the numbers on MHC class II reactive cells (**Fig 2**).

When epidermal sheets of TNCB treated mice were stained with MoAb against other antigens expressed on the surface of resident Langerhans cells (F4/80, CD11b, CD32, NLDC-145/DEC-205), an identical pattern of reduction was found. Moreover, double-labeling for these antigens and MHC class II demonstrated that all cells positive for CD11b, CD32, F/80 macrophage antigen, and NLDC were also positive for MHC class II and vice versa. In other words, there appeared to be no cells that had lost class II but still expressed one of the other antigens and vice versa.

For all three contact allergens we obtained ear swelling responses that were comparable with the published values (Phanuphak *et al*, 1974; Bergstresser *et al*, 1980; Cruz *et al* 1989) (data not shown).

Numbers of Langerhans cells do not decrease after epicutaneous application of a tolerizing or a nonsensitizing substance It has been shown that chemically closely related compounds exert different effects when applied epicutaneously. Whereas DNCB is a contact sensitizer, DCNB does not sensitize mice (Botham *et al*, 1987; Kolde and Knop, 1987). Similarly, DNTB that is structurally similar to the sensitizer DNFB induces specific tolerance (Kolde and Knop, 1987), at least at the concentration used in this study (Dearman *et al*, 1997). We therefore wondered whether these differential sensitizing potentials were reflected by a differential migratory behavior of Langerhans cells. Using anti-MHC class II (anti-I-A and anti-I-E) as well as anti-vimentin MoAb, we observed the expected numerical decrease of antibody-reactive cells in response to the known contact sensitizers (**Fig 1**). In contrast, skin painting with DNTB (1%) and the nonsensitizer DCNB (1%) left the Langerhans cell densities virtually unchanged. After 48 h the decrease in the number of labeled cells was not more than the decrease recorded in the acetone-olive oil control (**Fig 1*B***). Staining with anti-vimentin antibodies yielded identical results. The size and shape of Langerhans cells as well as anti-class II staining intensity on tolerogen and nonsensitizer treated epidermal sheets did not differ from control sheets. Evaluation by computer assisted image analysis confirmed the data (**Table I**).

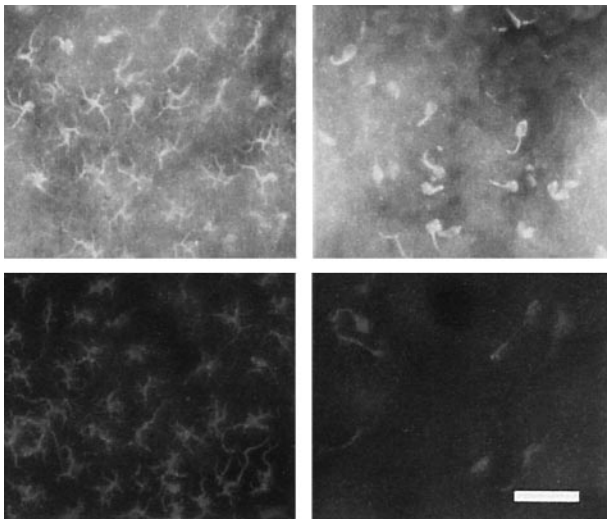

Figure 2. Vimentin reactive epidermal DC (i.e., Langerhans cells) decrease in response to contact allergens and in organ culture. Distribution of vimentin-reactive cells in epidermal sheets from untreated (*left panels*) *versus* TNCB treated (48 h; *upper right*) and cultured (48 h; *lower right*) skin. Evaluation by counting and morphometry showed that the reduction of Langerhans cells amounted to 36% after TNCB and >80% after culture. Note the larger size and the distorted morphology of Langerhans cells in contact allergen treated and cultured skin (*right panels*). *Scale bar*, 50 μm.

