British Journal of Pharmacology (1997) 121, 221–231

In vitro differentiation of human monocytes to macrophages: change of PDE profile and its relationship to suppression of tumour necrosis factor-α release by PDE inhibitors

†Florian Gantner, *Rochus Kupferschmidt, *Christian Schudt, †Albrecht Wendel & *,[1]Armin Hatzelmann

*Byk Gulden, Department of Biochemistry and †Biochemical Pharmacology, University of Konstanz, Konstanz, Germany

1 During *in vitro* culture in 10% human AB serum, human peripheral blood monocytes acquire a macrophage-like phenotype. The underlying differentiation was characterized by increased activities of the macrophage marker enzymes unspecific esterase (NaF-insensitive form) and acid phosphatase, as well as by a down-regulation in surface CD14 expression.

2 In parallel, a dramatic change in the phosphodiesterase (PDE) profile became evident within a few days that strongly resembled that previously described for human alveolar macrophages. Whereas PDE1 and PDE3 activities were augmented, PDE4 activity, which represented the major cyclic AMP-hydrolysing activity of peripheral blood monocytes, rapidly declined.

3 Monocytes and monocyte-derived macrophages responded to lipopolysaccharide (LPS) with the release of tumour necrosis factor-α (TNF). In line with the change in CD14 expression, the EC_{50} value of LPS for induction of TNF release increased from approximately 0.1 ng ml^{-1} in peripheral blood monocytes to about 2 ng ml^{-1} in macrophages.

4 Both populations of cells were equally susceptible towards inhibition of TNF release by cyclic AMP elevating agents such as dibutyryl cyclic AMP, prostaglandin E_2 (PGE_2) or forskolin, which all led to a complete abrogation of TNF production in a concentration-dependent manner and which were more efficient than the glucocorticoid dexamethasone.

5 In monocytes, PDE4 selective inhibitors (rolipram, RP73401) suppressed TNF formation by 80%, whereas motapizone, a PDE3 selective compound, exerted a comparatively weak effect (10–15% inhibition). Combined use of PDE3 plus PDE4 inhibitors resulted in an additive effect and fully abrogated LPS-induced TNF release as did the mixed PDE3/4 inhibitor tolafentrine.

6 In monocyte-derived macrophages, neither PDE3- nor PDE4-selective drugs markedly affected TNF generation when used alone (<15% inhibition), whereas in combination, they led to a maximal inhibition of TNF formation by about 40–50%. However, in the presence of PGE_2 (10 nM), motapizone and rolipram or RP73401 were equally effective and blocked TNF release by 40%. Tolafentrine or motapizone in the presence of either PDE4 inhibitor, completely abrogated TNF formation in the presence of PGE_2. Thus, an additional cyclic AMP trigger is necessary for PDE inhibitors to become effective in macrophages.

7 Finally, the putative regulatory role for PDE1 in the regulation of TNF production in macrophages was investigated. Zaprinast, at a concentration showing 80% inhibition of PDE1 activity (100 μmol l^{-1}), did not influence TNF release. At higher concentrations (1 mmol l^{-1}), zaprinast became effective, but this inhibition of TNF release can be attributed to a significant inhibitory action of this drug on PDE3 and PDE4 isoenzymes.

8 In summary, the *in vitro* differentiation of human peripheral blood monocytes to macrophages is characterized by a profound change in the PDE isoenzyme pattern. The change in the PDE4 to PDE3 ratio is functionally reflected by an altered susceptibility towards selective PDE inhibitors under appropriate stimulating conditions.

Keywords: Monocyte-derived macrophages; PDE inhibitors; prostaglandin E_2; tumour necrosis factor-α; cyclic AMP; PDE isoenzymes; LPS

Introduction

Among the large variety of cellular functions that are orchestrated by the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) protein kinase A (PKA) cascade, the anti-inflammatory effects caused by increased cyclic AMP levels have attracted considerable interest both in immunological and pharmacological research over the last decade. Intracellular cyclic AMP concentrations increase as a consequence of receptor-triggered adenylyl cyclase activation or, alternatively, by decreased activity of phosphodiesterases (PDE), which regulate breakdown of cyclic AMP and cyclic GMP, respectively. PDE are a growing group of enzymes classified into 7 distinct families (PDE1–PDE7) with several subtypes and splice variants (for recent reviews see Beavo *et al*., 1994; Bolger, 1994; Loughney & Ferguson, 1996). In inflammatory cells, the low K_m cyclic AMP-specific members of the PDE4 family are most prominently expressed and thus have attracted attention as a pharmacological target in the field of anti-inflammatory drug development. PDE4 selective inhibitors, such as the prototype rolipram, have been shown to inhibit a variety of leukocyte functions including inflammatory mediator release (reviewed in Dent & Giembycz, 1996). One of the most harmful endogenous pro-inflammatory cytokines is tumour necrosis factor-α (TNF) (Jones & Selby, 1989; Tracey & Cer-

[1]Author for correspondence at: Byk Gulden, Department of Biochemistry, POB 100310, D-78403 Konstanz, Germany.

ami, 1993; Strieter *et al*., 1993). Production of this cytokine has repeatedly been shown to be potently abrogated in the presence of PDE4 inhibitors *in vitro* (Semmler *et al*., 1993b; Schade & Schudt, 1993; Schudt *et al*., 1993b; 1995; Prabhakar *et al*., 1994; Seldon *et al*., 1995) and *in vivo* (Fischer *et al*., 1993; Sommer *et al*., 1995; Genain *et al*., 1995; Gantner *et al*., 1997), which is believed to contribute largely to the anti-inflammatory effects of these drugs, at least under acute inflammatory conditions. TNF has been shown to cause tissue destruction in various organs including nervous tissue (Sommer *et al*., 1995; Genain *et al*., 1995), liver (Tiegs *et al*., 1989; Leist *et al*., 1994; 1995a, b; Gantner *et al*., 1995a, b) and lung (Nash *et al*., 1991; Myatt *et al*., 1994; Windsor *et al*., 1994; Renzetti *et al*., 1996). Several cell types are known to be TNF producers when appropriately stimulated, but the most potent TNF producing cell populations belong to the monocyte/macrophage lineage (Beutler & Cerami, 1988; Gantner *et al*., 1996). In several organ-specific pathological situations tissue macrophages might be more critical as a cellular source of TNF than monocytes, since macrophages produce TNF directy at the site of its action. In the liver, for instance, Kupffer cells are known to be responsible for TNF production followed by TNF-dependent liver cell destruction in galactosamine/LPS-treated mice (Freudenberg *et al*., 1986; Leist *et al*., 1996). In the lung, a similar role is attributed to alveolar macrophages, which release TNF under conditions of airway disorders (Gosset *et al*., 1991; Ying *et al*., 1991; Broide *et al*., 1992; Vecchiarelli *et al*., 1992; Cembrzynsky-Nowak *et al*., 1993).

