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Expression and modulation of C5a receptor (CD88) on skin dendritic cells. Chemotactic effect of C5a on skin migratory dendritic cells

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SUMMARY

Although it is known that dendritic cells (DC) migrate in response to inflammatory stimuli, there is little information about the expression of receptors for chemotactic factors on DC. The present study has demonstrated by double immunostaining and flow cytometry of Langerhan's cell (LC)-enriched epidermal cell suspensions that a small subpopulation (5–6%) of epidermal resident LC (rLC) expresses receptors for C5a (C5aR). Epidermal rLC positive for C5aR show a round-shape morphology, were located next to the basement membrane, and express HLA-DR molecules higher than C5aR negative rLC. These observations suggest that rLC would express C5aR as part of their process of maturation during tissue trafficking. To investigate whether epidermal LC up-regulate C5aR along their differentiation pathway, LC were differentiated *in vitro* after culture in epidermal cell suspensions supplemented with granulocyte–macrophage colony-stimulating factor (GM-CSF). As a result, *in vitro* differentiated LC increased the expression of C5aR up to 69% of the DC population. In accordance with this observation, interdigitating DC of secondary lymphoid organs (lymph node and tonsil) also expressed C5aR. Migratory CD1a positive DC that spontaneously migrated out of dermal or split-skin organ explants were also positive for C5aR and were used for chemotaxis and chemokinesis assays in response to human recombinant C5a (rC5a). Optimum migration to rC5a was observed at 10^{-8} M with a sigmoidal dose–response curve. Checkboard analysis demonstrated that locomotion in response to rC5a was chemotaxis and not chemokinesis.

INTRODUCTION

Dendritic cells (DC) are leucocytes highly specialized in antigen processing and presentation to both naive and memory T cells.[1,2] In human skin, epidermal Langerhan's cells (LC) and dermal DC constitute a network of sentinel DC capable of uptaking and processing antigens as a previous step to present antigenic peptides to T cells. This involves the maturation of intra-epithelial LC and subsequent LC migration from epidermis to draining lymph nodes.[3–7] Several studies have reported DC activation and subsequent DC trafficking from non-lymphoid tissues (lung, skin, gut, heart and kidney) during the early steps of the acute inflammatory response or after the administration of lipopolysaccharide, tumour necrosis factor (TNF)-α or interleukin-1 (IL-1).[8–15] In skin grafts and skin organ explants, dermal injury alone and the subsequent non-specific inflammatory response are sufficient to induce intra-epithelial LC phenotypical changes and to stimulate the migration of epidermal LC.[16–21] However, little is known about the chemoattractants released during the acute phase of an inflammatory response that cause DC migration from peripheral tissues.

C5a is a glycopeptide generated during inflammation by cleavage of C5 that might be included in the list of putative mediators capable of inducing DC migration from non-lymphoid organs during the early phase of the inflammatory response.[22] C5a has been reported as chemoattractant for other myeloid cells such as neutrophils, eosinophils, basophils, monocytes, and lately for DC generated *in vitro* after treatment of peripheral blood mononuclear cells with granulocyte–macrophage colony stimulating factor (GM-CSF) plus

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Abbreviations: ABC/Px, avidin–biotin complex peroxidase; APAAP, alkaline phosphatase anti-alkaline phosphatase; ATCC, American Type Culture Collection; CD, cluster of differentiation; DC, dendritic cell; FCS, fetal calf serum; GM-CSF, granulocyte–macrophage colony stimulating factor; HLA, human leucocyte antigen; HSA, human serum albumin; ICAM-1, intercellular cell adhesion molecule 1; IL, interleukin; LC, Langerhan's cell; LFA-3, leucocyte function-associated antigen 3; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; rC5a, recombinant C5a; rLC, resident LC; TNF, tumour necrosis factor; Vth IWHLDA, Fifth International Workshop on Human Leucocyte Differentiation Antigens.

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IL-4.[22,23] C5a was demonstrated to be chemotactic even for non-myeloid cells as large granular lymphocytes and germinal centre B cells.[24,25] These biological effects are elicited via binding to a C5a specific receptor (C5aR, clustered as CD88) which belongs to the family of seven transmembrane domain receptors that transduces signal via guanosine triphosphate (GTP)-binding regulatory proteins.[26–28] C5aR is present on the surface of target cells and is expressed quite lately along the route of maturation of myeloid cells.[28,29]

The aim of the present work was to study the presence of C5aR on skin DC and to investigate how its expression on epidermal immature LC was modulated according to the state of cell differentiation. The role of C5a as chemoattractant for skin DC was tested by means of chemotaxis and chemokinesis assays performed with fully mature DC obtained by spontaneous migration from skin organ explants.

MATERIALS AND METHODS

Monoclonal antibodies (mAb)

Two mAb specific for C5aR S5/1 (immunoglobulin G2a: IgG2a from Dr M. Oppermann) and W17/1 (Ig1 from Dr M. Oppermann) obtained from the Blind Myeloid Panel of the Fifth International Workshop on Human Leucocyte Differentiation Antigens (Vth IWHLDA) (Boston, 1993) were used in the present study. Other mAb which recognize structures present on DC that are modulated after DC differentiation were used to monitor DC maturation *in vitro*, they included: anti-CD1a: fluoroscein isothiocyanate (FITC)-OKT6 (IgG1, from Ortho, NJ, USA); anti-human leucocyte antigen (HLA)-Class I molecules: W6/32 (IgG2a, from ATCC); anti-HLA-Class II molecules: L243 (IgG2a, from ATCC); anti-Fcγ-RII (CD32): IV.3 (IgG2b, from Dr Dinces, Vth IWHLDA); anti-CD40: G28.2 (IgG1, from Dr Ledbetter, Vth IWHLDA); anti-intercellular adhesion molecule (ICAM)-1 (CD54): F10.2 (IgG1, from Dr Bloem, Vth IWHLDA); anti-lymphocyte function-associated antigen (LFA)-3 (CD58): BRIC5 (IgG2a, from Dr Anstee, Vth IWHLDA); anti-B7.1 (CD80): BB1 (IgM, gift from Dr Clark); and anti-B7.2 (CD86): BU63 (IgG1, from Dr Hardie, Vth IWHLDA).

