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Effects of cilomilast on dendritic cell function in contact sensitivity and dendritic cell migration through skin

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Abstract

The phosphodiesterase 4 inhibitor cilomilast demonstrated strong inhibitory effects in a model of allergic contact dermatitis. In this study, we examined whether this inhibitory effect is at least partly due to modulation of dendritic cell function. Bone marrow-derived dendritic cells were pulsed with the sensitizer toluene-2,4-diisocyanate and administered subcutaneously to nonsensitized mice. Five days later, the mice were challenged with a low dose of toluene-2,4-diisocyanate onto the ears. In contrast to sham-treated mice, mice obtaining toluene-2,4-diisocyanate pulsed dendritic cells showed a significant increase in ear swelling. This swelling was not influenced when the dendritic cells were pre-incubated with cilomilast. When cilomilast was administered systemically simultaneously to the application of toluene-2,4-diisocyanate pulsed cells, there was an impaired allergic reaction provoked 5 days later. Additionally, a topical treatment with cilomilast resulted in a significant inhibition of skin dendritic cell migration. These results indicate that the antigen-presenting function of dendritic cells is not influenced by cilomilast but the dendritic cell T cell interaction and dendritic cell migration is modulated.

Keywords: Cilomilast; Phosphodiesterase 4 inhibitor; Dendritic cell; Skin, mouse; Matrix metalloproteinase 9; Toluene-2,4-diisocyanate; Zymography

1. Introduction

Dendritic cells are a subset of bone marrow-derived professional antigen-presenting cells that have the capacity to prime naive T cells and to initiate Ag-specific immune responses (Bancherau and Steinman, 1998). The phosphodiesterase 4 inhibitor cilomilast, currently evaluated for the treatment of asthma and chronic obstructive pulmonary disease (Giembycz, 2001), demonstrated inhibitory effects in a model of allergic contact dermatitis (Bäumer et al., 2002). We were interested to examine effects of cilomilast on dendritic cell function, as these cells play a pivotal role in the induction and elicitation of allergic contact dermatitis. Langerhans cells, epidermal dendritic cells, transport haptens (like toluene-2,4-diisocyanate) from skin through afferent lymphatic vessels to regional lymph nodes, where these haptens, bound to peptides are presented to T cells, which become specific T cells (induction). When the hapten is painted for a second time, it diffuses through the skin and could be loaded to Langerhans cells or dermal dendritic cells which present it to specific T cells which are then activated and response with cytokine production and activation of further inflammatory cells (elicitation; for review, see Krasteva et al., 1999). Human dendritic cells mainly show phosphodiesterase 1, phosphodiesterase 3 and phosphodiesterase 4 activity. During maturation from monocytes to dendritic cells the phosphodiesterase 4 activity decreases, while activities for phosphodiesterase 1 and phosphodiesterase 3 substantially increase (Gantner et al., 1999). Nevertheless, an inhibition of lipopolysaccharide-induced tumour necrosis factor α synthesis in dendritic cells by the highly selective phosphodiesterase 4 inhibitors roflumilast and cilomilast was demonstrated (Hatzelman and Schudt, 2001). Additionally, it was shown for Langerhans cells that their function is strongly influenced by an elevation of intracellular cAMP induced by calcitonin gene-related peptide (Asahina et al., 1995). The suppressing effect of cAMP-elevating agents including phosphodiesterase 4 inhibitors on

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murine bone marrow-derived dendritic cell function is also demonstrated by Kambayashi et al. (2001).

In the described study, murine bone marrow-derived dendritic cells were pulsed with the hapten toluene-2,4-diisocyanate and administered subcutaneously to mice to induce an allergic contact dermatitis in analogy to Krasteva et al. (1998) and Mizumoto et al. (2002). To examine effects of the phosphodiesterase 4 inhibitor cilomilast, dendritic cells were pre-incubated with 10 μM cilomilast, a concentration known to induce a distinct inhibition of $CD4^+$ T cell functions and lipopolysaccharide induced tumour necrosis factor α release in macrophages and monocytes (Hatzelman and Schudt, 2001).

A further experiment was performed to study effects of cilomilast on dendritic cell migration through skin based on the method of Ortner et al. (1996). Mouse ears, which were topically treated with cilomilast, were cultured for 3 days and the migrated cells were counted.

2. Materials and methods

2.1. Mice

Female BALB/c-mice were obtained from Charles River (Sulzfeld, Germany) at the age of 8 weeks (20 g body weight). All animals were healthy and were housed in groups of six mice per cage at 22 °C with a 12-h light/dark cycle. Water and a standard diet (Altromin, Lage/Lippe, Germany) were available ad libitum. The animal experiment had been registered by Bezirksregierung Hannover, Germany (Az. 509i-42502-98A839).