DC enter lymphatic vessels in skin organ culture and, to a lesser degree, after epicutaneous application of contact sensitizers Like Larsen *et al* (1990), we observed abundant accumulations of

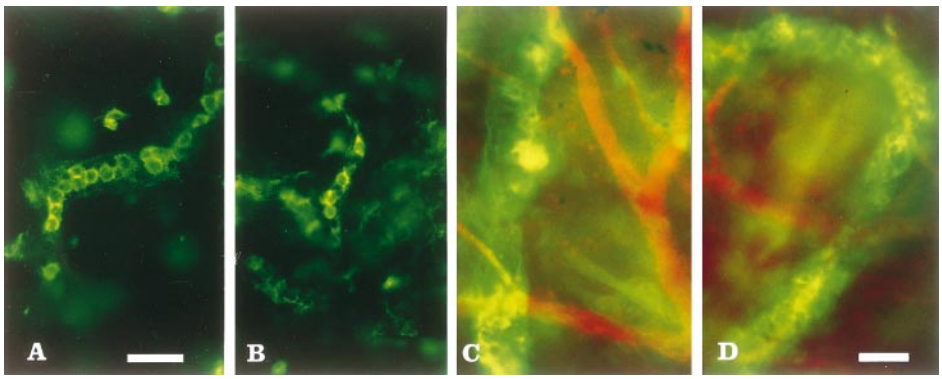

Figure 3. Migratory DC do not accumulate in dermal blood vessels. Dermal "cords," visualized by immunohistochemistry for MHC class II, form in untreated skin cultured for 3 d (*A*, *C*, *D*) and 3 d after *in vivo* application of a contact sensitizer (TNCB) (*B*). Note the characteristic nonrandom, string-like distribution of strongly MHC class II expressing cells. In (*C*) and (*D*) dermal sheets were double-labeled with MoAb against CD54/ICAM-1, identifying blood endothelia (*red fluorescence*) and class II, identifying DC (*green fluorescence*). There is no overlap between the two markers, suggesting that the "cords" (*green fluorescence*) do not represent blood vessels. *Scale bars*, (*A*, *B*) 75 μm; (*C*, *D*) 50 μm.

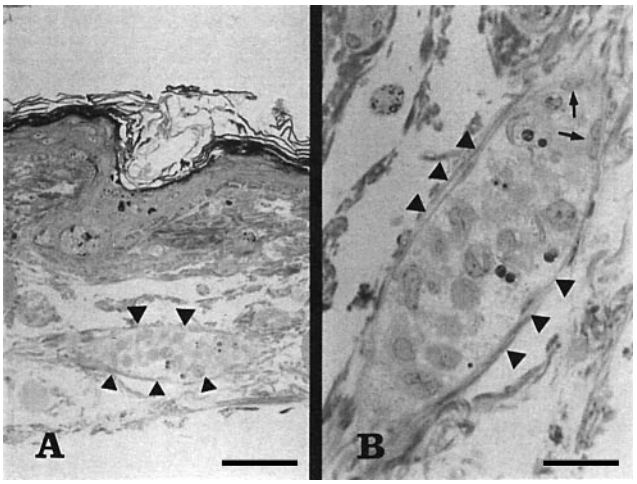

Figure 4. Morphology of migratory DC on semi-thin sections of cultured skin. Skin was cultured for 3 d and embedded for electron microscopy. Methylene blue stained 1 μm sections show cells with the typical morphology of DC (lobulated and excentric nuclei, light cytoplasm). They are located in wide clefts in the dermis, marked with triangles. Of the thin endothelial cell lining only the characteristic nuclei (*arrows*) that bulge into the lumen can be appreciated. (*B*) is a higher magnification of (*A*). *Scale bars*, (*A*) 50 μm, (*B*) 25 μm.

strongly class II cells in dermal sheets ("cords") after 2–3 d of culture (**Fig 3*A***). "Cords" also developed *in vivo* in contact sensitizer treated (TNCB) skin (**Fig 3*B***), though their frequency was markedly lower than in organ culture. In addition, we harvested typical mature DC from the culture medium as described (Larsen *et al*, 1990; Ortner *et al*, 1996). When the epidermis was removed from the dermis by dispase treatment and the dermal part was cultured by itself for 2–3 d, the same structures could be found. This shows that dermal DC also migrate via lymph vessels (see below).