LC-enriched epidermal cell suspensions

Epidermal cell suspensions were prepared by trypsinization of split-thickness skin specimens obtained from corrective plastic surgery (face). Briefly, skin was split-cut with a dermatome (1 mm thickness) and then incubated with 0·20% trypsin (Gibco, Grand Island, NY) in buffer (0·68% NaCl, 0·04% KCl, 0·1% glucose, 0·22% $NaHCO_2$, pH 7·2–7·4) for 20 min at 37°. After enzyme treatment, skin samples were immersed in RPMI-1640 supplemented with 20% heat-inactivated fetal calf serum (FCS) at 4°. Epidermal cells were detached from dermis by scraping and then blown in and out of a Pasteur pipette for 5 min. After filtration through sterile steal mesh (pore diameter: 35 μm), cells were washed three times in RPMI-1640 at 4° supplemented with 20% FCS. Cells were counted and viability was assessed with ethidium bromide and orange acridine. The suspension contained 0·5 to 1·5% LC, as judged by CD1a staining by flow cytometry, and viability was at least 85%. LC enrichment was achieved by Ficoll–Hypaque gradient (δ = 1·077 g/cm^3). After 15 min centrifugation at 300 g cells of the interface were collected and washed twice in RPMI-1640 with 20% FCS. This cell fraction consisted of 6 to 9% CD1a$^+$ cells, depending on the percentage of the starting CD1a$^+$ cells present in the bulk epidermal cell suspension.

In vitro *maturation of LC*

Bulk epidermal cell suspensions ($40–80 \times 10^6$ cells per skin sample) were cultured in RPMI-1640 supplemented with 15% heat-inactivated human AB serum, 20 mM/l Hepes buffer, 100 ng/ml of human recombinant GM-CSF (hrGM-CSF; Leucomax®, Schering-Plough, Republic of Ireland), 2 mM/l L-glutamine, 200 U/ml penicillin and 20 μg/ml gentamicin, in a CO_2 atmosphere of 5%, at 37°. After 72 hr of culture, the non-adherent fraction was harvested and subjected to a Ficoll–Hypaque density gradient (δ = 1·077 g/cm^3) as described, to eliminate debris, dead cells and most keratinocytes. Viability exceeded 80%, and the percentage of CD1a$^+$ cells ranged from 8 to 10%. Three experiments were performed with epidermal cells obtained from three different donors.

Migratory DC

Skin migratory DC were obtained from samples of corrective skin (face) surgery, as previously described.[17–21] Migratory DC were collected from whole skin, epidermis, or dermis by using different kinds of organ explants: (i) epidermal/dermal explants (split-skin explants); (ii) epidermal explants (epidermal sheets); and (iii) dermal explants (dermal sheets). Briefly, skin samples were rinsed in 70% ethanol, fixed on a cork plate and split-cut (0·5 mm thickness) by using a dermatome. To prepare epidermal/dermal explants, split-skin was cut into smaller squares (approx. 4 × 4 cm side) and cultured dermal side down in 100 mm tissue Petri dish (Falcon, Oxnard, CA) with RPMI-1640 culture media supplemented with 15% heat-inactivated FCS, 20 mM Hepes buffer, 2 mM glutamine, 20 μg/ml gentamicin, 200 U/ml penicillin, in a 5% CO_2 atmosphere and at 37°. To prepare epidermal and dermal explants, split-skin was incubated in 20 mM EDTA, pH 7·2–7·4, 2 hr at 37°. After that, epidermal sheets were microdissected from the underlying dermis by simple traction with fine forceps under stereomicroscopic observation.

During culture, split-skin explants and dermal sheets were maintained with the epidermal face at the air–liquid interface by means of a steel mesh. Otherwise, split-skin explants would have coiled up and sunk to the bottom of the dish and, as consequence, epidermis would have detached from dermis after 2–3 days of culture. Epidermal sheets were cultured without the mesh because they spread out and floated spontaneously at the air–liquid interface when placed on the culture medium with the dermal side down.

Flow cytometric analysis

LC-enriched epidermal cells suspensions obtained immediately after preparation, *in vitro* differentiated LC, and migratory DC obtained from skin explants were successively incubated with: (i) pooled human AB serum (1/10) 30 min, to block Fc receptors; (ii) primary mAb at optimal concentrations (0·5–1 μg/0·1 ml) for 60 min; (iii) R-phycoerythrin (R-PE)-conjugated goat anti-mouse immunoglobulin (Dako, Denmark) for 30 min; (iv) normal mouse serum (1/10) during 15 min, in order to block any residual free goat anti-mouse immunoglobulin binding sites; and (v) FITC-conjugated anti-CD1a for 45 min. All incubations and washings were done at 4° with RPMI-1640

supplemented with 5% FCS and 0·1% Na_3N. Cells labelled successively with irrelevant isotype-matched primary mAb, R-PE-conjugated goat anti-mouse immunoglobulin, and FITC-conjugated irrelevant IgG1 were used as negative controls. Fluorescence intensity was analysed by fluorescence activated cell sorting (FACStar^{plus}; Becton Dickinson, Mountain View, CA). Dead cells were excluded by gating with propidium iodide.

Immunoperoxidase technique

For light microscopy immunolabelling studies, tissue samples were embedded in Tissue-Tek OCT (Miles Laboratory Inc, Elkhart, IN), frozen in isopentane chilled in a bath of liquid nitrogen, and stored at −20°. Four micron sections were cut and mounted on poly-L-lysine coated (Sigma, St Louis, MO) slides. Cells migrated from organ explants were harvested from culture wells and spun onto glass slides using a Shandon cytocentrifuge (5 min at 230 ***g***). Slides were air-dried, fixed in cold (4°) acetone for 5 min, and stored until use.