2.2. Dendritic cell generation from bone marrow cultures

Large numbers of highly pure dendritic cells were generated according to Lutz et al. (1999, 2002) with slight modifications. Briefly, bone marrow was flushed from femurs of the hind limbs with ice cold PBS (phosphate-buffered saline) and taken into RPMI 1640 (Biochrom, Berlin, Germany). 10% fetal calf serum (Biochrom) and 50 μM 2-mercaptoethanol (Sigma, Deisenhofen, Germany) were added. 2×10^6 cells were seeded in 10 ml medium on a petri dish ($Cell^+$, Sarstedt, Nümbrecht, Germany). The medium contained 20 ng/ml granulocyte-macrophage colony-stimulating factor (Sigma). On day 3, 10-ml fresh medium supplemented with 200 ng granulocyte-macrophage colony stimulating factor was added. At days 6, 8 and 10, 50% of the medium was collected, centrifuged and the cell pellet resuspended in 10-ml fresh medium containing 200 ng (day 10: 100 ng) granulocyte-macrophage colony stimulating factor.

The purity of dendritic cells was determined by using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Cells were transferred into microtiter plates and washed twice in PBS containing 1% bovine serum albumin and 0.1% NaN_3. Cell washing was repeated after incubation with the first and the second antibody. Every antibody was incubated for 30 min at 4 °C. Dendritic cells were identified as positive for CD 11c (hamster immunoglobulin (Ig) G_1, PE (phycoerythrin) conjugated, Pharmingen, Hamburg, Germany) and major histocompatibility complex II (I-A/I-E, rat IgG_{2a}, biotin-conjugated, Pharmingen). The isotypes were hamster IgG_1 (PE conjugated) and rat IgG_{2a} (biotin-conjugated). Biotinylated antibodies were detected with Red 670-streptavidin (Gibco, Gaitherburg, MD, USA).

FACS analysis of the day 11 cell suspension demonstrated a high yield of CD 11c and major histocompatibility complex class II positive cells (Fig. 1). These data are in accordance with observations of Lutz et al. (1999).

2.3. Pre-incubation with cilomilast

After 10 days of cultivation, the cells were placed in a 12-well plate (4×10^5 cells/well) and incubated with 10 μM cilomilast (Arzneimittelwerke Dresden, now: elbion, Radebeul, Germany) for 24 h.

2.4. Hapten pulsing of dendritic cells

Dendritic cells were collected on day 11 of culture and placed in a 12-well plate (4×10^5 cells/well). The cells were taken in RPMI 1640 without fetal calf serum containing 100

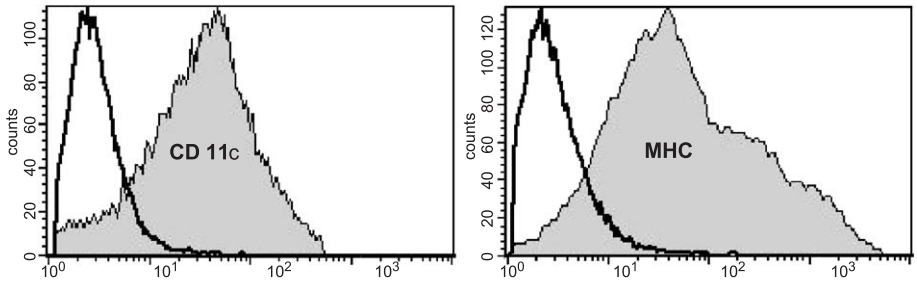

Fig. 1. FACS analysis of bone marrow-derived dendritic cells after 11 days of culture. The high expression of CD 11c (filled histogram) demonstrates the purity of dendritic cells. The expression of MHCII is comparable to Lutz et al. (1999). Open histograms represent isotype controls.

μg/ml toluene-2,4-diisocyanate. The cells were incubated for 30 min at 37 °C. After incubation, the cells were washed twice in RPMI + 10% fetal calf serum and PBS successively.

2.5. Immunisation with dendritic cells and the mouse ear swelling test

BALB/c mice were treated with 2×10^5 hapten pulsed dendritic cells in 200 μl PBS subcutaneously (abdominal skin) to prime for contact sensitivity. To test, whether the reaction of toluene-2,4-diisocyanate pulsed cells was specific, a sample of the dendritic cells were snap-frozen in liquid nitrogen and thawed twice. These necrotic cells were administered at the same dose (2×10^5) to naive mice. For further experiments, sham-treated mice received unpulsed dendritic cells, which were incubated in the same dimethylsulfoxide (DMSO) concentration as toluene-2,4-diisocyanate pulsed dendritic cells.

In an additional experiment, cilomilast (25 mg/kg) was administered intraperitoneally to five mice 24 and 1 h before, 10 min, 4 h, 8 h and 24 h after the application of toluene-2,4-diisocyanate pulsed dendritic cells. The sham-treated group ($n = 5$) received the same dose of toluene-2,4-diisocyanate pulsed dendritic cells.

After 5 days, the reaction was challenged by topical administration of 0.5% toluene-2,4-diisocyanate onto the mice ears (10 μl each on the inner and outer surface). Ear swelling was measured using a cutimeter (model 7309, Mitutoyo, Neuss, Germany). The swelling was calculated by comparison of the values before challenge with those of 24 h after challenge.