Double-labeling of dermal sheets from skin cultured for 2–3 d for MHC class II (identifying DC and thus "cords") and CD54/ICAM-1 (identifying blood vessel endothelia; Kuhlman *et al*, 1991), revealed that the "cords" were CD54 negative. Conversely, CD54-positive structures with the typical morphology of blood vessels did not contain DC (**Fig 3*C***, ***D***). These findings rule out the possibility that dermal "cords" might be blood vessels.

On semi-thin sections of cultured skin specimens embedded for electron microscopy, accumulations of DC in the dermis became readily apparent. **Figure 4** shows considerable numbers of conspicuous cells in wide clefts that correspond to the immunohistochemically detectable "cords." The cells exhibit features typical for DC, such as long and thin cytoplasmic processes ("veils"), lobulated and excentrically localized nuclei, and a light cytoplasm. They are surrounded by a very thin endothelial lining of which, on semi-thin sections, only the characteristic nuclei that protrude into the lumen can be seen.

Electron microscopy provided the ultrastructural proof that the "cords" in cultured murine skin were in fact lymphatic vessels. The dermal clefts were lined by a thin sheet of endothelial cytoplasm. These endothelial cells rested on a discontinuous, in most places absent, basement membrane (**Fig 5**). Anchoring filaments extending from the ablumenal plasma membrane into the underlying connective tissue were observed (Gerli *et al*, 1991). Weibel-Palade bodies, which are characteristic for blood endothelial cells (Ryan, 1989), were not detected. Occasionally the endothelial lining was interrupted. Tight junctional specializations as seen in blood endothelia did not occur. Pericytes were not present; erythrocytes were not found. The cells within the lumena of the vessels displayed all signs of mature DC. They extended thin and long cytoplasmic, organelle-free sheets ("veils") in all directions. The nuclei were strongly lobulated. The electron-light cytoplasm contained few typical electron-dense lysosomes but many small, peripherally located electron-lucent ("empty") vesicles and a variable number of multivesicular organelles that, by mere morphology, corresponded to MHC class II containing organelles or "MIIC" (Kleijmeer *et al*, 1994). In profiles of 25 different DC an average of four (range 0–12) such organelles were found (**Fig 5**). Furthermore, DC displayed typical profiles of smooth endoplasmic reticulum. Birbeck granules were found in about one-fourth of DC; however, as compared with resident epidermal Langerhans cells their numbers were much less and they looked small and stunted.

Maturation of DC during migration We attempted to define the maturational state of migrating DC in organ cultures by immunohistochemistry for molecules that are differentially expressed on/in immature and mature DC (**Table II**), i.e., MoAb 2A1 and MoAb GL1 (anti-CD86) as markers for maturity; MoAb In1 (invariant chain) as an indicator for immaturity. In untreated epidermis, Langerhans cells *in situ* express MHC class II (mostly intracellularly; Kleijmeer *et al*, 1994; Mommaas *et al*, 1995) and invariant chain, but no CD86 nor the antigen recognized by MoAb 2A1. MHC class II expression in organ cultures is upregulated within a few hours (Larsen *et al*, 1990). **Figure 6** (*top panels*) shows that after 48 h of organ culture Langerhans cells *in situ* have acquired the expression of CD86; they are negative for 2A1 and they still express invariant chain. A similar expression pattern of Langerhans cells *in situ* was observed 48 h after the application of a contact sensitizer (3% TNCB; not shown): all Langerhans cells remaining in the epidermis expressed invariant chain; many Langerhans cells, though not all, became $CD86^+$. At the same time, most of the strongly MHC class II-positive cells (i.e., DC) in the dermal "cords" of cultures are positive for both maturation markers, CD86 and 2A1 (**Fig 6**, *bottom panels*), used in this study. Many of them still contain the invariant chain. Finally, the phenotype of DC that emigrated into the culture medium over the culture period of 2–3 d corresponded to that of mature DC, i.e. $CD86^+/2A1^+$. Yet, invariant chain was still expressed in a variable but substantial percentage of DC (≈40% of all MHC class II+ cells in three experiments: 20%/18%/82%). This was reflected in the capacity of migrant DC to process a native protein antigen much better than corresponding Langerhans cells cultured for 3 d (**Fig 7**). In contrast to cultured isolated Langerhans cells, Birbeck granules could be easily identified in DC within lymph vessels (≈25%; 25 profiles of DC in three different experiments) and in DC that had emigrated into the culture medium (≈10%; 50 profiles of DC in one experiment).