For single-labelling procedures epidermal sheets, cryostat sections and cytospins were successively incubated as follows: (i) pooled human AB serum (1 : 10, 20 min, room temperature) to inhibit nonspecific binding by blocking Fc receptors; (ii) primary mAb (0·5–1 μg/0·1 ml) overnight, 4°; (iii) biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (Dako); and (iv) avidin–biotin complex peroxidase (ABC/Px) (Dako). Peroxidase activity was detected by incubation with the substrate 3-amino-9-ethylcarbazole (AEC) (Sigma). Endogenous peroxidase activity was blocked immediately before ABC/Px incubation by successive passages in 70% ethanol, 1% H_2O_2 in methanol, and 70% ethanol. Tissue sections and cytospins were counterstained with hematoxylin and mounted in phosphate-buffered saline (PBS)/glycerol (1 : 10). Murine irrelevant immunoglobulins of the same isotype of primary mAb were used as controls.

In some experiments double staining were performed by incubating epithelial sheets or cryostat sections with mAb S5/1, overnight at 4°, followed by biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin, and alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako). The reaction was developed with New Fuchsin (Sigma) to obtain an end product red colour. For the second marker, samples were exposed to normal mouse serum (1 : 10) 15 min at room temperature, to block any residual free goat anti-mouse immunoglobulin binding sites, and then successively incubated with either biotinylated anti-CD3 or biotinylated anti-HLA-DR followed by ABC/Px. The second reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) to obtain an end product brown colour and mounted in PBS/glycerol (1 : 10).

Chemotaxis assay

In vitro locomotion of migratory DC was assayed by the micropore filter technique. Briefly, 8×10^4 migratory DC in 0·5 ml of RPMI-1640, 10 mM Hepes with 0·5% human serum albumin (HSA) were seeded in the upper compartment of the chemotactic chambers (Nunc, Denmark) placed in a 24-well culture plate (Corning, USA). The upper and lower compartments were separated by a polycarbonate filter with a 8 μm pore size. The lower compartment was filled with 0·5 ml control medium (RPMI-1640, 10 mM Hepes with 0·5% HSA) or with different concentrations of recombinant human complement C5a (rC5a) (Sigma) diluted in the same medium. Chambers were incubated for 2 hr at 37° in a 5% CO_2 moist atmosphere. At the end of incubation the upper chamber with the filter were removed and the number of DC present in the lower compartment was assessed by flow cytometry using a cytometer (Cytoron; Ortho Diagnosis, NJ) equipped with the software program 'Research'. Checkboard analysis of chemokinetic activity of DC in response to rC5a was performed placing graded concentration of chemotactic factor in the upper compartment along with the cells to be tested. All experiments were performed in triplicate.

In some experiments polycarbonate filters were removed from the upper chamber, rinsed in PBS, fixed in 4% formalin at 4°, 20 min, and then exposed to PBS with 5% pooled human AB serum (1 : 10, 20 min, room temperature). Polycarbonate membranes were successively incubated with: (i) mAb anti-HLA-DR (1 μg/0·1 ml, overnight, 4°); (ii) biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (Dako); and (iii) ABC/Px (Dako). Peroxidase activity was developed with the substrate DAB (Sigma). Endogenous peroxidase activity was blocked immediately before ABC/Px incubation as described. After labelling, filters were rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and mounted on slides.

Statistical analysis

Analysis of variance (ANOVA) was used for statistical analysis of the data, with individual group means compared using *post hoc* Student Newman Kules analysis. $P < 0.05$ was considered significant.

RESULTS

C5aR is expressed by a subpopulation of epidermal LC

Epidermal cell suspensions enriched in LC were analysed for the expression of C5aR by two-colour immunofluorescence flow cytometry. LC were identified by their positivity for CD1a. The percentage of CD1a positive cells was further enriched from 6–9% to 100% by electronic gating. A small subset (< 6%) of the epidermal population of CD1a positive cells were also positive for C5aR (Fig. 1). Similar results were obtained using either the mAb S5/1 or the mAb W17/1, both specific for the extracellular domain of the C5aR.[30]

In normal epidermis there is a small subset of LC (approx. 10% of the whole population of epidermal LC), which expresses much higher levels of HLA-DR, and may be considered the *in situ* equivalent of the LC maturation observed after short term culture *in vitro*.[31] The relationship between the positivity for C5aR and the state of *in situ* maturation of LC was investigated by the analysis of the HLA-DR and C5aR double immunofluorescence on LC-enriched epidermal cell suspensions. As can be seen in Fig. 2, the subset of LC C5aR positive stained much brighter with anti-HLA-DR mAb than the subpopulation of LC C5aR negative.

Preparation of LC-enriched epidermal cell suspensions requires that thin fragments of skin are exposed to trypsin. Proteolytic treatment of this type might be expected to trim off trypsin-sensitive cell surface epitopes. To test whether the low number of C5aR positive LC was due to sensitivity of C5aR epitopes to trypsinization, epidermal sheets and cryostat