Whereas much data exist on the effect of PDE inhibitors on human monocyte functions (Molnar-Kimber *et al*., 1993; Seldon *et al*., 1995; Souness *et al*., 1996), few have been published on human alveolar macrophages (Schudt *et al*., 1993b). The reason for this may be due to the fact that monocytes are easy to obtain in large numbers, whereas human alveolar macrophages are not. Recently, we have compared the PDE activity profile of freshly prepared peripheral blood monocytes and human alveolar macrophages. Whereas monocytes mainly contained PDE4 activity, the most prominent PDE activity in alveolar macrophages was found to be PDE1, followed by PDE3 and PDE4 (Tenor *et al*., 1995a). In preliminary experiments we noticed that monocytes acquired a PDE profile very similar to that of alveolar macrophages when kept in culture for about one week. Since such an *in vitro* system permits the generation of large numbers of macrophages, we were interested to study the phenotypical changes of cultured monocytes in more detail. In addition, we wondered whether the change of PDE isoenzyme pattern was functionally reflected by comparing the influence of non-selective and PDE3- as well as PDE4-selective inhibitors on the regulation of LPS-stimulated TNF production in the two cell populations.

Methods

Monocyte isolation and culture

For each experiment 250 ml of peripheral venous blood was drawn from a single volunteer. Alternatively, cells were isolated from 500 ml buffy coats stored overnight at 4°C (Blutbank Münsterlingen, CH). Citrate (0.31%) was used as an anti-coagulant. The blood was diluted 1.6 fold with PBS (pH 7.4) before centrifugation at $220 \times g$ at 20°C for 20 min.

The cell pellet was layered on a Percoll gradient ($\rho = 1.077$ g ml^{-1}) and the interphase containing the peripheral blood mononuclear cells (PBMC) was obtained following a $800 \times g$ centrifugation. Cells were washed twice in elutriation medium (PBS, 2% heat-inactivated human AB serum, 2 mmol l^{-1} EDTA, 5 mmol l^{-1} glucose, pH 7.4) before countercurrent centrifugal elutriation of the cells by a J2-MC centrifuge equipped with a JE-6B rotor (Beckman, U.S.A.).

The monocyte containing fraction (platelet-free, 75–85% pure) was obtained at a flow rate of 39 ml min^{-1} and a rotor speed of 3000 r.p.m.

Cells were left to adhere on culture dishes (Primaria 3072, Becton Dickenson, NJ, U.S.A.) for 1 h at 37°C, non-adherent cells were removed and the medium was exchanged for fresh RPMI 1640 (containing 10% heat-inactivated human AB serum (Sigma, Deisenhoften, Germany) and antibiotics (penicilline, 5000 iu ml^{-1}; streptomycin, 5 mg ml^{-1}). Monocytes (5×10^6) were cultured in a volume of 10 ml per plate in an incubator run at 37°C. Medium was exchanged every 2–3 days. Flow cytometry confirmed that the monocyte population was 90–95% pure at the beginning of culture.

Flow cytometry

Freshly prepared monocytes (day 0 of culture) or cultured monocytes (day 6) were removed from culture dishes after two washing steps (cold PBS) by vigorous pipetting and by the use of cell lifters (Costar 3008, Cambridge, MA, U.S.A.). Cells were washed twice in PBS and resuspended in staining buffer (PBS containing 2% FCS) at a density of $5 \times 10^5 - 1 \times 10^6$ cells ml^{-1}. Fluorescent dye-labelled antibodies (Ab) (Immunotech, Hamburg, Germany) directed against various surface markers were added for 30 min on ice. The incubation was performed in the dark and unbound Ab was then removed by centrifugation in staining buffer. Expression of surface markers was analysed by flow cytometry (Coulter, Hamburg, Germany).

Sonication of cells

Culture dishes were washed twice in PBS containing Ca^{2+} (0.5 mmol l^{-1}) and Mg^{2+} (0.5 mmol l^{-1}). Cells were harvested and disrupted by sonication for 2 min (Branson sonifier 250, output control 1) and homogenates were centrifuged at $15800 \times g$ for 5 min, the supernatant was decanted (see below, PDE activities) and the pellet fraction was stored separately at −20°C until further use.

Determination of cellular protein content

For protein determination a commercially available kit (Pierce Chemical Company, U.S.A) based on the specific reaction of bicinchoninic acid (BCA) with cuprous ion (Cu^{1+}) was used according to the manufacturer's protocol (Micro BCA Protein Assay Reagent Kit Nr. 23225).

DNA determination

DNA was measured according to the method of Burton (1956). Samples ($15800 \times g$ pellets, see above) were resuspended in PBS/0.5 N perchloric acid, hydrolysed at 70°C for 30 min and then cooled to room temperature before diphenylamine (3% in acetate) was added. After overnight incubation, colour development was measured at 595 nm and DNA content was calculated based on a standard curve with calf thymus DNA (type I from Sigma Deisenhofen, Germany).

Acid phosphatase

Acid phosphatase activity was determined as described by Becker *et al*. (1987). Samples ($15800 \times g$ supernatants) were analysed for enzymatic activity with *p*-nitrophenolphosphate (50 mmol l^{-1} in citrate buffer, pH 4.0) as a substrate. After incubation at 37°C for 60 min the reaction was stopped by addition of NaOH (0.1 N) and colour formation was determined spectrophotometrically at 405 nm. A *p*-nitrophenol dilution curve ranging from 0.65–40 mmol l^{-1} was used as a standard; heat-inactivated enzyme was used as a blank.

Unspecific esterase

Unspecific esterase activity was determined as described by Sonne *et al*. (1991) with 100 μl α-naphthylacetate (5 mg dissolved in 1 ml methanol plus 25 ml PBS containing 20 mmol l^{-1} HEPES and 0.2% BSA) as a substrate solution.

After incubation of the samples ($15800 \times g$ supernatants) in the absence and presence of NaF (0.6 mmol l^{-1}) at 37°C for 30 min the reaction was stopped by addition of sodium acetate buffer (100 mmol l^{-1}, pH 8.0) containing sodium barbitale (10 mmol l^{-1}), SDS (4%) and fast red (0.2%). Colour formation was determined spectrophotometrically at 490 nm. An α-naphthol dilution curve (50 mmol l^{-1} in ethanol) ranging from 0.012–1.6 mmol l^{-1} was used as a standard; heat-inactivated enzyme was used as a blank.

Lactate dehydrogenase

LDH was determined by use of a commercially available kit (Sigma, Deisenhofen, Germany) according to the manufacturer's instructions.