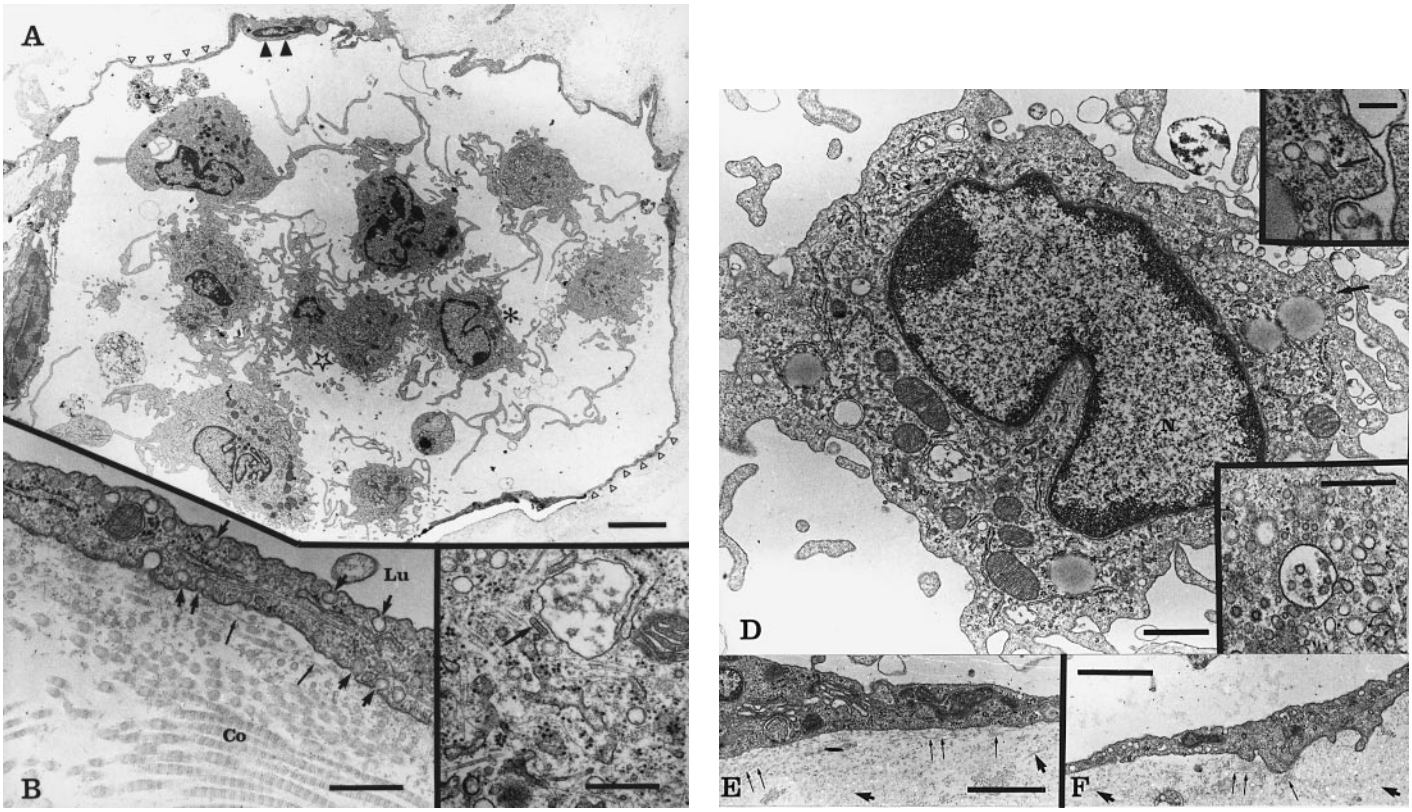

Figure 5. Ultrastructure of migratory DC within a lymph vessel ("cord"). Skin was cultured for 3 d and embedded for electron microscopy. In (*A*) many cells displaying the ultrastructural features of DC can be seen inside a wide lumen of a vessel that is surrounded by a thin monolayer of endothelial cells (*open arrowheads*). A nucleus of an endothelial cell protrudes into the lumen (*closed arrowheads*). DC possess irregular nuclei and extend many thin cytoplasmic processes ("veils"). The star and asterisk mark the cells enlarged in (*C*) and (*D*), respectively. (*B*) Note the lack of a basement membrane on the ablumenal side of the endothelial cell, anchoring filaments (*thin arrows*) and numerous pinocytotic vesicles (*bold arrows*). (*C*) High power of the cytoplasm of a DC. Smooth endoplasmic reticulum and one runted Birbeck granule (*arrow*) become apparent. (*D*) Ultrastructure of another DC in the lumen of the lymph vessel. Note the tennis racket-shaped Birbeck granule (*arrow* and enlarged in the *upper inset*). An organelle (from another DC) morphologically corresponding to an MIIC is shown in the *lower inset*. Parts (*E*) and (*F*) show more prominent accumulations of typical anchoring filaments (*thin*, *long arrows*; *short arrows* point at longitudinally as well as transversely sectioned collagen fibrils) on the ablumenal side of a lymph vessel. Lu, lumen; Co, collagen fibrils; N, nucleus. (*A*) *scale bar*, 3.5 μm; (*B*, *C*) lower inset in (*D*), *scale bar*, 0.5 μm; (*D*) *scale bar* = 1 μm; upper inset in (*D*), *scale bar* = 0.2 μm; (*E*, *F*) *scale bar* = 1 μm.