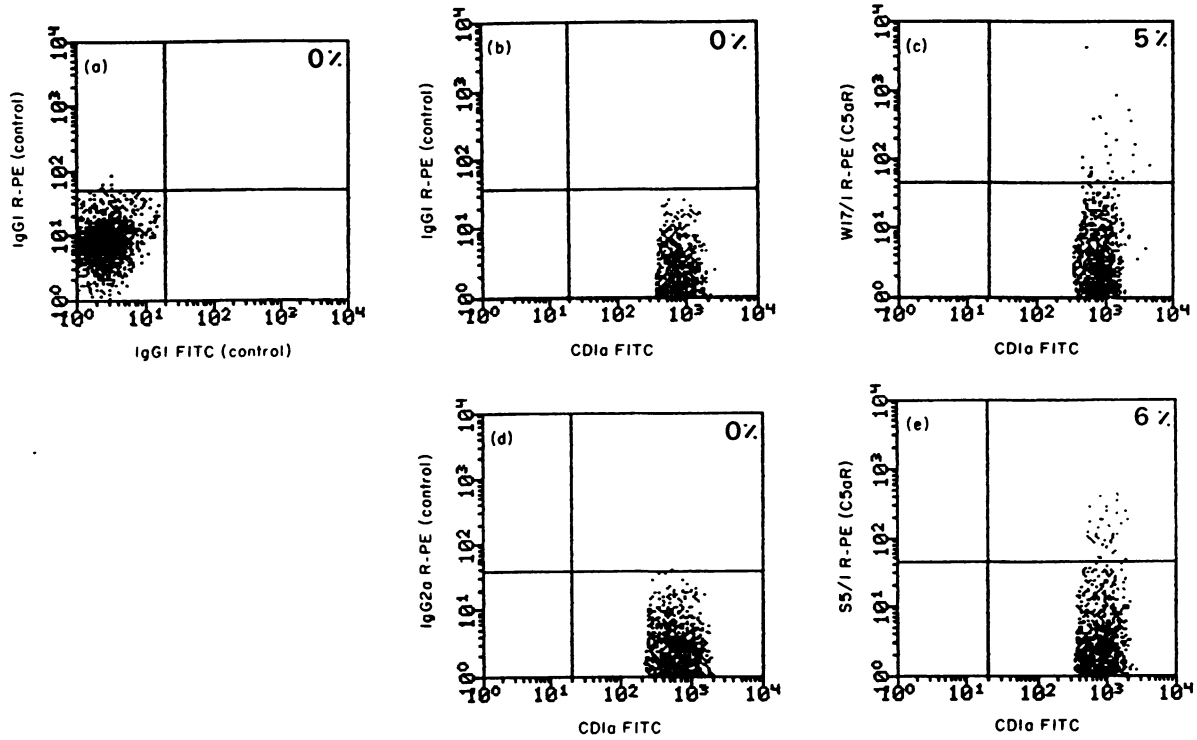

Figure 1. Expression of C5aR by human epidermal LC. Epidermal cell suspensions were obtained from skin and stained for simultaneous expression of C5aR (vertical axis) and CD1a (horizontal axis) and analysed by flow cytometry. (a) Vertical and horizontal markers were set to include >98% of negative cells in the left lower quadrant when isotype control IgG1 was used instead of primary mAb. (b–e) By means of electronic gating only the population of epidermal CD1a positive cells (e.g. LC) was analysed. (b and d) Vertical markers were set to include >99·5% of CD1a positive cells in right lower compartment when isotype controls IgG1–R–PE and IgG2a–FITC were used. (c and e) A small subset of CD1a positive cells coexpress C5aR.

sections of normal skin were immunostained by ABC/Px to detect the expression of C5aR. A low number of C5aR positive cells was observed in epidermal sheets (<3 cells/mm^2 of epidermis, five different experiments). Cells that carried C5aR were round-shaped (in some cases with cytoplasmic tail) and located at the same plane of focus than the basal and parabasal keratinocytes, next to the basement membrane (Fig. 3). Double immunostaining demonstrated that this cells co-expressed highly HLA-DR molecules.

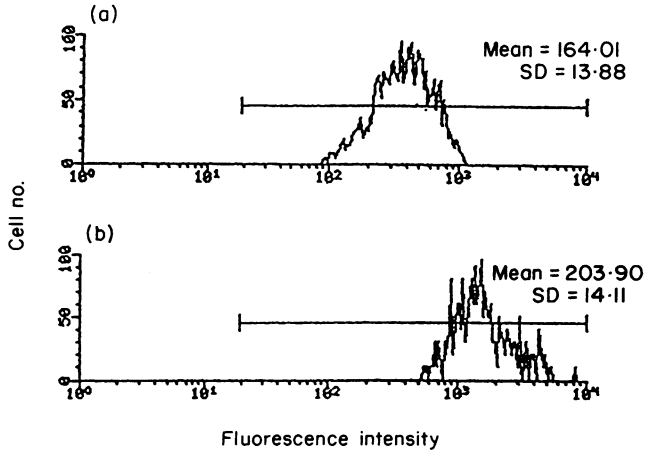

Figure 2. Expression of HLA-DR on C5aR positive versus C5aR negative epidermal LC. Epidermal cell suspensions were obtained from skin and stained for simultaneous expression of C5aR and HLA-DR and analysed by flow cytometry. Range bars were set to include <1% of positive cells when isotype control IgG1 was used instead of primary mAb. Histograms (a) and (b) illustrate respectively the expression of HLA-DR on C5aR negative LC and on C5aR positive LC. Subpopulations of LC were artificially purified by electronic gating.

C5aR expression during *in vitro* maturation of LC

In order to corroborate the putative relationship found

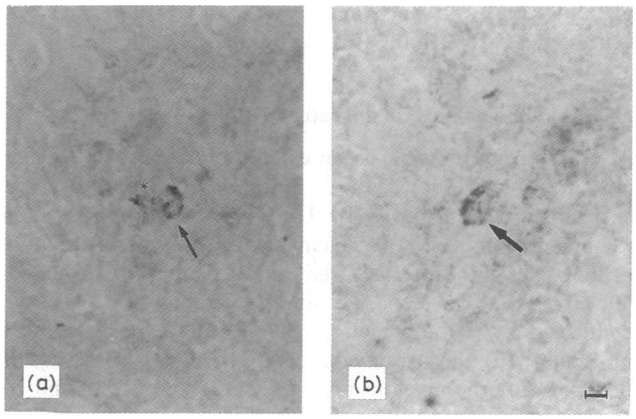

Figure 3. (a and b). Detail of an epidermal sheet immunostained for C5aR. Two cells positive for C5aR (arrows) located at level of basal and parabasal keratinocytes can be observed. One of them shows a short cytoplasmic tail or dendrite (a, asterisk). (APAAP, Bar 10 μm).

