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Suppression of tumor necrosis factor-α production by interleukin-10 is enhanced by cAMP-elevating agents

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Abstract

The pro-inflammatory peptide tumor necrosis factor-α (TNF) stimulates production of the anti-inflammatory cytokine interleukin-10 by monocytes which in turn inhibits the synthesis of TNF. This inhibitory effect of interleukin-10 may contribute to the balance of pro- and anti-inflammatory cytokines in several diseases, e.g., chronic inflammatory bowel disease. In the present study we addressed the question whether interleukin-10 in combination with other TNF-suppressing agents leads to enhanced suppression of TNF synthesis. We investigated the inhibitory potency of interleukin-10 in combination with rolipram, a specific type IV phosphodiesterase inhibitor, or with cicaprost, a stable prostacyclin analogue in lipopolysaccharide-stimulated human peripheral blood mononuclear cells. Peripheral blood mononuclear cells were stimulated with 10 ng/ml lipopolysaccharide in the absence or presence of interleukin-10 or one of the cAMP-elevating agents. First, we confirmed the TNF-suppressing effect of interleukin-10, rolipram and cicaprost alone and determined the IC_{50} for these substances. Second, for the combination of interleukin-10 with one of the cAMP-elevating substances we were able to demonstrate enhanced TNF inhibition. Of these, the combination of interleukin-10 and rolipram revealed an additive effect. The maximal TNF synthesis of 5.5 ± 1.1 ng/ml after lipopolysaccharide stimulation alone was inhibited by 0.1 ng/ml interleukin-10 to 2.7 ± 0.6 ng/ml TNF and by 100 nM rolipram to 3.1 ± 0.6 ng/ml TNF. Both substances combined suppressed TNF synthesis to 1.5 ± 0.3 ng/ml. After stimulation with *Staphylococcus epidermidis* we could demonstrate a more pronounced inhibition of TNF synthesis by interleukin-10 compared to rolipram which was more effective after stimulation with lipopolysaccharide. Finally, the additive inhibitory effect of interleukin-10 and rolipram could be confirmed on the level of TNF mRNA. The results obtained in the present investigation could form a prerequisite to study the combination of interleukin-10 and cAMP-elevating agents in in vivo models of acute or chronic inflammatory diseases.

Keywords: TNF-α (tumor necrosis factor-α); Interleukin-10; cAMP-elevating agent

1. Introduction

Tumor necrosis factor-α (TNF) plays a central role as a pro-inflammatory mediator. TNF is a growth factor for T and B cells (Kehrl et al., 1987; Yokota et al., 1988), an activator of macrophages, and it induces the production of other cytokines such as interleukin-1 (Dinarello et al., 1986) and granulocyte-macrophage colony stimulating factor (Broudy et al., 1996). These pro-inflammatory effects suggest inhibition of TNF synthesis as a therapeutical approach in different diseases such as rheumatoid arthritis, septic shock or chronic inflammatory bowel disease (Elliott et al., 1994; Schreiber et al., 1995; Tracey and Cerami, 1994). Recently, successful therapy of patients with rheumatoid arthritis by an anti-TNF antibody has been reported (Elliott et al., 1994). However, repeated application of antibodies was limited by side-effects. This underlines the need for alternative strategies to inhibit the synthesis or the effect of TNF.

Interleukin-10 has initially been identified as a T_{H2} cell product which inhibits T_{H1} cell proliferation, development and function (Fiorentino et al., 1989). As recently demonstrated, interleukin-10 is produced by various other cells, including activated B cells, B cell lymphoma cells (O'Garra et al., 1992; Suda et al., 1990), monocytes and macrophages, keratinocytes (Enk and Katz, 1992), and mast cells (Moore et al., 1993). According to its original

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name, cytokine synthesis inhibitory factor (CSIF), interleukin-10 inhibits the production of interleukin-1β, interleukin-6, interleukin-8, and TNF in monocytes and macrophages (Bogdan et al., 1991; De Waal Malefyt et al., 1991; Fiorentino et al., 1991; Oswald et al., 1992), of interleukin-4 and interleukin-5 in T_{H2} cells (Moore et al., 1993) and of interferon-γ and TNF in natural killer cells (Hsu et al., 1992). Furthermore interleukin-10 induces proliferation of B cells and differentiation of cytotoxic T cells (Chen and Zlotnik, 1991; O'Garra et al., 1992).