Table II. DC mature during migration. Expression of maturation markers and functional properties of migrating DC in skin organ cultures[a]

	MHC class II	Invar. chain	CD86	MoAb 2A1	Pr[b]	S[c]
Freshly isolated Langerhans cells *in vitro*	++[d]	+	–	±	+++	±
Langerhans cells *in situ* in uncultured epidermis	+[d,e]	+	–	–	nt[f]	nt
Langerhans cells *in situ* in epidermis from 2–3 d cultured skin	+++	+	+	–	nt	nt
Langerhans cells and dermal DC *in situ* in dermis from 3 d cultured skin/"cords"	+++	+	++	+++	nt	nt
Emigrated Langerhans cells and dermal DC *in vitro* ("crawl-out" cells)	+++[d]	++	++	+++	++	+++
Cultured Langerhans cells *in vitro*	+++[d]	–	++	+++	±	+++

[a]Antigen expression on epidermal and dermal sheets from skin cultured for 3 d was determined by immunohistochemistry. For comparison, staining patterns and functional data of freshly isolated and 3 d cultured epidermal Langerhans cells are included. Fluorescence staining intensities are presented in an arbitrary scale (– to +++).
[b]Pr, antigen processing capacity, as determined by means of peptide specific T cell hybridomas (Koch *et al*, 1995).
[c]S, sensitizing capacity for resting T cells, as determined in the allogeneic mixed leukocyte reaction (Schuler and Steinman, 1985; Lenz *et al*, 1993; Ortner *et al*, 1996).
[d]Surface expression.
[e]Data from Kleijmeer *et al* (1994) and Mommaas *et al* (1995).
[f]nt, not testable.