2.6. Histological examination

3 mice of each group (toluene-2,4-diisocyanate, $n = 6$) were sacrificed for histological examination. The ears were fixed in 4% formaldehyde and embedded in paraffin or Technovit (Kulzer, Friedrichsdorf, Germany).

2.7. Inhibition of cytokine synthesis in bone marrow derived dendritic cells

Murine bone marrow-derived dendritic cells were cultured as described above. At day 10, dendritic cells were placed in a 96-well plate (5×10^4 cells/well) and incubated with 10 μM cilomilast or vehicle (DMSO) for 24 h ($n = 5$ each group). After pre-incubation, the cells were stimulated by addition of 5 μl lipopolysaccharide (*Escherichia coli*, O127:B8, Sigma) working solution (1 μg/ml lipopolysaccharide, final concentration). Twenty-four hours later, the supernatant was collected and stored at −20 °C until determination of tumour necrosis factor α and interleukin 1 β by enzyme linked immunosorbend assay (ELISA, DuoSet and Quantikine, respectively, R&D Systems, Wiesbaden, Germany). Three independent experiments were performed.

2.8. Skin dendritic cell migration assay

Mice ears were treated with 20 μl cilomilast (3% = 600 μg; in acetone/DMSO 9:1) or with the vehicle (10 μl on both, the inner and outer surface of the ears). The concentration of cilomilast was selected based on in vivo results (Bäumer et al., 2002). Two hours later, the mice were sacrificed. The ears were rinsed in 75% ethanol and air-dried for 10 min. The cartilage-free dorsal halves of split mouse ear skin were cultured in 24-well size plates based on a method of Ortner et al. (1996). Directly before cultivation, the ear halves were treated again with 10 μl cilomilast or vehicle. The halves laid epidermal side up onto tables made of sieves (size: 100 mesh, Sigma) and three small screws (stainless steel, lengths: 5 mm). 1.3 ml RPMI 1640 + 10% fetal calf serum (penicillin 50 I.U./ml, streptomycin 50 μg/ml, Sigma) was added to each well so that the epidermis was slightly covered with media. As a chemotactic factor for dendritic cells, macrophage inflammatory protein-3 β was added (50 ng/ml, R&D Systems). It was already demonstrated by Kellermann et al. (1999) that macrophage inflammatory protein-3 β improves the yield of migrated cells. This was confirmed by own observations. The tables were changed daily to new wells and new media (including macrophage inflammatory protein-3 β). Migrated cells from each ear (day 2 and day 3) were pooled and counted with a hemocytometer (Neubauer, VWR, Darmstadt, Germany). The viability of the cells was assessed by trypan blue exclusion. Four independent experiments ($n = 6$ each group) were performed. To exclude cytotoxic effects of cilomilast, the cell viability of six cilomilast-treated and six sham-treated mouse ears was measured by means of methylthiazoletetrazolium assay. At the end of the migration assay (day 3), ear halves were placed in 1 ml TKM (Tris, potassium chloride, magnesium chloride) buffer containing 0.1 ml methylthiazoletetrazolium (Sigma) solution and were homogenised (Ultra Turrax, Ika, Staufen, Germany). After an incubation period of 2 h (37 °C), 1 ml 0.1 N HCl in 2-propanol was added to the homogenate and the samples were centrifuged. After centrifugation, the supernatant of each homogenate (containing the coloured formazan) was measured photometrically (570 nm) in triplicate (MRX, Dynatech Deutschland, Denkendorf). After the protein content was measured (Biorad, Munich, Germany), the extinction was related to a concentration calibration line.

2.9. Preparation of epidermal sheets for immunohistochemistry

The preparation and the evaluation of epidermal sheet was performed according to Ratzinger et al. (2002). In short, skin was floated on 0.5 M ammonium thiocyanate (Riedel de Haën, Hannover, Germany) for 10 min at 37 °C. The epidermis was separated from the dermis and immediately fixed in cold acetone. The dendritic cells were detected with the primary major histocompatibility complex class II anti-

body (monoclonal anti-mouse major histocompatibility complex class II (I-A/I-E), rat IgG2b, Beckton Dickinson, Heidelberg, Germany) in a 1:200 dilution. Labelling of the antibodies was visualised by using biotinylated rabbit-anti-rat immunoglobulin G (1:500, DAKO, Hamburg, Germany) and coupling the antibodies to a fluorochrom (carbocyanin 3, 1:4000, Jackson Immunoresearch Laboratories, PA, USA) using a conventional streptavidine–biotin technique. Analyses were performed by using Kontron KS 400 image analysis system. The density of Langerhans cells was counted under the microscope using ×40 magnifications and a calibrated grid. Sixteen randomly chosen areas per ear were analysed (Ratzinger et al., 2002). Six cilomilast-treated and seven vehicle-treated ears were analysed.