PDE activities

Samples ($15800 \times g$ supernatants) were further separated by a $100{,}000 \times g$ centrifugation step (1 h, 4°C). The supernatant was decanted (soluble fraction) and the remaining pellet was resuspended in an equal volume of buffer (particulate fraction). PDE activity was determined as described by Thompson & Appleman (1979) with some modifications (Bauer & Schwabe, 1980). In brief, the enzyme containing fractions were assayed in a final volume of 200 μl containing 60 mmol l^{-1} Tris HCl pH 7.4, 5 mmol l^{-1} $MgCl_2$, 0.5 μmol l^{-1} cyclic AMP or cyclic GMP (28000 c.p.m. [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) and were incubated in the presence and absence of activators or inhibitors for 30 min at 37°C. The reaction was terminated by the addition of 50 μl of 0.2 N HCl and the assay mixture was left on ice for further 15 min. *Crotalus atrox* snake venom (0.5 mg ml^{-1}) was added for 15 min at 37°C and the assay mixture was then loaded on QAE-Sephadex A-25 columns (1 ml bed volume) and eluted with 2 ml ammonium formate (30 mmol l^{-1}; pH 6.0). The radioactivity in the eluate was counted in a liquid scintillation counter. PDE isoenzyme activity calculations in the soluble as well as in the particular cell fractions were performed by making use of activators and the PDE family-selective inhibitors motapizone (PDE3), rolipram (PDE4) and zaprinast (PDE5). Calculations were performed as described by Tenor *et al*. (1995a).

TNF determination

Monocytes or monocyte-derived macrophages were incubated in 96 well plates at a density of 5×10^4 cells per 200 μl with or without LPS. At the time points indicated, supernatants were removed and frozen at −80°C until further use. Total TNF was determined by a commercially available ELISA kit (Immunotech, Hamburg, Germany) according to the manufacturer's protocol.

Chemicals

Rolipram (4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone) was a generous gift from Schering AG (Berlin, Germany), and motapizone (4,5-dihydro-6-[4-(11-imidazol-1-yl)-2-thienyl]-5-methyl-3-pyridazinone) from Nattermann (Cologne, Germany). Zaprinast was purchased from Sigma Chemie (Deisenhofen, Germany). RP73401 (Souness *et al*., 1995) and tolafentrine (Schudt *et al*., 1993a) have been synthesized at the chemistry facilities of Byk Gulden (Konstanz, Germany). HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]) and dodecylsulphate Na-salt (SDS) were obtained from Serva (Heidelberg, Germany). LPS (*S. abortus equi*), PGE_2 and all other reagents were purchased from Sigma Chemie (Deisenhofen, Germany).

Statistics

Unless otherwise stated, data are expressed as means ± s.d. (standard deviation) of at least three independent experiments. Statistical significances were determined with the unpaired Student's *t* test if applicable or with the Mann-Whitney test if variances were non-homogeneous (U-test < 0.05) by use of commerically available statistic programmes (GraphPad Software, San Diego, CA).

Results

PDE profile of cultured monocytes

During culture of peripheral blood monocytes in 10% human AB serum, the PDE activity profile changed dramatically. Based on specific activity, a marked increased in PDE1 activity, (hardly detectable at day 0), was noted from day 2 onwards which was maximal at day 6 to day 8 and slightly decreased until the end of culture (day 10). PDE3, which contributed 15–20% to the total cyclic AMP hydrolysing activity in freshly prepared monocytes (day 0), also significantly increased, but with somewhat slower kinetics compared to PDE1. In contrast, PDE4, the most prominent PDE enzyme present in monocytes (at day 0), was down-regulated very rapidly within 48 h and then remained unchanged over the remaining culture time investigated. PDE2 and PDE5 activities were almost undetectable in monocytes and did not change during culture (Figure 1).

In general, the majority of PDE1 (>90%) and PDE4 (>70%) activities were found in the soluble fraction, whereas PDE3 activity was mainly membrane-bound (>90%). These ratios did not change during culture.

Thus, a PDE activity profile had emerged during culture of monocytes that was very similar to that found in human alveolar macrophages (Tenor *et al*., 1995a).

Monocytes acquire a macrophage-like phenotype in culture

The macrophage-like PDE pattern prompted us to look for other biochemical markers typical for these cells. We investigated the time course of enzyme activities of acid phosphatase (Goldberg & Barka, 1962), unspecific esterases (NaF-sensitive and NaF-insensitive forms) as well as lactate dehydrogenase (LDH) as a constitutive control enzyme. In parallel

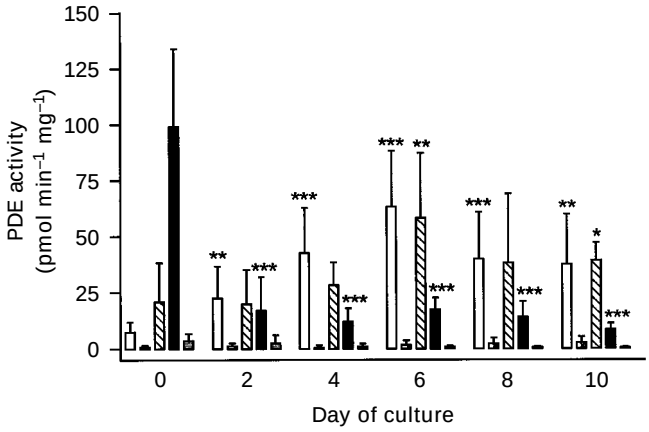

Figure 1 PDE activity profile of monocytes (day 0) during differentiation towards macrophages. Monocytes were plated in culture dishes, left to adhere and then kept in culture for 0 to 10 days. At the time points indicated, cells were harvested and PDE activities were determined in the cytosolic and particulate fraction, respectively. Data are expressed as mean total activities (soluble plus particulate) ± s.d. from 6–11 independent preparations. Open columns, PDE1; cross-hatched columns, PDE2; hatched columns, PDE3; solid columns, PDE4; stippled columns, PDE5. Statistically significant PDE activity changes compared to the corresponding control values at day 0 are represented by $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

with the PDE activity changes, the specific activities of acid phosphatase and NaF-insensitive esterase significantly increased during *in vitro* differentiation (Figure 2). The ratio between NaF-sensitive (not shown) to NaF-insensitive (macrophage-selective, Sun, 1985) unspecific esterase dropped, whereas total unspecific esterase activity remained stable from day 2 onwards (data not shown). LDH activity at the end of culture was similar to that measured in peripheral blood monocytes. Notably, marked changes in total protein content were noted during differentiation. Within 24 h, total cell numbers significantly declined, probably due to preparative stress and ensuing lysis of about 50% of the plated cells. From day 5 onwards cell numbers had recovered and reached the numbers that had been seeded at the beginning of culture. This suggests that monocytes/macrophages were still capable of proliferating. In parallel, the cells became firmly adherent to the plastic dishes and a dramatic increase in total protein/DNA-ratio of the cells was noted, which is in accordance with the increase in cell surface that was visible by microscopic inspection.