On the basis of these results interleukin-10 has been tested as an alternative to treat TNF-mediated disorders. Interleukin-10 protected Bagg albino/c mice from lethal endotoxemia after intraperitoneal injection of endotoxin (Howard et al., 1993). A clinical trial of local interleukin-10 application in chronic inflammatory bowel disease showed suppression of the proinflammatory cytokines TNF and interleukin-1β and an improvement of endoscopic score (Schreiber et al., 1995).

Various cAMP-elevating agents such as rolipram and cicaprost have been demonstrated to inhibit TNF synthesis (Greten et al., 1996). Rolipram, a specific type IV phosphodiesterase inhibitor, and cicaprost, a prostacyclin analogue, have both been studied in vitro and in animal models (Eigler et al., 1996; Eisenhut et al., 1993; Schade, 1989; Semmler et al., 1993; Turner et al., 1993). Pharmacokinetics for rolipram have been studied in large clinical trials.

Based on these results it seemed promising to investigate the combination of the endogenous mediator interleukin-10 with cAMP-elevating agents in the suppression of TNF synthesis. In the present study we (i) compare the TNF-suppressing capacity of interleukin-10 and of rolipram and cicaprost when acting by themselves; (ii) we study the combination of cAMP-elevating agents and interleukin-10; (iii) we compare the effect of interleukin-10 and rolipram using *Staphylococcus epidermidis* as stimulus; and (iv) we investigate the effect of the combination of rolipram and interleukin-10 at the TNF mRNA level by Northern blot analysis.

2. Materials and methods

2.1. Preparation of mononuclear cells

Heparinized blood was drawn from healthy fasting volunteers, who had been without medication for at least 2 weeks. The peripheral blood mononuclear cell fraction was obtained by gradient centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany), as described previously (Böyum, 1968; Endres et al., 1991). As a modification of the protocol, isolation was performed in tubes containing a horizontal porous filter disc over the Ficoll layer (Leucosep tubes, Greiner, Frickenhausen, Germany) in order to facilitate layering of blood. Cells were dispensed into 12×75 mm polypropylene culture tubes (Falcon Becton-Dickinson, Lincoln Park, NJ, USA). RPMI 1640 culture medium was supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicilline and 100 μg/ml streptomycin (all from Sigma, Munich, Germany). The cells were suspended at 5.0×10^6 cells/ml in this medium supplemented with 2% heat-inactivated sterile human serum (from a donor with AB blood group).

2.2. Preparation of monocytes

Peripheral blood mononuclear cells were prepared as described above. Monocytes were isolated in accordance to the protocol of Manié et al. (1993). A 2 h adherence period in a 350-mm diameter tissue culture dish (Falcon, Becton-Dickinson, UK) with a final concentration of 5.0×10^6 cells/ml was carried out at 37°C in a humified 5% CO_2 atmosphere. Plates were then washed five times with medium. Adherent cells consisted of 95% monocytes as controlled by light microscopy (300 cells counted). To compare the results obtained with monocytes peripheral blood mononuclear cells were treated in an identical manner apart from the washing procedure for the peripheral blood mononuclear cells. Monocytes were incubated for 20 h in complete medium in the presence or absence of rolipram and the different stimuli.

2.3. Preparation of the compounds

Rolipram (racemate of 4-[3′-cyclopentyloxy-4′-methoxyphenyl]-2-pyrolidone, from Schering, Berlin, Germany), supplied in powder form, was dissolved in RPMI medium by vigorous vortexing. Cicaprost (Schering) was directly diluted in RPMI medium containing 2% sterile heat-inactivated human serum. Interleukin-10 (Boehringer-Mannheim, Mannheim, Germany) provided in RPMI medium, supplemented with 5% sterile heat-inactivated human serum, was diluted in RPMI medium containing 2% human serum. 62.5 μl of the inhibitors rolipram, adrenaline or cicaprost and 62.5 μl of interleukin-10 were pipetted into wells of a 48-well culture plate (Falcon Becton-Dickinson).

2.4. Endotoxin assay

To exclude contaminations with endotoxin all substances used were tested in the limulus amoebocyte lysate assay according to the manufacturer's protocol (Chromogenix, Charlston, SC, USA) and were found endotoxin negative (endotoxin content less than 6.0 pg/ml).