2.10. Zymography

Ear halves (six each group) were homogenised in liquid nitrogen. The homogenates were taken in 150 μl PBS buffer and the samples were mixed intensively. After centrifugation (3000 × *g*, 10 min, 4 °C), the supernatant was collected and the protein content was quantified (Bio Rad, Munich, Germany). Each sample was diluted to a concentration of 2.25 mg/ml protein. 20 μl of each sample was subjected to electrophoresis on a 3.9% acrylamid stacking gel/7.5% acrylamid separating gel (Pro Sieve 50, Biozym, Hess. Oldendorf, Germany) containing 1 mg/ml gelatine in the presence of SDS under nonreducing conditions according to Corbel et al. (2002). After electrophoresis, gels were washed twice with 2.5% Triton X 100, rinsed with water, and incubated at 37 °C overnight in 50 mM Tris, 5 mM $CaCl_2$ and 200 mM NaCl. Gels were stained with Coomassie Brilliant Blue (Merck, Darmstadt, Germany) and destained in a solution of 30% methanol and 10% acetic acid (all salts and solutions: Merck). Gelatinase activities appeared as clear bands against a blue background. Molecular weights of gelatinolytic bands were estimated using recombinant protein molecular weight markers (10–250 kDa, Bio Rad). Three gels (each containing four samples) were performed. Quantification of the gelatinolytic bands was performed by densitometry. The image acquisition was done with a HP scan jet 7400 c scanner by using PhotoImpact 4.0 program (Ulead systems). The densitometry was determined by means of Scion Image software for PC (Scion, Frederick, MD, USA). Grey values of the background and of the gelatinolytic band were transformed into optical densities OD = log10 [255/grey value]). The optical density of the background was subtracted from the optical density of the gelatinolytic band. This yields negative values as the gelatinolytic bands have lower optical densities as the background.

Table 1
Effect of cilomilast on lipopolysaccharide-induced tumour necrosis factor α and interleukin 1 β release in murine bone marrow-derived dendritic cells

	Control	LPS	LPS + cilomilast
TNFα (pg/ml)			
Expt. 1	250 ± 88	5708 ± 523	3270 ± 541[a]
Expt. 2	280 ± 35	4208 ± 339	2184 ± 111[a]
Expt. 3	464 ± 64	5217 ± 631	3366 ± 257[a]
IL-1 β (pg/ml)			
Expt. 1	<4	68 ± 9	49 ± 8[b]
Expt. 2	<4	71 ± 17	29 ± 6[a]
Expt. 3	<4	26 ± 15	16 ± 13

Mean ± S.D. of three independent experiments ($n = 5$ each group). Preincubation with cilomilast (10 μM) results in a significant inhibition of lipopolysaccharide induced cytokine production (except for IL 1 β in experiment 3) measured in the supernatant 24 h after lipopolysaccharide stimulation.

[a] $P < 0.01$.

[b] $P < 0.05$.

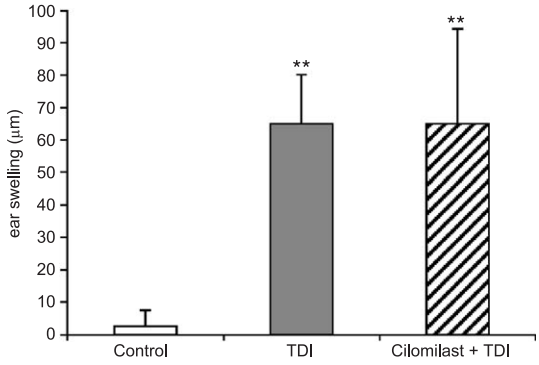

Fig. 2. Ear swelling provoked by toluene-2,4-diisocyanate pulsed dendritic cells. Dendritic cells (2×10^5) were administered subcutaneously to naive BALB/c mice. Dendritic cells were pulsed with vehicle (DMSO), toluene-2,4-diisocyanate (100 μg/ml) or toluene-2,4-diisocyanate and cilomilast (10 μM). Five days later, mice were challenged by ear painting with toluene-2,4-diisocyanate (0.5%). The swelling was measured 24 h after challenge (*$P < 0.05$, $n = 6$ each group, control: $n = 4$).

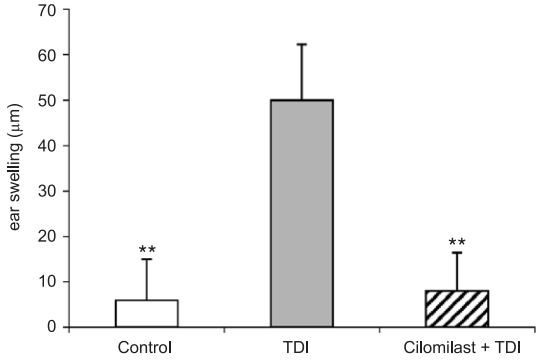

Fig. 3. Effect of systemically administered cilomilast on ear swelling provoked by toluene-2,4-diisocyanate pulsed dendritic cells. Dendritic cells (2×10^5) were administered subcutaneously to naive BALB/c mice. Dendritic cells were pulsed with vehicle (DMSO, white bar) or toluene-2,4-diisocyanate (100 μg/ml, grey and hatched bar). One group (hatched bar) was treated repetitiously systemically with cilomilast (25 mg/kg). Five days later, mice were challenged by ear painting with toluene-2,4-diisocyanate (0.5%). The swelling was measured 24 h after challenge (*$P < 0.05$, $n = 5$ each group).