In addition to the biochemical and morphological criteria, we looked for changes of surface marker expression on monocytes during culture. For this purpose we analysed freshly prepared monocytes and cells that were kept in culture for 6 days. As shown in Figure 3, a tremendous down-regulation of the monocyte marker CD14 and, to a lesser extent, of MHCII molecules was noted. As expected, the common leukocyte antigen CD45 and the myeloid marker CD13 remained unchanged. Furthermore, the absence of CD1a expression indicates that no differentiation towards dendritic cells, which also originate from $CD14^+$ monocytes (Kiertscher & Roth, 1996; Zhou & Tedder, 1996; reviewed in Peters *et al*., 1996), had occurred. The percentage of positive cells (Figure 3) was further verified by a decrease of mean fluorescence values of CD14 and MHCII stained cells at day 6 compared to freshly isolated monocytes (not shown).

Overall, the changes in morphology, enzyme activities and surface marker expression strongly suggest that peripheral blood monocytes under our experimental culture conditions differentiate towards macrophage-like cells.

LPS-stimulated release of TNF

We first addressed the question of whether *in vitro* differentiated monocytes were still capable of responding to LPS

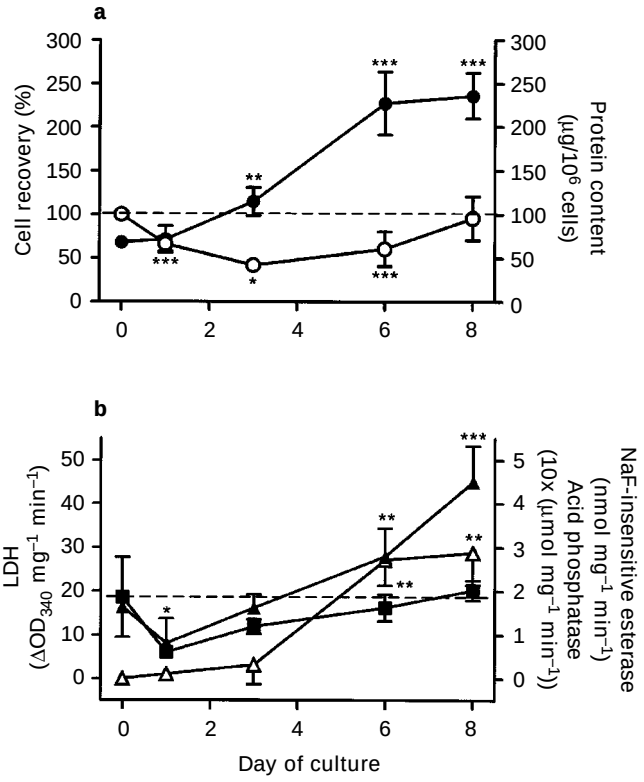

Figure 2 Change of biochemical markers during culture of monocytes. Cultured monocytes were harvested at the time points indicated, protein content (●) was determined, cell recovery (○) calculated based on DNA contents, and activities of LDH (■), unspecific esterase (NaF-insensitive form) (△) and acid phosphatase (▲) assessed. Data are expressed as mean values (vertical lines show s.d.) on a per cell basis (a) or as specific enzyme activities (b) from 6–11 independent preparations. $*P<0.05$, $**P<0.01$ and $***P<0.001$, statistically significant activity changes compared to control values at day 0.

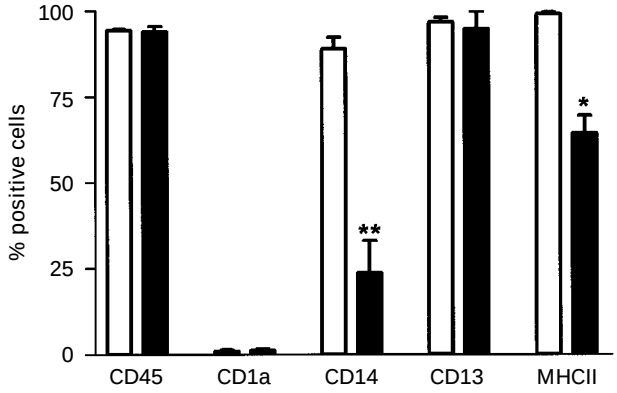

Figure 3 Change of surface marker expression by culture of monocytes. Surface marker expression was analysed on freshly isolated monocytes at day 0 (open columns) and on monocyte-derived macrophages at day 6 (solid columns) by the use of fluorescence-dye labelled Ab directed towards various leukocyte markers. Data represent mean values ± s.d. of four independent experiments. $*P<0.05$, $**P<0.1$ versus monocyte control.

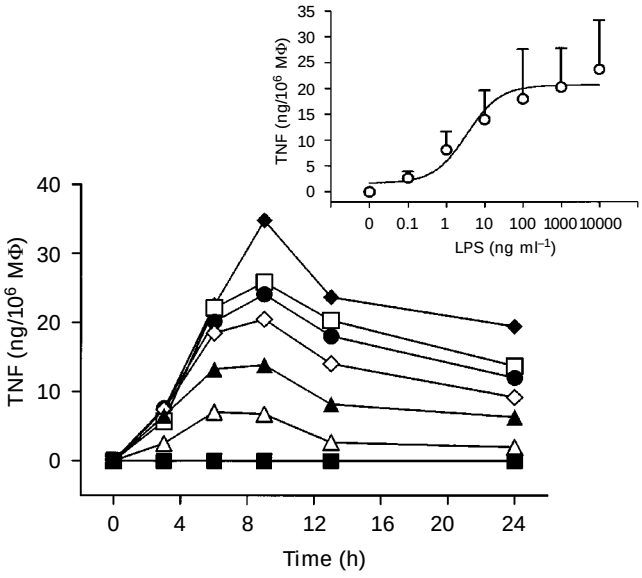

Figure 4 LPS-induced TNF release from monocyte-derived macrophages. Monocytes were plated on 96 well plates (5×10^4 cells in 200 µl) and medium was changed every 2–3 days. At day 6, the monocyte-derived macrophages (MΦ) were stimulated by various concentrations of LPS: (◆) 10,000 (□) 1000, (●) 100, (◇) 10, (▲) 1, (△) 0.1 ng ml^{-1}; (■) control curve in the absence of LPS. The kinetics of TNF release over a time period of 24 h is shown in the main graph. Data are mean values from 5 experiments. For reasons of clarity, s.d. is not given. Inset: TNF production as assessed 13 h after stimulation (mean values ± s.d. (vertical lines) of 8 independent preparations) is depicted as a fitted concentration-response curve (non-linear regression).

with the release of TNF, since these cells showed reduced expression of CD14, the main LPS receptor (Figure 3). As depicted in Figure 4, a time- and concentration-dependent TNF release of LPS-stimulated monocyte-derived macrophages was noted. Independent of the stimulus concentration used, TNF in the supernatant increased up to 9 h following stimulation and then decreased until the end of the experiment.