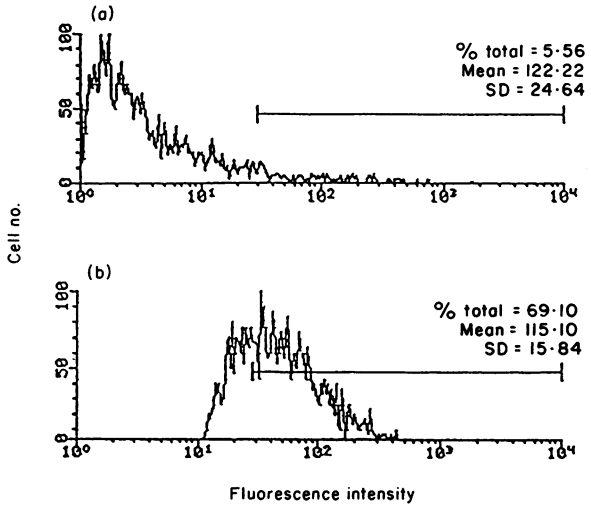

Figure 4. Changes in the expression of C5aR on LC during differentiation after short-term culture. LC were cultured for 3 days in an epidermal cell suspension and then processed for double staining using primary mAb specific for C5aR (S5/1), revealed by PE-conjugated anti-mouse immunoglobulin, followed by FITC-conjugated anti CD1a. Histograms only illustrate LC population purified by electronic gating according to CD1a positivity. Range bars were set to include < 1% of positive cells when isotype control IgG2a was used instead of primary mAb. (a and b) Expression of C5aR on epidermal LC before (a) and after (b) LC *in vitro* differentiation.

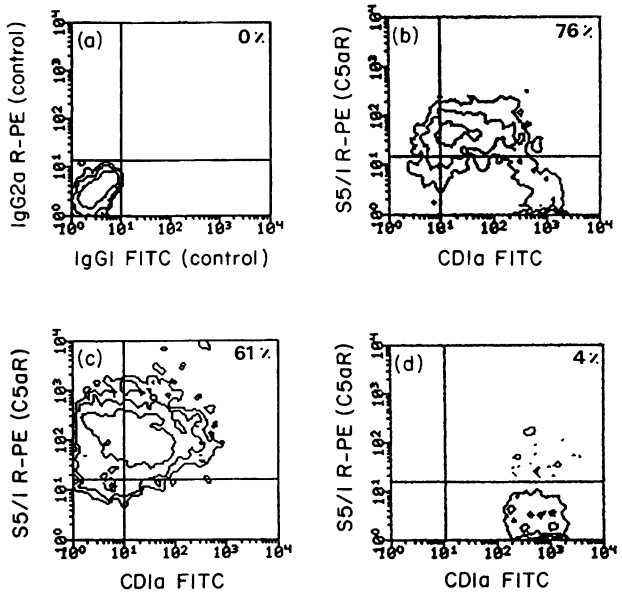

Figure 5. Expression of C5aR by DC migrated from different kind of skin organ explants. Migratory DC were harvested after 48 hr culture of skin organ explants, double immunostained for the expression of C5aR (vertical axis) and CD1a (horizontal axis), and analysed for flow cytometry. Migratory lymphocytes were excluded by their lower FSC and SSC on a FSC versus SSC dot plot. (a) Vertical and horizontal markers were set to include > 98% of negative cells in the left lower quadrant when isotype control IgG1 and IgG2a were used. (b) DC migrated from epidermal/dermal explants are composed by two different subsets of DC: (i) DC $CD1a^{high}$ C5aR negative, and DC $CD1a^{low}$ C5aR positive. (c) DC migrated for dermal explants are all $CD1a^{low}$ C5aR positive (d) DC migrated from epidermis are $CD1a^{high}$ and only a minority of cells express C5aR.

between the state of epidermal maturation of LC and the expression of C5aR, epidermal LC were induced to differentiate *in vitro* upon 72 hr culture in bulk epidermal cell suspensions supplemented with hrGM-CSF.[32–34] A control panel of mAb was used to confirm *in vitro* maturation of LC. LC up-regulated major histocompatibility complex (MHC) class I and II and increased several fold the expression of the adhesion molecules ICAM-1 (CD54) and LFA3 (CD58). The expression of CD1a and CD32 antigens decreased their intensity but were still present. The co-stimulatory signals B7.1 (CD80) and B.72 (CD86) as well as the CD40 molecule, all of them negative or expressed weakly by a low number of fLC, were expressed by 100% of LC. Upon culture, LC up-regulated their expression of C5aR (Fig. 4). Approximately 70% of CD1a positive cells expressed C5aR with the two mAb tested.

C5aR expression on skin migratory DC

After culturing the skin organ explants for 48 hr, 60% of the migratory cells showed DC morphology. Migratory DC enrichment was achieved by Universal Separation Medium (Sigma) gradient (δ = 1·067 g/cm^3). After 20 min centrifugation at 300 *g* cells of the interface were collected and consisted of 80–90% of DC. The dendritic lineage of migratory DC was confirmed by: (i) the high levels of MHC Class I and II molecules; (ii) the expression of CD1a and CD1c; (iii) the positivity for the myeloid markers CD13 and CD33 and the absence of the monocyte/macrophage markers CD14 and CD68, and the granulocyte markers CDw65, CD66acde and CD66b; and (iv) by ultrastructure (data not presented).