2.11. Statistical evaluation

The results are presented as mean ± S.D. (ear swelling, cytokine concentration) or as median boxes (migration assay). Statistical significance was determined using Mann–Whitney *U*-test for two independent samples. For more than two samples, a Kruskal–Wallis one-way analysis followed by a multiple comparison method (Dunn's test)

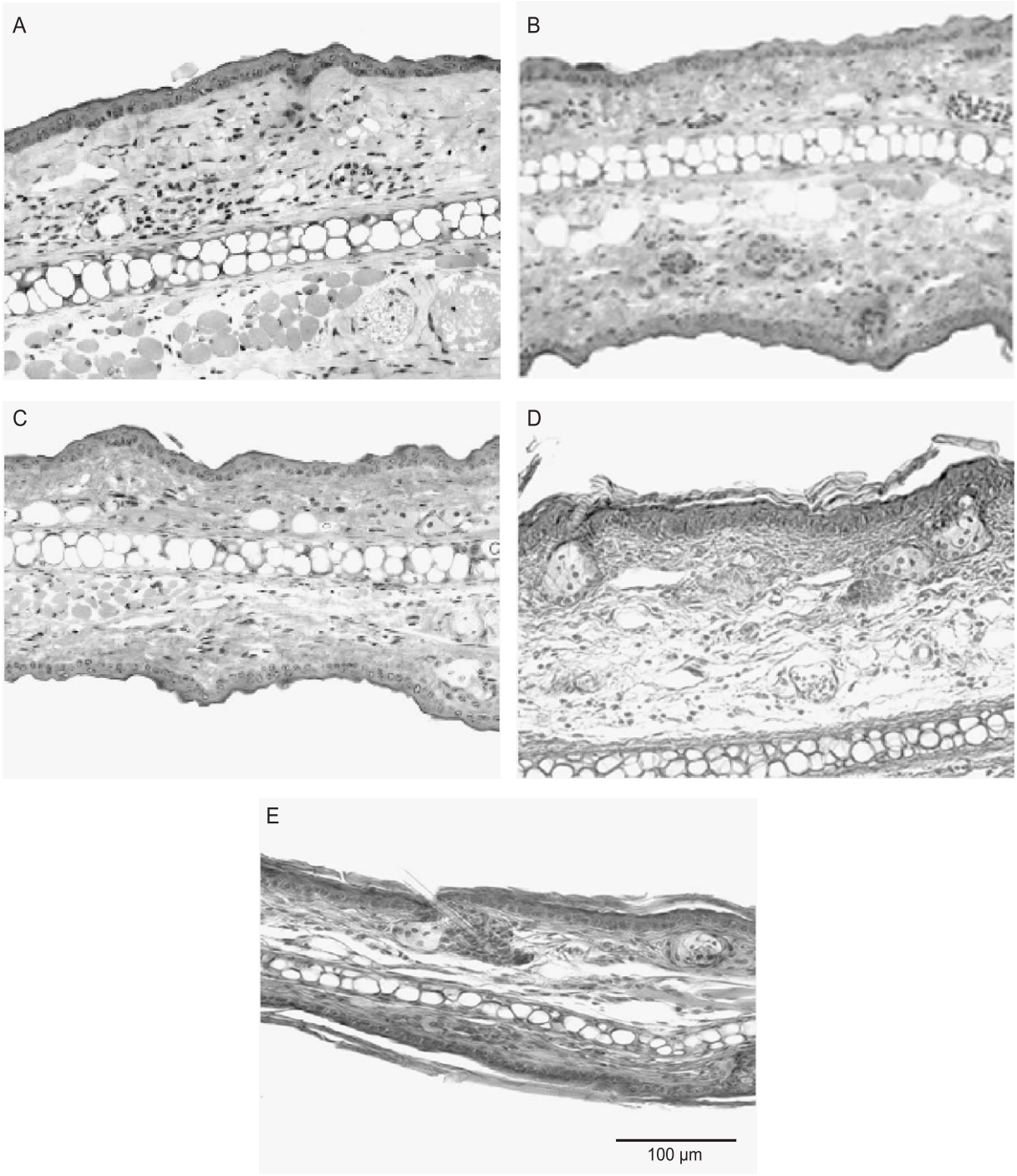

Fig. 4. Histopathology of skin reaction in sensitized mice. Mice received toluene-2,4-diisocyanate pulsed dendritic cells (A, B, D, E) or sham-treated dendritic cells (C). Five days later, the allergic reaction was provoked with 20 µl 0.5% toluene-2,4-diisocyanate onto the ear skin. Mice who received sham-treated dendritic cells show only a moderate reaction 24 h after toluene-2,4-diisocyanate administration (C). Mice injected with toluene-2,4-diisocyanate pulsed dendritic cells show a distinct inflammatory reaction (A, D). A pre-incubation of dendritic cells with cilomilast does not attenuate the reaction (B). A systemic treatment of mice with cilomilast simultaneously to the administration of toluene-2,4-diisocyanate pulsed dendritic cells results in a distinct inhibition of the allergic response measured 5 days later (E). Sections A–C are embedded in Technovit, D and E are embedded in paraffin.

was performed. The density of MHCII positive cells in murine epidermis was evaluated by a one-way ANOVA (analysis of variance) followed by a Dunnett's test.