We next compared freshly isolated monocytes and cultured cells at day 6 derived from the same individuals. The 6–7 h isolation procedure of monocytes did not allow us to analyse the kinetics of TNF release, in contrast to the experiment described above. Therefore, we focused on 13 h, i.e. overnight incubation, and measured TNF in the supernatant of monocytes exposed to increasing concentrations of LPS. In contrast to monocyte-derived macrophages (day 6; Figure 4), freshly prepared monocytes sometimes spontaneously released TNF indicating activation due to the isolation procedure. When exposed to LPS, basal TNF release from monocytes was drastically augmented. As shown in Figure 5, 1 ng ml^{-1} LPS was sufficient to induce nearly maximal TNF production. After correction for cell numbers, LPS was one order of magnitude more potent on monocytes compared to macrophages with regard to induction of TNF synthesis. This observation fits well with the diminished expression of the LPS receptor CD14 on macrophages (Figure 3).

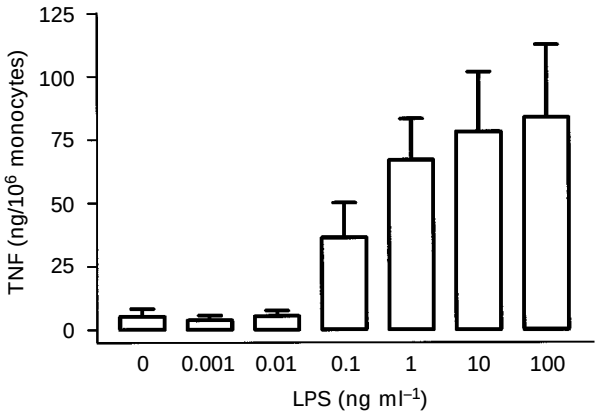

Figure 5 LPS-induced TNF release from freshly isolated human monocytes. Monocytes were seeded on 96 well plates (5×10^4 cells in 200 μl) and left to adhere for 1 h before medium was exchanged. Cells were stimulated by addition of various LPS concentrations and TNF was assessed in the supernatant after overnight incubation for 13 h. Data represent mean values ± s.d. of 5 independent concentration-response curves carried out in cells derived from 5 individuals.

Modulation of TNF production by cyclic AMP

As outlined above, both freshly prepared monocytes as well as *in vitro*-derived macrophages were able to release TNF following LPS stimulation. Our next aim was to investigate whether both cell populations still were susceptible towards pharmacological modulation of the second messenger cyclic AMP. In order to raise cellular cyclic AMP we used a cell permeable equivalent, dibutyryl (db)-cyclic AMP, the adenylyl cyclase activator, forskolin, and PGE_2. The effects of these cyclic AMP elevating drugs were compared to dexamethasone, a commonly used anti-inflammatory agent. As depicted in Figure 6, all agents produced a concentration-dependent inhibition of TNF production in monocytes and in macrophages. The maximal inhibition of TNF release by the cyclic AMP elevating drugs was more pronounced compared to dexamethasone, the latter inhibiting TNF production by only 50–60%. Compared to db-cyclic AMP, the cyclic GMP analogue db-cyclic GMP was a comparatively weak inhibitor of LPS-induced TNF formation.

These data clearly demonstrate that elevation of intracellular cyclic AMP is an appropriate pharmacological approach to suppress completely TNF generation of LPS-stimulated monocytes/macrophages.

Modulation of TNF production by PDE inhibitors

Our next aim was to clarify whether the changed PDE pattern that was acquired by monocytes during culture would be reflected by a change in the inhibitory profile of family selective PDE inhibitors.

We used a submaximal LPS concentration of 1 ng ml^{-1} to study the inhibition of TNF production. The maximum inhibition of TNF release by the non-selective PDE inhibitor theophylline was 60% at its highest concentration (1 mmol l^{-1}) used. The PDE4 selective compounds rolipram and RP73401 both were highly effective in suppressing TNF release from LPS-stimulated monocytes (80% inhibition at concentrations that were selective for PDE4). In contrast, a very weak effect of the PDE3 selective inhibitor motapizone was observed (10–15% suppression at 1 μmol l^{-1}). However, combined use of motapizone (1 μmol l^{-1}) and rolipram or RP73401 resulted in an additive inhibition of TNF production, i.e. a complete abrogation of TNF release. In line with this observation the mixed PDE3/4 inhibitor tolafentrine was more effective than the PDE4 selective compounds (Figure 7).

These results demonstrate that PDE4 plays a dominant functional role in the regulation of LPS-induced TNF release from human monocytes. In contrast, PDE3 only plays a minor role. Thus, the PDE pattern measured in these cells (Figure 1, day 0) is functionally reflected by the inhibition of TNF synthesis. Accordingly, the effect of PDE4 inhibition should be less pronounced in macrophages, since this PDE isoenzyme was down-regulated during culture (Figure 1). Indeed, rolipram and RP73401 only weakly affected TNF generation of LPS-stimulated macrophages. The PDE3 inhibitor motapizone (1 μmol l^{-1}) was completely ineffective when used alone, but increased the effect of the PDE4 inhibitors (Figure 8a). The unspecific inhibitor theophylline and the PDE3/4 inhibitor tolafentrine maximally inhibited TNF release by up to 60% (Figure 8a). The observation of a complete abrogation of TNF production in macrophages by db-cyclic AMP (Figure 6) led us to hypothesize that the cyclic AMP signal generated in macrophages in the presence of PDE3/4 inhibitors was not sufficiently high to block fully TNF production in response to LPS. To test this assumption, we used PGE_2 as a costimulus in order to increase further intracellular cyclic AMP. Indeed, PGE_2 at a concentration (10 nmol l^{-1}) hardly effective when used alone (Figure 6), strongly augmented the TNF suppressing action of the PDE inhibitors: inhibition of TNF release by PDE4 selective compounds and by the PDE3 inhibitor motapizone was 30–40%, and by the mixed PDE3/4 inhibitor tolafentrine or by the combined use of PDE3 and PDE4 inhibitors nearly 100% (Figure 8b).

PDE1 is not functionally involved in the regulation of TNF release

Due to the unavailability of a mono-selective PDE1 inhibitor, we used zaprinast to elucidate the role of PDE1 in TNF generation by macrophages. We determined the IC_{50} values of zaprinast for PDE1, PDE3 and PDE4, respectively, in the homogenate of *in vitro*-differentiated macrophages (PDE5 was not present). In parallel, a concentration-response curve of zaprinast with regard to inhibition of LPS-stimulated TNF release from macrophages, was carried out. Zaprinast significantly blocked TNF synthesis at 1 mmol l^{-1}. It lacked efficacy at 100 μmol l^{-1} (Figure 8a), although at this concentration zaprinast selectively blocked PDE1 activity (Table 1).