The expression of C5aR on migratory DC was demonstrated both by flow cytometry and by immunocytochemistry on cytospin preparations (Figs 5 and 6). Based on the CD1a and C5aR positivity two subpopulations of skin migratory DC were distinguished: (i) a predominant population composed of $CD1a^{low}$ C5aR positive DC; and (ii) a minority subset that

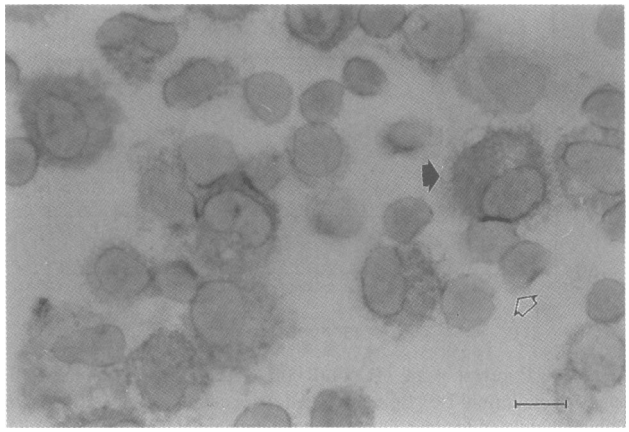

Figure 6. Cytospin preparation of migratory DC and lymphocytes harvested 48 hr culture of skin organ explants and immunostained for C5aR expression. DC show cytoplasmic expression of C5aR (black arrow), by contrast lymphocytes are negative (open arrow). (ABC/Px, Bar 10 μm).

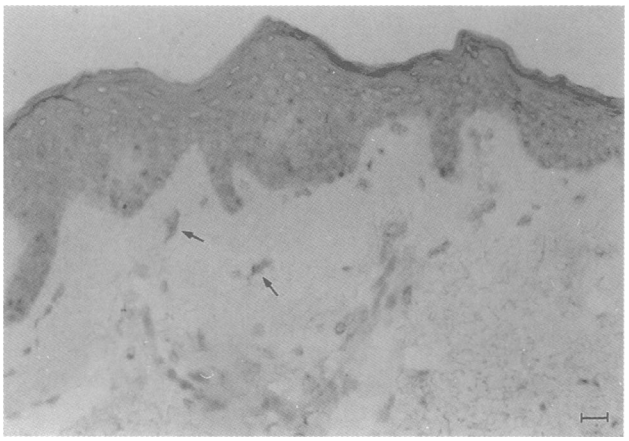

Figure 7. Vertical section of normal skin immunostained for C5aR. Cells with DC morphology and positive for C5aR are scattered mainly in the interstitial dermis (arrows) and around vessels. No C5aR positive epidermal LC are observed. (ABC/Px, Bar 20 μm).

comprised CD1a^{high} C5aR negative DC. Culture of dermal explants devoid of epidermis demonstrated that the CD1a^{low} C5aR positive DC subset was dermis derived. In contrast, the CD1a^{high} C5aR negative DC were only observed after culturing epidermal sheets (devoid of dermis) suggesting as putative origins epidermis, skin appendages or hair follicles.

Vertical cryostat sections of skin immunostained by ABC/Px showed lack of positive cells for C5aR in epidermis and the presence of C5aR positive cells with DC morphology located preferentially in the upper dermis, around small vessels and scattered in the interstitium (Fig. 7).

Chemotaxis of migratory DC

After locomotion assays, cells migrated to the lower compartment of chemotaxis chamber were collected, analysed and counted by flow cytometry. In preliminary experiments and according to the positivity of migrated cells for either CD1a or CD3, two different gates on a forward scatter (FSC) versus side scatter (SSC) dot plot were defined: (i) 'DC gate' (R1 = CD1a positive CD3 negative); and (ii) T lymphocyte gate (R2 = CD1a negative CD3 positive) (Fig. 8). Only cells located in R1 were considered as DC that had passed the filter and were counted by flow cytometry.

Locomotion of migratory DC in response to different concentration of rC5a is depicted in Fig. 9. Optimum migration to rC5a was found at 10^{-8} M with a sigmoidal dose–response curve. The maximal migratory response was observed 2 hr after placing migratory DC in the upper chamber (Fig. 9). Immunostaining of polycarbonate membranes removed from chemotaxis chambers showed cells with DC morphology (cytoplasmic veils or flaps) and high expression of HLA-DR crossing the membrane by protruding a long and thick cytoplasmic processes through the filter pores (Fig. 10).

To investigate whether locomotion of migratory DC through the polycarbonate filter toward the lower chamber depended on the presence of a gradient of C5a between the lower and the upper compartments of the chamber, a limited series of checkboard analysis as described by Zigmond and Hirsch was performed.[35] Checkboard assay allows one to

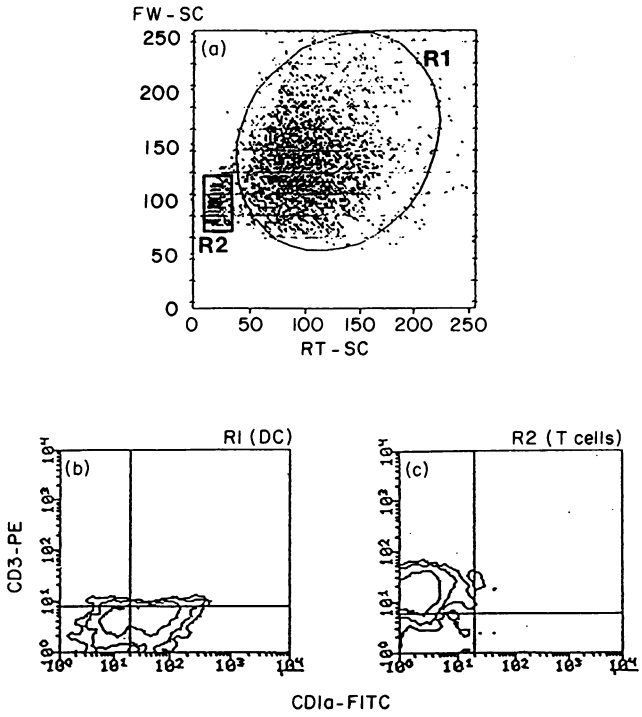

Figure 8. Flow cytometric analysis of migrating cells that after passing the polycarbonate membrane, were collected from the lower compartment of the chemotaxis chamber, and were double immunostained for CD1a and CD3. According to their FSC, SSC, and positivity for either CD3-PE or CD1a-FITC, two different regions were defined on a FSC versus SSC dot plot (a): (i) R1 or 'DC gate' that included large, granular, CD1a positive and CD3 negative cells (b); and (ii) R2 or 'T-lymphocyte gate' composed of small, non-granular, CD1a negative and CD3 positive cells (c). Only those cells gated in R1 on a FSC versus SSC dot plot were considered DC that had passed the membrane and counted by flow cytometry.