3. Results

3.1. Inhibition of cytokine synthesis in bone marrow-derived dendritic cells

To study if the bone marrow-derived dendritic cells respond to the pre-incubation of 10 μM cilomilast in vitro, dendritic cells were stimulated with lipopolysaccharide. Tumour necrosis factor α as well as interleukin 1 β was measured in the supernatant. Lipopolysaccharide stimulation without cilomilast results in a significant increase of these cytokines, whereas pre-incubation with cilomilast (10 μM) inhibited this enhanced secretion significantly (Table 1).

3.2. Toluene-2,4-diisocyanate pulsed cells immunize naive mice

Dendritic cells pulsed with toluene-2,4-diisocyanate induce a significant ear swelling when challenged 5 days later (increase of ear thickness: 54 ± 16 μm). There is no response to the challenge, when the snap frozen pulsed cells were administered (increase of ear thickness: 3 ± 7 μm).

Administration of dendritic cells incubated with the vehicle (DMSO) instead of toluene-2,4-diisocyanate also did not result in significant swelling (Fig. 2). Incubation with cilomilast (10 μM) does not attenuate the response to toluene-2,4-diisocyanate challenge (Fig. 2). Histological examination of the ears demonstrates that the ear swelling occurs with an edema and influx of inflammatory cells

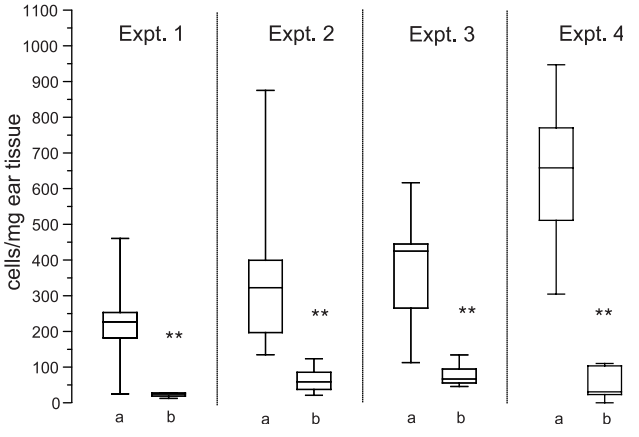

Fig. 5. Migration of skin dendritic cells from mouse ear explants. The mice were treated with 3% cilomilast (20 μl in acetone/DMSO 9:1; B) or vehicle (A). After 2 h, the mice were sacrificed and the dorsal ear halves were cultured for 3 days. The ears were moved to a new well (and new medium) daily. After 3 days, the cells from days 2 and 3 were harvested and counted; $^{**}P<0.01$, $n=6$ for each group and experiment, box represent 25–75%, whiskers represent the range.

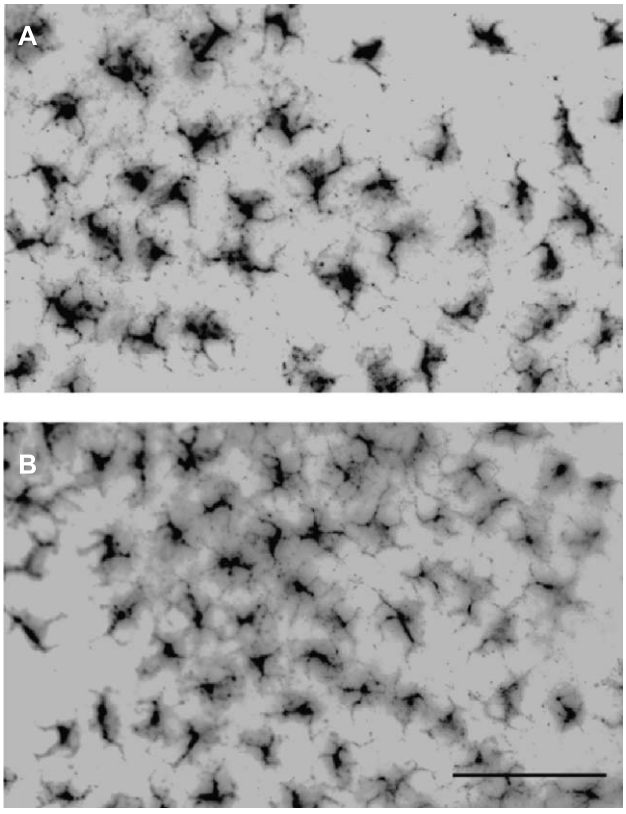

Fig. 6. Effect of cilomilast on Langerhans cell density in murine skin (epidermis) cultured for 3 days as described in Materials and methods. There are markedly less cells in vehicle-treated epidermis (A) compared to cilomilast treated (B) mouse ears (bar represents 50 μm).

(Fig. 4). Repetitive systemic treatment with cilomilast (25 mg/kg) simultaneously to the administration of toluene-2,4-diisocyanate pulsed dendritic cells results in a significant inhibition of the challenged ear swelling on day 5 after treatment (Figs. 3 and 4).