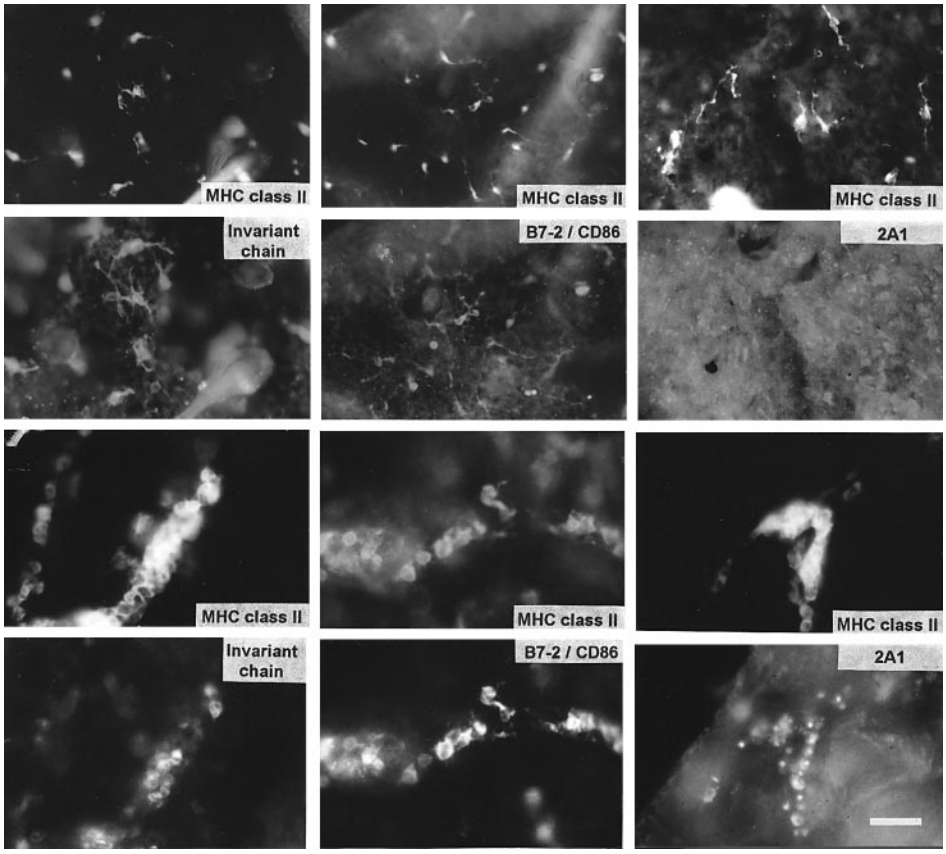

Figure 6. Migratory DC express maturation markers as well as signs of immaturity *in situ*. Epidermal (*top two rows*) and dermal (*bottom two rows*) sheets from skin cultured for 48 h were double-immunolabeled for MHC class II and the indicated molecules. Langerhans cells in the epidermis and DC within "cords" express invariant chain (*left column*) and CD86 (*middle column*). Maturation marker 2A1, however, is expressed only on DC within "cords" (*right column*, *bottom pictures*) but not on Langerhans cells in the epidermis (*right column*, *top pictures*). *Scale bar*, 25 μm.

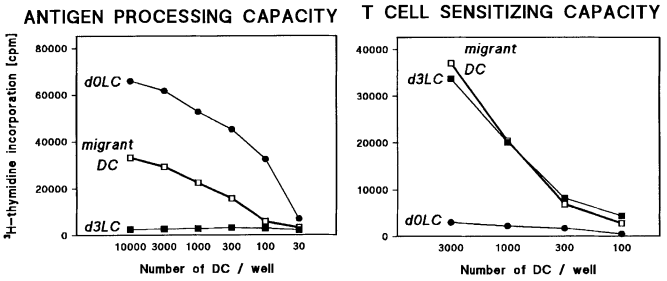

Figure 7. Populations of DC that have emigrated into the culture medium retain the capacity to process native protein antigen. Antigen processing capacity of BALB/C DC that had emigrated into the culture medium during the culture period of 3 d was measured by the response of a hen egg lysozyme specific T cell hybridoma. Compared with a "classical" population of mature DC, i.e., Langerhans cells that had been cultured for 3 d together with keratinocytes (■), migratory DC (□) retain a considerable capacity to process the protein and present it to the hybridoma (*left panel*). When tested for their T cell sensitizing capacities in an allogeneic mixed leukocyte reactions, migrant DC behave like typical mature DC (*right panel*). This experiment is representative of three experiments.