From these experiments, we conclude that PDE1 is not functionally involved in the regulation of LPS-induced TNF

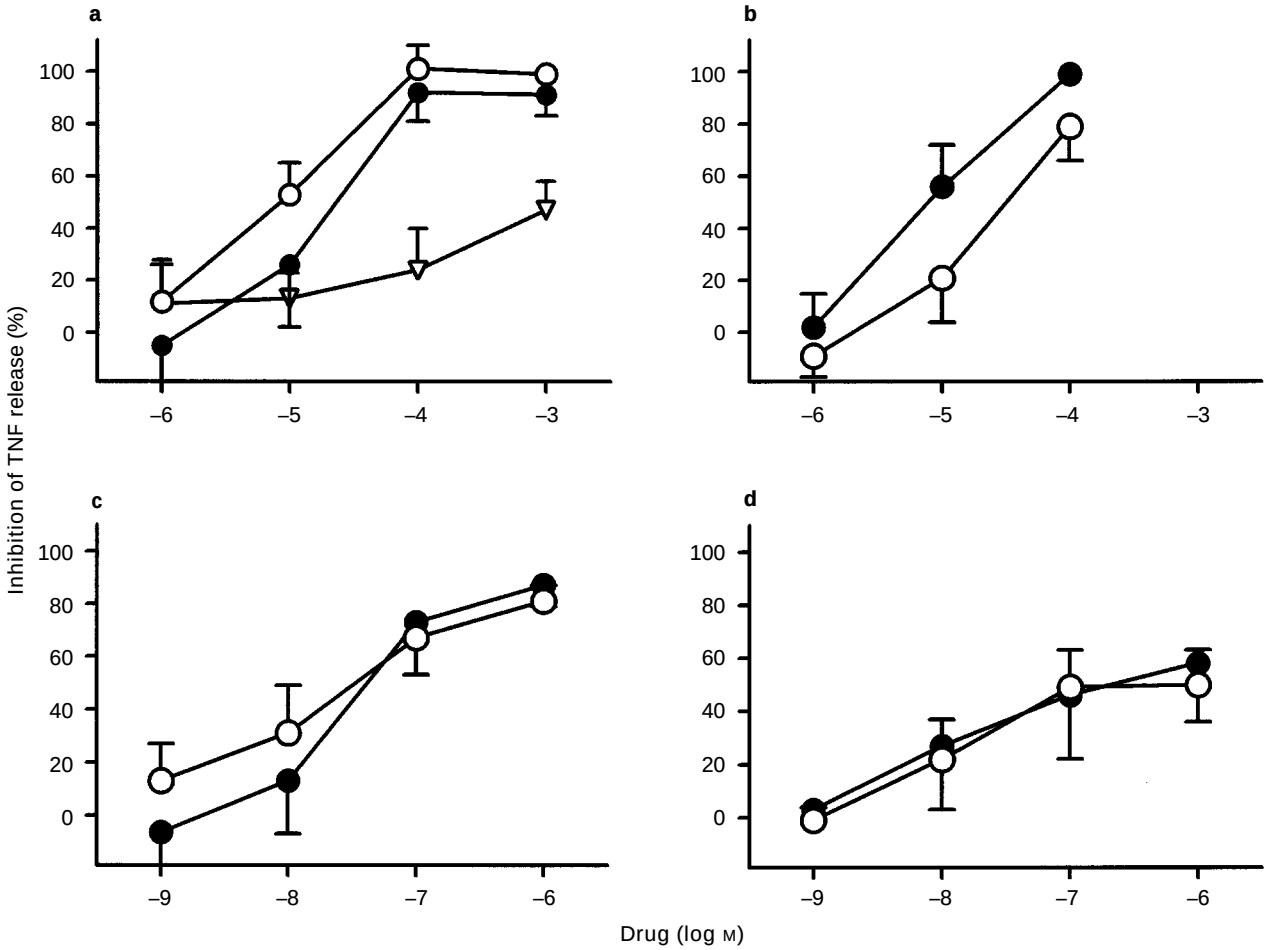

Figure 6 Inhibition of LPS-induced TNF release in human monocytes and monocyte-derived macrophages by cyclic AMP elevating drugs, (a) dibutyryl cyclic AMP, (b) forskolin, (c) prostaglandin E_2, and (d) dexamethasone. (○) Monocytes (day 0) or (●) macrophages (day 6) were seeded on 96 well plates at identical densities (4×10^5 cells in 200 μl) and incubated with medium (control values), various drugs (a) dibutyryl-cyclic GMP (▽) was tested in monocytes only) at the concentrations indicated or the same volume of solvent (0.1% DMSO) for 30 min before LPS (1 ng ml^{-1} for monocytes, 1000 ng ml^{-1} for macrophages) was added. Drug effect was calculated based on the amount of TNF secreted in absence of any compound (control values, set at 100%). Data are means of 4–8 separate experiments run in duplicate; vertical lines show s.d.

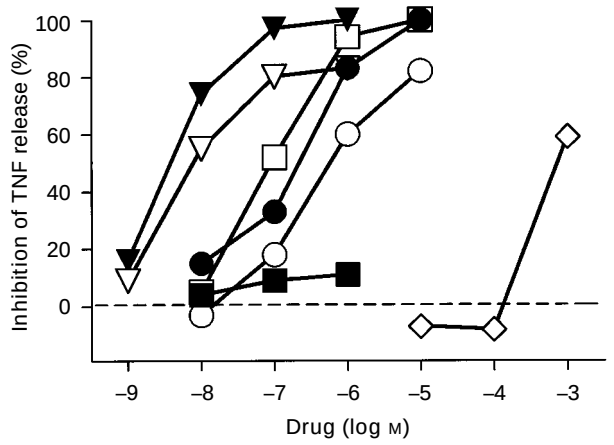

Figure 7 Suppression of LPS-stimulated TNF synthesis by PDE inhibitors, (■) motapizone, (○) rolipram, (●) rolipram + motapizone (1 μM), (▽) RP73401, (▼) RP73401 + motapizone (1 μM), (□) tolafentrine and (◇) theophylline, in human monocytes. Monocytes were seeded on 96 well plates (4×10^5 cells in 200 μl) in the absence (0.1% DMSO as solvent control) or presence of PDE inhibitors for 30 min before LPS (1 ng ml^{-1}) was added. Drug effect was calculated based on the amount of TNF secreted in the absence of any compound (control values, set at 100%). Data are means of 8–12 separate experiments run in duplicate. For reasons of clarity s.d. values are not shown, which were in the range of 2–18%.

release from human macrophages. This is further supported by the fact that inhibition of PDE3 and PDE4 in the presence of PGE_2 was sufficient to abrogate fully TNF release in these cells.