3.3. Skin dendritic cells migration assay

Topical treatment with cilomilast (3%) lead to significant reduction of cell migration from mouse ear explants compared to vehicle-treated ears (Fig. 5). Determined by phase contrast microscopy, nearly all of the cells exhibit the hairy morphology typical of mature skin dendritic cells. Cytotoxic effects of cilomilast were excluded by the viability test. There is no difference in methylthiazoletetrazolium turnover between cilomilast-treated and vehicle-treated mouse ears (vehicle: 115.2 ± 37.7 μg formazan/mg tissue vs. cilomilast: 113.2 ± 27.2 μg formazan/mg tissue).

The Langerhans cell density was significantly lower in vehicle-treated compared to cilomilast-treated epidermis (cilomilast: 1231 ± 84 cells mm^{-2} vs. vehicle: 994 ± 103 cells mm^{-2} (mean ± S.D.; $P<0.05$) and Fig. 6). These results correlate well to those of Ratzinger et al. (2002) who count a mean Langerhans cell density of approximately 1100 cells mm^{-2} and measure a significant decrease by

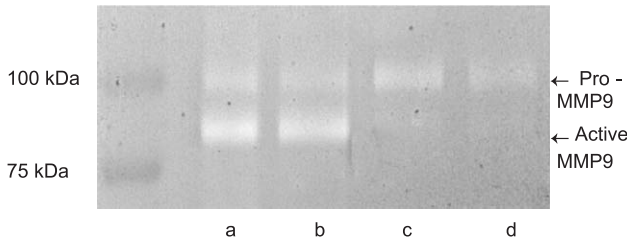

Fig. 7. Gelatine zymogram for matrix metalloproteinase activity. Lanes a and b are samples from two vehicle-treated mouse ears 3 days after cultivation (migration assay). Lanes c and d are samples from two cilomilast-treated mouse ears. The pro-matrix metalloproteinase-9 (92 kDa) and active matrix metalloproteinase-9 (~ 80 kDa) bands are indicated at the right side. The kDa specifications indicate the molecular weight standard (left lane). One representative zymogram from a total of three.

intradermal injection of tumour necrosis factor α to almost 800 cells mm^{-2}.

3.4. Zymography

A zymography of mouse ears was performed as former studies indicate a central role for matrix metalloproteinase 9 activity for dendritic cell migration (Kobayashi, 1997, 1999). Gelatinase B (matrix metalloproteinase 9) activity was markedly reduced in cilomilast treated ear halves 3 days after cultivation (Fig. 7). Apart from the 92-kDa band (pro-matrix metalloproteinase 9), the band for the active matrix metalloproteinase 9 (approximately 80 kDa) is nearly not obvious in cilomilast-treated mouse ears. The optical density (mean ± S.D.) for vehicle-treated mouse ears was -0.20 ± 0.06 compared to -0.03 ± 0.02 for cilomilast-treated mouse ears.

4. Discussion

Major aim of this study was to examine the participation of dendritic cells in the strong inhibitory effect of the phosphodiesterase 4 inhibitor cilomilast in a model of allergic contact dermatitis. In a former study, BALB/c mice were sensitized to the hapten toluene-2,4-diisocyanate by epicutaneous sensitization. When administered topically 2 h before a challenge of mice ears with toluene-2,4-diisocyanate, cilomilast inhibited the inflammatory response significantly in sensitized BALB/c mice, confirmed by a significant decrease of interleukin 1 β and interleukin 4 measured in mice ears (Bäumer et al., 2002).

According to Gantner et al. (1999), prominent phosphodiesterase isoenzymes in human dendritic cells are the phosphodiesterase 3 and 4. Therefore, it could be postulated that a pre-incubation of dendritic cells with cilomilast at a high dose (10 μM) would have modulatory effects on dendritic cells function. This concentration leads to 35% inhibition of lipopolysaccharide induced tumour necrosis factor α release in human monocyte-derived dendritic cells when the cells were pre-incubated for 30 min (Hatzelman and Schudt, 2001). Our results obtain a little higher inhibition of cytokine secretion. This may be due to the long pre-incubation of 24 h. The inhibitory effect of cilomilast was dose dependent (tested for tumour necrosis factor α: 1–30 μM, not shown). As 10 μM cilomilast exhibit the strongest inhibitory action in vitro, which was not enhanced by administration of 30 μM cilomilast, it was decided to take 10 μM for the in vivo studies.

To determine the effect of cilomilast on dendritic cell function, it was decided to pulse murine bone marrow-derived dendritic cells with the hapten toluene-2,4-diisocyanate which was also used for active cutaneous sensitization (Bäumer et al., 2002). The magnitude of the response (mean ear swelling of 60 μm) 24 h after toluene-2,4-diisocyanate painting onto the ears is comparable to that of Mizumoto et al. (2002) for the hapten trinitrochlorobenzene but less than that achieved by Krasteva et al. (1998) for dinitrofluorobenzene.