DISCUSSION

Pathway of migration By immunohistochemistry and electron microscopy we found a reduction of epidermal Langerhans cells and typical string-like accumulations of DC in the dermis ("cords") in response to hapten stimuli as well as upon skin organ culture. These data complement existing experimental evidence for the migration of Langerhans cells to the lymphoid organs, and allow important conclusions to be drawn. (i) Our data prove that migratory cutaneous DC accumulate in and migrate through dermal lymphatic vessels. Blood vessels are not a preferred pathway in the skin. This is analogous to our previous findings in human skin organ cultures (Lukas *et al*, 1996). The dermal pathway of DC migration as originally defined by the work of Larsen *et al* (1990) leads indeed through the afferent lymphatics. (ii) Our data provide additional evidence that a substantial number of epidermal Langerhans cells physically leave the epidermis during organ culture or the induction phase of contact hypersensitivity. They render it unlikely that the decrease of immunohistochemically detectable Langerhans cells in response to hapten treatment is merely due to a loss of immunoreactivity of surface molecules. We base this conclusion on the facts that (i) immunostaining for the cytoskeletal protein vimentin revealed similar reductions in Langerhans cell densities as immunostaining for cell surface molecules, and (ii) six different surface molecules (MHC class II, F4/80, CD11b, CD32, DEC-205, ADPase) decreased concomitantly. Thus, these findings broaden the experimental basis for the concept that hapten-laden DC that have been recovered from lymph nodes draining the sites of epicutaneous hapten application are derived from cutaneous DC (Macatonia *et al*, 1987). Still, diffusion of free or protein-bound hapten into the nodes cannot be ruled out. This may be biologically relevant in a situation (low doses of hapten/low zone tolerance) where hapten carriage by Langerhans cells does not appear to play a role (Steinbrink *et al*, 1996).

Induction of migration In the contact hypersensitivity model the emigration of Langerhans cells from the epidermis as well as the formation of "cords" occurred specifically in response to contact sensitizing agents but not to nonsensitizing (Botham *et al*, 1987) compounds. The specificity in migratory behavior is paralleled by sensitizer specific changes in the ultrastructural reaction patterns of Langerhans cells (Kolde and Knop, 1987; Becker *et al*, 1994; Steinbrink *et al*, 1996). There is also evidence that epicutaneously applied haptens induce specific cytokine production patterns in epidermal cells. Most notably, IL-α and MIP-2 in keratinocytes (Enk and Katz, 1992), and IL-1β are upregulated in Langerhans cells (Enk and Katz, 1992). TNF-α is induced nonspecifically in keratinocytes (Enk and Katz, 1992). These cytokines were shown to be involved in migration of DC in contact hypersensitivity (Enk *et al*, 1993; Cumberbatch *et al*, 1994) and in skin organ cultures (unpublished observations). Their expression in skin organ cultures has not yet been studied in detail. Messenger RNA for granulocyte-macrophage colony stimulating factor has shown to be elevated in murine skin organ cultures (Larsen *et al*, 1994).

Derivation of migrating DC in the dermis The observation of Birbeck granules in about one-fourth of "cord" DC strongly indicates that part of these DC are epidermis derived. We had previously observed the same in human skin organ cultures (Lukas *et al*, 1996). Dermal DC (Lenz *et al*, 1993; Nestle *et al*, 1993; Duraiswamy *et al*, 1994) probably also migrate to the nodes via the same route because "cords" were also found in cultures of epidermis depleted dermis. The functional competence of dermal DC for the induction of contact hypersensitivity has been demonstrated (Streilein, 1989; Tse and Cooper, 1990). We conclude that in whole skin cultures the DC population in the dermal lymphatics is composed of both Langerhans cells and dermal DC.