Discussion

In a recent study (Tenor *et al*., 1995a), we noted that alveolar macrophages or *in vitro*-derived macrophages express a markedly different PDE activity profile compared to peripheral blood monocytes. This prompted us to (i) analyse in detail the changes in PDE profile and other parameters during differentiation of monocytes towards macrophages, and (ii) investigate the pharmacological consequences of the altered PDE isoenzyme activities with particular emphasis on susceptibility to suppression of TNF release by selective PDE inhibitors.

The morphological and biochemical changes that we observed in differentiating monocytes are in line with our objective to generate macrophage-like cells *in vitro*. CD14, a surface marker for both monocytes and macrophages, was dramatically down-regulated during culture. With regard to lung macrophages, this observation is in accordance with the findings of other groups (Perez-Arellano *et al*., 1993; Hoogsteden *et al*., 1993), and supports the view that under our culture conditions macrophages of an alveolar type may arise.

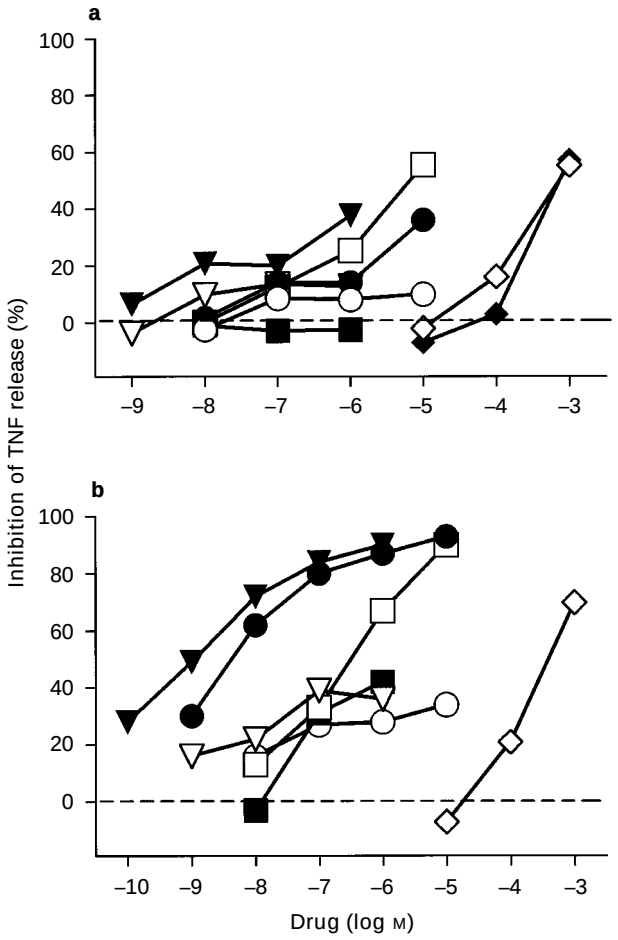

Figure 8 Suppression of LPS-stimulated TNF synthesis by PDE inhibitors, (○) rolipram, (●) rolipram + motapizone (1 μM), (▽) RP73401, (▼) RP73401 + motapizone (1 μM), (□) tolafentrine, (◇) theophylline, (■) motapizone and (◆) zaprinast, in human monocyte-derived macrophages. Monocytes were differentiated to macrophages within 6 days (see legend of Figure 1) on 96 well plates (4×10^5 cells in 200 μl). They were then incubated in the absence (a) or presence (b) of PGE_2 (10 nM) with PDE inhibitors for 30 min before LPS (100 ng ml^{-1}) was added. Drug effect was calculated based on the amount of TNF secreted in the absence of any compound (control values in presence of 0.1% DMSO, set at 100%). Data are means of 4–8 separate experiments run in duplicate. For reasons of clarity s.d. values are not shown, which were in the range of 9–26%.

Importantly, the myeloid markers CD45 and CD13 remained stable, and the monocyte-derived cells were devoid of any CD1a expression.

Several methods have been described for the generation of macrophages *in vitro*, and depend upon the contribution of a variety of factors possibly influencing the differentiation process, such as serum (content as well as source) and growth factors (Kaplan & Gaudernack, 1982; Bauer *et al*., 1988; Testa *et al*., 1989; Lepe-Zuniga & Klostergard, 1990; Gessani *et al*., 1993). Both increased (Gessani *et al*., 1993; Lepe-Zuniga & Klostergard, 1990) and decreased (Burchett *et al*., 1988) capacity to release TNF of human macrophages compared to monocytes has been demonstrated. The lower expression of the LPS receptor CD14 in monocyte-derived macrophages under our conditions might explain the higher EC_{50} for LPS to induce TNF release (see Figures 4 and 5).

A pre-requisite for the usefulness and validity of our system was the susceptibility of both monocytes and macrophages towards the inhibition of TNF release by a rise in cyclic AMP levels. Identical inhibition curves in both cell populations were found by db-cyclic AMP, forskolin and PGE_2, suggesting that

Table 1 Inhibition of PDE activities in the homogenate of human monocyte-derived macrophages by zaprinast

PDE activity	IC_{50} (μmol l^{-1})
PDE1	36
PDE3	295
PDE4	646

Concentration-dependent inhibition curves were carried out in the homogenate of cultured monocytes on day 6. Zaprinast was used at concentrations ranging from 1 μmol l^{-1} to 1 mmol l^{-1}. Mean values from three independent experiments are given.

the signalling cascades involving cyclic AMP are not modulated during differentiation towards macrophages. Negative regulation of TNF synthesis by cyclic AMP has been repeatedly obtained in monocytes and macrophages (Renz *et al*., 1988; Taffet *et al*., 1989; Severn *et al*., 1992; Semmler *et al*., 1993a, b; Prabhakar *et al*., 1994; Reinstein *et al*., 1994; Seldon *et al*., 1995), although the mechanisms of TNF down-regulation by cyclic AMP are not understood in detail. Both cyclic AMP effects on transcription and/or on posttranscriptional levels of the TNF gene have been suggested (Burchett *et al*., 1988; Taffet *et al*., 1989; reviewed in Spriggs *et al*., 1991), and it is not clear whether cyclic AMP acts directly via an AP-1/CRE-like promoter sequence found on the human TNF gene (Newell *et al*., 1994) or, alternatively, acts indirectly by increasing interleukin-10 (IL-10) production (Platzer *et al*., 1995; Arai *et al*., 1995; Kambayashi *et al*., 1995b. Jilg *et al*., 1996). IL-10 may lead to TNF suppression by attenuation of nuclear factor-κB (NF-κB) activation (Satriano & Schlondorf, 1994), an important step in the LPS-triggered cascade leading to TNF production (Shakov *et al*., 1990; Ishikawa *et al*., 1995).