distinguish chemotaxis (directional cell locomotion requiring a chemoattractant gradient) from chemokinesis (undirectional cell migration in the absence of a gradient) by varying the concentrations of chemoattractant above and below the filter in the migration chamber. Results obtained by means of checkboard analysis are shown in Table 1. The values presented

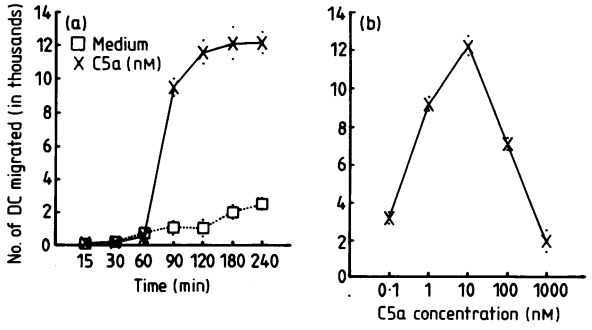

Figure 9. (a) Time course of migratory response to migratory DC to rC5a. Migration of 8×10^4 DC seeded in the upper compartment of the chemotaxis chamber was assessed at different time intervals after exposure to a rC5a gradient (10^{-9} M) or diluent. (b) Dose–response curve of migratory DC in response to different concentrations of rC5a after 120 min of culture.

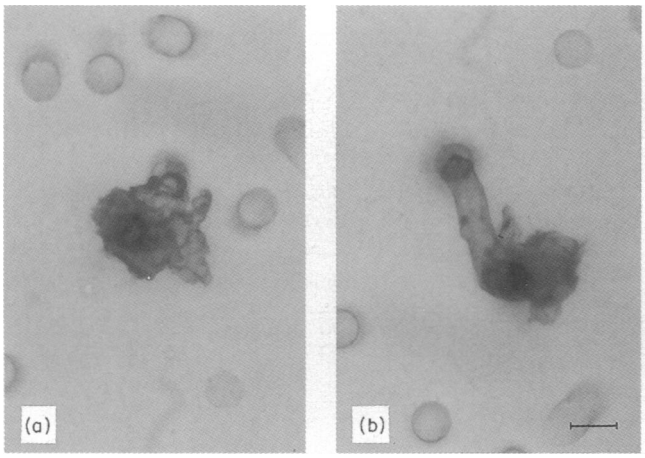

Figure 10. Immunostaining of a polycarbonate membrane that shows cells with DC morphology with wide cytoplasmic veils (a) and strong HLA-DR positivity passing through the filter pores by means of cytoplasmic processes (b). (ABC/Px, Bar 10 μm).

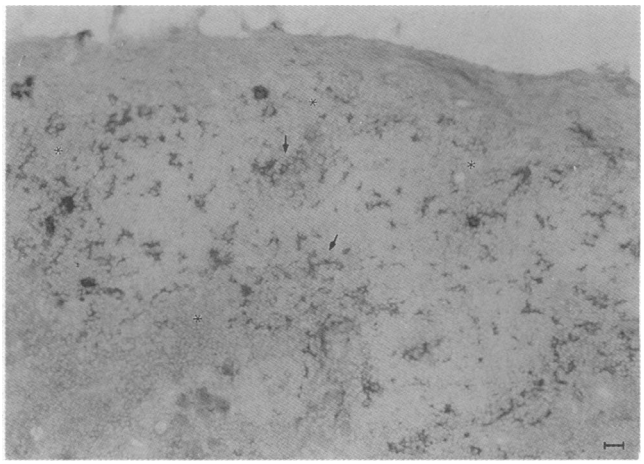

Figure 11. Vertical section of the cortical area of a lymph node immunostained for C5aR (red) and CD3 (light brown). Cells with DC morphology and located in T-cell areas (asterisks) (e.g. interdigitating DC) express C5aR (arrows). Scattered tingible body macrophages positive for C5aR can be seen inside the follicles (APAAP–AEC in red, followed by ABC/Px-DAB in brown, Bar 20 μm).

along the diagonal, where there is no gradient, show only a modest augmentation of DC migration (chemokinetic effect) as compared to a baseline control, although it failed to reach the $P < 0{\cdot}05$ level of significance. DC had a significant migratory response ($P < 0{\cdot}01$) when the concentration of rC5a was higher in the lower compartment (positive gradient), demonstrating that DC migration was caused by chemotactic effect of rC5a.

C5aR positivity on interdigitating DC of secondary lymphoid organs

Frozen sections of lymph nodes and tonsils from three different donors were labelled with anti-CD3 and anti-C5aR mAb by double immunostaining in order to determine the expression of C5aR on Interdigitating DC. As can be seen in Fig. 11 cells with DC morphology and located in T cells areas of lymph nodes (e.g. interdigitating DC) are positive for C5aR. In non-T cell areas of the cortex (B follicles) only tingible body macrophages expressed C5aR. Follicular DC did not show positivity for C5aR. Similar results were observed in tonsil.