To confirm that the pulsed dendritic cells lead to an active sensitization, a pilot study demonstrated that inactivated and killed cells (by two freeze/thaw cycles) are not able to induce a toluene-2,4-diisocyanate specific allergic contact sensitivity. A pre-incubation with a high dose of cilomilast does not influence the allergic response. Therefore, it is concluded that the antigen presenting function of dendritic cells is not modulated by the phosphodiesterase 4 inhibitor. The allergic response is dramatically attenuated, when toluene-2,4-diisocyanate pulsed dendritic cells were administered to mice who were treated with cilomilast systemically. In accordance with in vitro results (Gantner et al., 1999; Hatzelman and Schudt, 2001), this observation leads to the assumption that the T cell response is modulated by cilomilast. Nevertheless, it has to be considered that phosphodiesterase 4 inhibitors modulate the function of various cell types involved in inflammatory and immunological processes. So it is difficult to discriminate the action of cilomilast on dendritic cells from that on other immune cells in vivo.

The distinct decrease of migration of skin dendritic cells in ear explants might be due to reduced gelatinase (matrix metalloproteinase 9) activity in cilomilast treated ears. It was recently demonstrated that matrix metalloproteinase 2 and matrix metalloproteinase 9 are necessary for the migration of dendritic cells in human and murine skin (Ratzinger et al., 2002). The zymography demonstrated a decrease of active matrix metalloproteinase 9 in cilomilast-treated mouse ears, which might at least partly explain the inhibition of migration. Whether the matrix metalloproteinase 9 in vehicle-treated mouse ears was produced by keratinocytes (Makela et al., 1998), Langerhans cells (Kobayashi, 1997) or fibroblasts (Kohyama et al., 2002) remain to be elucidated. Kobayashi detects matrix metalloproteinase 9 activity in Langerhans cells and in Langerhans cells depleted epidermal cells (activated by hapten painting). So the source of matrix metalloproteinase 9 may be Langerhans cells and sheer craft activated keratinocytes (by the manipulation for

cultivation as the epidermal sheets were dragged during the separation with forceps). As phosphodiesterase 4 is expressed in keratinocytes (Chujor et al., 1998), these cells may be a target for the phosphodiesterase 4 inhibitory action of cilomilast. Additionally, it was demonstrated that cilomilast inhibits the matrix metalloproteinase 9 activity in tumour necrosis factor α activated human fetal lung fibroblasts (Kohyama et al., 2002).

An inhibition of matrix metalloproteinase 9 activity by another phosphodiesterase 4 inhibitor (RP 73401) was shown in a mouse model ovalbumin-induced airway reactivity (Belleguic et al., 2000) and a model of acute lung injury (Corbel et al., 2002). It was demonstrated via zymography that the bronchoalveolar lavage of RP 73401-treated mice consists of significant less matrix metalloproteinase 9 activity compared to sham-treated mice. However, it has to be noted that the inhibition of matrix metalloproteinase 9 activity in both models is accompanied by a significant reduction of neutrophil influx, and that therefore the main source for matrix metalloproteinase 9 is missing in RP 73401-treated mice (Belleguic et al., 2000; Corbel et al., 2002). The advantage of the used ear explants is that an effect of inflammatory cells like neutrophils can be excluded.

The significant inhibition of migration of epidermal dendritic cells from ear explants by cilomilast may have clinical consequences as this mechanism might be involved in the attenuated allergic response observed in a model of allergic contact dermatitis (Bäumer et al., 2002). The role of matrix metalloproteinase 9 activity in contact hypersensitivity is discussed contrary. The development of contact hypersensitivity is not impaired in matrix metalloproteinase 9-deficient mice (Wang et al., 1999). On the other hand, Kobayashi et al. (1999) could demonstrate by an intradermal injection of anti-matrix metalloproteinase 9 monoclonal antibody a markedly inhibition of hapten-induced decrease in Langerhans cell number in the epidermis and the accumulation of dendritic cells in the regional lymph node. They also conclude that matrix metalloproteinase 9 plays a key role in the morphological and phenotypic maturation of dendritic cells in the skin. Additionally, the broad acting matrix metalloproteinase inhibitor batimastat almost completely abrogated the inflammatory reaction in a model of contact sensitivity (Mattei et al., 2002).

The described study demonstrates that the modulatory effect of the toluene-2,4-diisocyanate-induced inflammatory reaction by cilomilast is not predominantly mediated through a direct modulation of the dendritic cells function but by an influence on dendritic cells migration and a modulation of the dendritic cell–T cell interaction. This is in accordance with in vitro studies. Gantner et al. (1999) conclude from dendritic cell-mediated T cell proliferation tests that T cells are the major target cell populations of phosphodiesterase inhibitor action. Antigen processing is unlikely to be inhibited by phosphodiesterase 4 inhibitors in dendritic cells but in vitro tests with murine dendritic cells are under way. The inhibition of migration of dendritic cells by cilomilast has to be confirmed in models of allergic diseases like contact sensitivity. The involvement of matrix metalloproteinase 9 and the origin of matrix metalloproteinase 9 will be elucidated in further studies.

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