In accordance with a study by Seldon *et al*. (1995), dexamethasone was less effective in suppressing TNF release (maximally 60% inhibition) compared to cyclic AMP elevating agents indicating that at least some glucocorticoid-insensitive activation steps are induced by LPS. Whereas there is general agreement on the presence of PDE4 as the predominant PDE isoenzyme in human peripheral blood monocytes, the presence of PDE3 and PDE1 activities remains controversial (Tenor *et al*., 1995a; Seldon *et al*., 1995; Souness *et al*., 1996). Preliminary PCR analyses revealed that among the PDE4 subtypes, PDE4A, PDE4B and PDE4D are expressed (Gekeler *et al*., unpublished observation), which is in accordance with recent observations (Verghese *et al*., 1995; Souness *et al*., 1996). Following differentiation to macrophages, PDE4 expression was reduced to the PDE4A subtype (Gekeler *et al*., unpublished observation), which may explain the rapid decline in total PDE4 activity within 24 h.

The functional PDE-inhibitor studies performed in LPS-stimulated monocytes perfectly reflect the enzymatic PDE activity pattern. In addition, the IC_{50} values measured for these inhibitors with regard to the inhibition of PDE4 catalysed breakdown of cyclic AMP (data not shown) fit entirely with their potency in suppressing TNF generation. A minor contribution of TNF regulation by cyclic AMP was attributable to PDE3, since motapizone (PDE3 selective) exerted an additive effect of 10–15% and the mixed PDE3/4 inhibitor tolanfentrine completely abrogated TNF formation in monocytes (see Figure 7). Exactly the same inhibition curves were found by Souness *et al*. (1996), underlining a minor though significant functional role of PDE3 in the regulation of monocyte responses. In this respect, monocytes differ from human eosinophils (Dent *et al*., 1994; Hatzelmann *et al*., 1995) or neutrophils (Schudt *et al*., 1991), which do not contain PDE3 activity and consequently their functional responses are not influenced by PDE3 inhibitors. Monocytes also differ from human T lymphocytes, where the existence and important functional role of PDE3 is well characterized *in vitro* (Robiseck *et al*., 1989; 1991; Essayan

et al., 1994; Tenor *et al*., 1995b; Schmidt *et al*., 1995; Gantner *et al*., 1995c; Banner *et al*., 1995; Giembycz *et al*., 1996) and *in vivo* (Gantner *et al*., 1997).

Monocyte-derived macrophages do respond to PDE inhibitors, which is in line with their susceptibility towards other cyclic AMP-elevating drugs, but blocking a single PDE family was not sufficient to evoke a significant suppression of TNF synthesis.

However, upon addition of exogenous PGE_2 a synergistic effect was noted. Thus, both PDE3 and PDE4 inhibitors significantly diminished LPS-induced TNF formation, and combined inhibition of both isoenzymes fully abrogated TNF release in the presence of PGE_2 (Figure 8b). These data suggest that an additional cyclic AMP trigger is necessary for PDE inhibitors to become effective in macrophages, a conclusion that is in agreement with results described by Sinha *et al*. (1995).

Numerous studies exist that describe autocrine PGE_2 release as an additional mechanism contributing to cyclic AMP-mediated effects (Kunkel *et al*., 1986; Kambayashi *et al*., 1995a). Therefore, altered endogenous synthesis of PGE_2 would have been an alternative possibility to explain why PDE4 inhibitors suppressed TNF production in monocytes but not in macrophages. However, no evidence for the involvement of endogenous PGE_2 was found. The presence of indomethacin (10 μmol l^{-1}), an inhibitor of PGE_2 synthesis by blocking cyclo-oxygenase, affected neither basal nor LPS-induced TNF release into the supernatant of monocytes or macrophages (data not shown). Essentially the same result was obtained by Seldon *et al*. (1995) in a similar set of experiments performed with the cyclo-oxygenase inhibitor, flurbiprofen.

In contrast to monocytes, PDE3 inhibitors are equally effective as PDE4-selective drugs in macrophages. This clearly shows that the inverse effects on PDE activities during differentiation, i.e. a marked downregulation of PDE4 paralleled by a significant increase in PDE3 activities, were mirrored by a greater impact of PDE3 in regulating TNF synthesis by cyclic AMP in macrophages. This notion provides a rationale for the development of mixed PDE3/4 inhibitors for the treatment of airway diseases such as asthma, where alveolar macrophages and TNF have been suggested to be causally involved (Gosset *et al*., 1991; Ying *et al*., 1991; Broide *et al*., 1992; Vecchiarelli *et al*., 1992; Cembrzynsky-Nowak *et al*., 1993). PDE3/4 synergism may reduce the risk of side effects caused by each monoselective inhibitor alone, since it can be speculated that the required doses of dual inhibitors of PDE3 and PDE4 isoenzymes will be markedly lower, an assumption that has been discussed in detail by Hatzelmann *et al*. (1996).

Although drastically upregulated in macrophages, no evidence for a functional role of PDE1 in the regulation of TNF was noted. The IC_{50} value of zaprinast for the macrophage PDE1 enzyme activity strongly argues against any significant contribution of PDE1 to the regulation by cyclic AMP of TNF synthesis; the reduced TNF levels measured at 1 mmol l^{-1} zaprinast were most likely due to inhibition of PDE3 and PDE4 isoenzymes by this compound. The question arises, which cyclic AMP or cyclic GMP mediated effects might be regulated by PDE1. At the moment, we cannot give a satisfactory answer to this question, but it is tempting to speculate that PDE1 might be involved in the process of differentiation and proliferation of macrophages. A critical role for PDE1 in the regulation of proliferation in leukaemic cells has been suggested (Epstein *et al*., 1987; Hurwitz *et al*., 1990) and, recently, the PDE1B1 subtype was identified as a key player in the mitogenesis of both leukaemic cells and peripheral blood leukocytes from healthy donors (Jiang *et al*., 1996).

In conclusion, the results of this study show that macrophages significantly differ from monocytes both in PDE isoenzyme profile and in functional inhibition exerted by PDE inhibitors. This observation provides an important basis for better predictions on the actions of PDE inhibitors on airway macrophages and for a rational design of appropriate isotype specific drugs.

The technical assistance of H. Goebel is gratefully acknowledged. The authors thank Dr V. Gekeler for performing PCR analyses, and Drs H. Amschler and D. Flockerzi for the synthesis of RP73401 and tolafentrine, respectively. Further thanks are addressed to Dr S. Postius for help with the flow cytometry, to C. Dickhaut for help with the statistics, and to Dr M. Leist for critically reading the manuscript. This work was supported by a postdoctoral grant from the Ministerium für Wissenschaft und Forschung Baden-Württemberg (720.61-11/21).

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(Received December 3, 1996
Revised February 3, 1997
Accepted February 10, 1997)