2.5. Cell stimulation

Lipopolysaccharide (from *Escherichia coli* 055:B5; Sigma) was freshly diluted from a frozen aliquot with the supplemented RPMI medium containing 2% endotoxin-free

human albumin (Behringwerke, Marburg, Germany). Lipopolysaccharide, 125 μl, was added into wells of the 48-well culture plate containing 125 μl of diluted compounds as described above. Subsequent addition of 250 μl peripheral blood mononuclear cell suspension with final concentrations of 10 ng/ml lipopolysaccharide and 2.5×10^6 cells/ml gave a final volume of 500 μl. A 20 h incubation period at 37°C in 5% CO_2 and fully humidified air was terminated by freezing the plates at −70°C to obtain combined lysate plus supernatant.

2.6. Measurement of TNF

TNF was determined by specific radioimmunoassay as described (Endres et al., 1988; Van der Meer et al., 1988). In order to rationalize sample processing a 96-microtube plate system with single polypropylene tubes (Sarstedt, Nümbrecht, Germany) was used. The sample (50 μl) was added to 50 μl of diluted polyclonal anti-TNF rabbit antiserum and 50 μl 1% rabbit immunoglobulin G and was incubated overnight. Bolton Hunter-labeled ^{125}I-TNF (50 μl) (NEN/Du Pont, Bad Homburg, Germany) was added on the second day. After another overnight incubation, 250 μl of second antibody (sheep anti-rabbit immunoglobulin G) in 6% polyethylene glycol was added. TNF concentrations were calculated from a standard curve of human recombinant TNF (supplied by the National Institute for Biological Standards and Control, Potters Bar, UK) ranging from 0.02 to 10 ng/ml.

2.7. Northern blot analysis

Peripheral blood mononuclear cells (10 ml) were prepared as described above and stimulated with heat-killed *S. epidermidis* (optical densitiy at 570 nm = 1×10^{-3}/cm) at a concentration of 4×10^6 cells/ml for 4 h in the presence of 100 nM rolipram, 1.0 ng/ml interleukin-10 or a combination of both in 50 ml polypropylene tubes (Greiner, Frickenhausen, Germany). *S. epidermidis* had been prepared as described (Wakabayashi et al., 1991) and was freshly diluted from a frozen aliquot. Cells were harvested by centrifugation at $2000 \times g$ for 5 min and total RNA was extracted with phenol/chloroform/isoamylalcohol, with a second precipitation step. 16 μg/lane of total RNA, as determined photometrically (Genequant, Pharmacia LKB, Freiburg, Germany) at 260 nm, was run on a 1.2% agarose glyoxyl gel and fixed onto nylon membranes as described (Chomczynski and Sacchi, 1987; Sambrook et al., 1989). Total RNA was stained with methylene blue in Na-acetate to control for equal amounts of RNA on all lanes, and photographs of the blots were prepared with instant film (Polaroid positive/negative films, No. 665). The membranes were prehybridized for 2 h at 42°C (50% formamide, 5× standard saline citrate, 50% Denhardt's solution, herring sperm DNA, 10% sodium dodecyl sulfate) and then hybridized with an [α-^{33}P]dCTP (Redivue, Amersham, Amersham, UK) random-labelled cDNA (Rediprime kit, Amersham) probe for human TNF (American Type Culture Collection, Rockville, MD, USA, No. 53165). The hybridized blots were exposed to a β-ray sensitive film (Hyperfilm-βmax, Amersham) for up to 10 days at room temperature. Films were developed by hand with the appropriate solutions (Agfa-Gevaert, Antwerp, Belgium). The films were scanned (Image Master DTS, Pharmacia LKB) and optical density was analyzed using standard software (Image Master, Pharmacia LKB).

2.8. Statistical analysis

Results are given as means ± S.E.M. The paired two-tailed Student's *t*-test was performed for comparisons of means. Differences were considered statistically significant for $P < 0.050$. All statistical analyses were performed using Stat-View 512 software (Abacus Concepts, Calabasas, CA, USA).