DISCUSSION

In the present study, we have shown by flow cytometry that a small population (5–6%) of epidermal LC expresses C5aR. Resident LC (rLC) are considered relatively immature epidermal DC in transit to the T-cell areas of secondary lymphoid organs. They lack certain adhesion molecules and co-stimulatory signals necessary to cluster and then to stimulate T cells.[32,33] However, in normal epidermis a minority of rLC (5–15% of epidermal rLC) show the following features as compared with the remaining rLC:

(i) a higher level of HLA-DR and CD11c;
(ii) positivity for CD40;
(iii) a lower level of Fcγ-RII (CD32);
(iv) a location in a lower position in epidermis close to the basement membrane;
(v) a very strong immunostimulatory capacity in mixed leucocyte reactions;
(vi) a higher level of cytosolic calcium.[31,36–38]

These features have led to consider that such subset of epidermal rLC is composed by LC that have differentiated *in situ* and are about to leave the epithelium.[31,38] By double immunostaining and flow cytometry we were able to determine that the subpopulation of epidermal LC positive for C5aR co-expresses higher levels of HLA-DR than C5aR negative LC, and therefore, it can be included as part of the subset of mature epidermal rLC. The localization of these C5aR positive cells in proximity to the epidermal/dermal basement membrane, and their round-shape morphology (sometimes with a small cytoplasmic tail) support the hypothesis that they are migratory cells on their way out of the epidermis. By means of flow cytometric analysis of epidermal cell suspensions and immunostaining of epidermal sheets, it should not be ruled out that the C5aR positive cells, despite their proximity to the epidermal/dermal junction, might have already crossed the basement membrane and remained attached to the dermal side of the epidermal sheet. This possibility could explain the absence of C5aR positive LC in epidermis in vertical sections of skin. In this case C5a would play a role in LC locomotion in a later

Table 1. Unidirectional migration of DC in response to rC5a

Dose in lower compartment	Dose in upper compartment		
	0	10^{-9} M	10^{-8} M
0	1485 + 138	822 + 12	1038 + 70
10^{-9} M	5840 + 215	1654 + 72	1474 + 145
10^{-8} M	9355 + 313	5960 + 267	1703 + 132

state of DC differentiation, immediately after rLC leave the epidermis.

The upregulation of C5aR along the differentiation of LC was confirmed by the *in vitro* maturation of epidermal LC after a short-term culture with keratinocytes supplemented with GM-CSF. Cultured LC express on their cell surface all the molecules necessary for antigen presentation for both naive and memory T cells (i.e. high levels of MHC class I and class II molecules, the adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58), and the co-stimulatory signals B7.1 (CD80) and B7.2 (CD86)), and resemble the interdigitating DC of secondary lymphoid organs.[32,33] In accordance with these results we confirmed the previous observation that interdigitating DC located in T-cell areas of lymphoid organs were C5aR positive.[39]

The absence of detectable levels of C5aR on immature LC and the late expression of C5aR during LC differentiation is in accordance with the demonstration on cells of myeloid lineage that C5aR is a late-appearing marker. In fact, C5aR is absent in the immature myeloid cell lines KG-1, HL-60, TH-P1 and U937, and it is expressed lately during the maturation of granulocytes and monocytes in bone marrow.[28,29,40] The appearance of C5aR on fully mature myeloid cell is also supported by the observation that lines U937 and HL-60 differentiated with dibutyryl cyclic adenosine monophosphate (AMP) express C5aR.[26,28]

According to phenotypic and ultrastructural studies by Romani *et al.*, DC emigrated from epidermal/dermal explants comprised at least two different subsets: (i) epidermal rLC ($CD1a^{high}$, CD36 negative, Lag negative, Birbeck granules abundant); and (ii) dermal DC ($CD1a^{low}$, CD36 positive, Lag negative, Birbeck granules absent).[17] By using a similar method we demonstrated here, that in contrast to epidermal LC, the majority of dermis-derived DC expressed C5aR. The expression of high levels of CD1a and the presence of abundant Birbeck granules in epidermal-derived LC is against the idea that epidermis-derived LC are differentiated LC ready to migrate from epidermis.[17] It is likely, that epidermis-derived LC detach spontaneously from air follicles and epidermal appendages without a previous process of differentiation and, therefore, without up-regulation of C5aR. By contrast, $CD1a^{low}$ dermal DC show features of fully mature DC with high expression of adhesion molecules, HLA-class I and II antigens, costimulatory molecules, and majority of cells positive for C5aR.

The low number of DC in blood, lymph and peripheral organs along with the difficulty to disrupt solid tissues and to obtain a sheer population of DC have hampered the studies of chemotaxis on these cells. The short-term culture of skin explant organ offers a new approach to obtain enough number of DC to carry out this kind of assays. The results presented here demonstrated that skin-derived DC migrate unidirectionally through polycarbonate filters in response to rC5a in a sigmoidal dose–response curve. In this regard, we have confirmed here a recent report that DC generated *in vitro* after culture of peripheral blood mononuclear cells (PBMC) supplemented with GM-CSF and IL-4 also migrate unidirectionally in response to rC5a.[23]

As it was previously demonstrated for neutrophils, monocyte and large granular lymphocyte chemotaxis, supersaturating concentrations of C5a (in the present study concentrations higher than 10^{-7} M) showed and inhibitory effect on DC chemotaxis.[22,24]

The up-regulation of the C5aR on DC during their maturation might represent one of the links between innate and adaptive immunity. Immature rLC require the presence of IL-1, TNF-α and GM-CSF in order to differentiate into fully mature antigen presenting cells. As part of this process LC might acquire C5aR. It is well known that IL-1, TNF-α and C5a are all generated during the early phases of an inflammatory response and that epidermal rLC express IL-1R and 75 kD TNFR.[41] In this scene the up-regulation of the C5aR on LC might be one of the components that might trigger LC migration and the subsequent adaptative immunity during an inflammatory process, as recently postulated by Katz and co-workers.[42]

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