Maturation during migration We found that migratory DC display the morphology and several phenotypical features of mature DC, already within the epidermal environment. This confirms the work of others who had noted that emigrating murine DC in the "cords" abundantly express costimulatory molecules recognized by the CTLA-4 molecule (CD80 and/or CD86; Larsen *et al*, 1994); as do DC that can be recovered from the culture medium and that have arrived there from the epidermis and/or dermis, presumably via passage through "cords" (Inaba *et al*, 1994; Larsen *et al*, 1994; Pope *et al*, 1995). In addition, our data suggest a sequence of phenotypical events in the maturation of DC within the cutaneous environment (**Table II**). The first signs of maturation are the upregulation of MHC class II (Larsen *et al*, 1990) and the de novo expression of CD86 (still in the epidermis). This is followed by the appearance of perinuclear 2A1 staining (in the "cords"), and finally the loss of invariant chain. Pierre *et al* (1997) have studied these changes in the same skin organ culture model at a more refined level by using confocal microscopy. They additionally observed a translocation of MHC class II molecules from intracellular endosomal/lysosomal compartments in immature ("early") DC to the plasma membrane in mature ("late") DC. These data underscore the notion that migration and maturation of DC are tightly linked processes that are triggered by the same stimuli.

Aiba and Katz (1990) have demonstrated that Langerhans cells isolated from the epidermis 1 d after the application of contact sensitizers express signs of functional maturity, i.e., an increased capacity to stimulate resting T cells in an allogeneic mixed leukocyte reaction; they still retain a considerable activity to process native protein antigens, though. It seems that these cells have not yet completely developed into fully mature DC. Populations of DC-containing cells at different stages of maturation are not unusual. We have previously observed it in Langerhans cells from standard 3 d epidermal cell cultures (Koch *et al*, 1995). Three additional observations indicate that some features of maturation might lag behind: (i) 1–2 d after the application of contact allergens the remaining resident Langerhans cells had upregulated MHC class II and, in part, CD86, but still expressed CD32, F4/80 macrophage antigen, ADPase, and—most notably—invariant chain. Similarly, in cultured skin, populations of maturing migratory DC within lymph vessels (high MHC class II, $CD86^+$, in part $2A1^+$) still displayed invariant chain. In contrast, in Langerhans cells cultured for 2–3 d these molecules can only be detected in minor subpopulations (invariant chain; Koch *et al*, 1995) or not at all (CD32, F4/80, ADPase; Schuler and Steinman, 1985) by immunohistochemistry. (ii) We readily found Birbeck granules in DC located in the lymphatic vessels and, to a lesser degree, in DC that had migrated into the culture medium. In cultured epidermal Langerhans cells, however, this was virtually impossible (Schuler and Steinman, 1985). (iii) The expression of invariant chain in a substantial percentage of emigrated Langerhans cells/DC may explain why these cell populations displayed a relatively high capacity to process native protein antigens as compared with the standard population of mature DC, namely cultured Langerhans cells (Koch *et al*, 1995). It may also help explain the finding of substantial antigen processing activity in populations of migratory rat lymph-derived DC (Liu and MacPherson, 1995).

Comparison of contact hypersensitivity *versus* skin organ culture model We were able to show similarities between the two models in terms of kinetics and pathways of DC migration. The triggering of migration and maturation by inflammatory cytokines also appears to be similar in the contact hypersensitivity model (Enk and Katz, 1992; Cumberbatch and Kimber, 1995) and in the skin organ culture model (manuscript in preparation). This would emphasize the relevance and importance of the latter model (Steinman *et al*, 1995) for studying aspects of contact hypersensitivity. A major difference is the extent to which dermal "cords" are formed: many "cords" are formed in skin organ cultures but few are formed in the *in vivo* contact hypersensitivity model (Silberberg-Sinakin *et al*, 1976; Schuler *et al*, 1991). A reason for this might be that in the organ cultures the lymphatic vessels are artificially (partly) clogged, which would result in the observed accumulation of DC. *In vivo*, however, the lymph flow is not obstructed and therefore "cords" do not become obvious that frequently. We believe that skin organ culture will prove useful for investigating the interplay of cytokines and adhesion molecules involved in migration.

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