3. Results

3.1. Dose-dependent suppression of tumor necrosis factor-α synthesis by interleukin-10

Addition of interleukin-10 led to a dose-dependent suppression of TNF synthesis (Fig. 1). Peripheral blood mononuclear cells incubated with 10 ng/ml lipopolysaccharide alone reached a maximal TNF production of 2.9 ± 0.8 ng/ml. Significant suppression of TNF to 1.6 ± 0.3 ng/ml was observed at an interleukin-10 concentration of 0.1 ng/ml and above. Incubating cells with 100 ng/ml interleukin-10 achieved almost complete suppression of TNF synthesis to 0.1 ± 0.0 ng/ml. The mean IC_{50} was calculated at 0.4 ng/ml of interleukin-10 ($n = 6$ donors;

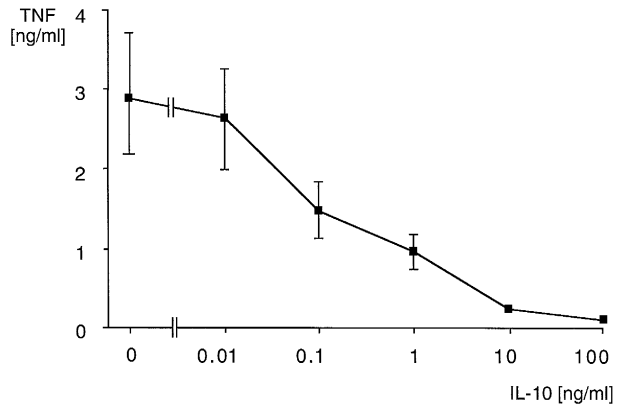

Fig. 1. Interleukin-10 dose dependently suppresses lipopolysaccharide-induced TNF production. Human peripheral blood mononuclear cells (2.5×10^6/ml) were stimulated for 20 h with 10 ng/ml lipopolysaccharide in the presence of interleukin-10 at different concentrations. Total (i.e., intracellular and extracellular) TNF synthesis was measured by radioimmunoassay. Values represent means ± S.E.M. of six experiments.

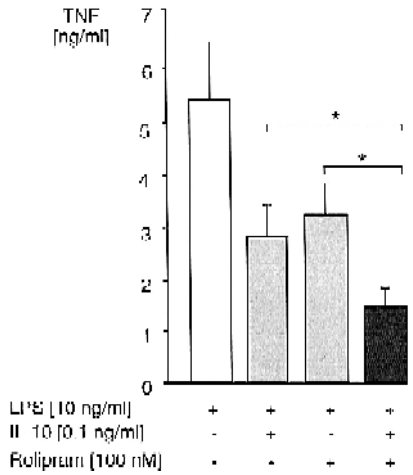

Fig. 2. Combined suppressive effect of interleukin-10 and rolipram on TNF synthesis. Human peripheral blood mononuclear cells were stimulated with 10 ng/ml lipopolysaccharide for 20 h in the presence of interleukin-10 (0.1 ng/ml) or rolipram (100 nM) alone or in combination. Columns represent means ± S.E.M. of six different donors. Asterisk (*) indicates statistically significant difference at $P = 0.040$ for comparison of the combination (interleukin-10 plus rolipram) with rolipram alone and at $P = 0.006$ for comparison of the combination with interleukin-10 alone.

means ± S.E.M.). Cells incubated without lipopolysaccharide did not produce measurable amounts of TNF.

3.2. Additive suppression of TNF production by the combination of interleukin-10 and rolipram

Rolipram, the specific type IV phosphodiesterase inhibitor, was examined for its TNF-suppressing effect tested in combination with interleukin-10. The concentration of rolipram alone which suppressed TNF synthesis to 50% (IC_{50}) was determined at 100 nM. This is similar to earlier experiments in our laboratory and to values reported in the literature (Semmler et al., 1992). Peripheral blood mononuclear cells stimulated with 10 ng/ml lipopolysaccharide alone showed a maximal TNF accumulation of 5.5 ± 1.1 ng/ml (Fig. 2). Addition of 0.1 ng/ml interleukin-10 led to a marked suppression of TNF synthesis (2.7 ± 0.6 ng/ml, $P = 0.024$). Rolipram, 100 nM, inhibited TNF to 3.1 ± 0.6 ng/ml ($P = 0.003$). The application of both TNF inhibitory agents, interleukin-10 and rolipram, achieved an additive effect in TNF suppression to 1.5 ± 0.3 ng/ml with a significance when compared to the effect of interleukin-10 ($P = 0.009$) or rolipram ($P = 0.040$) alone ($n = 6$ donors; means ± S.E.M.).

To further evaluate the additive effect on TNF suppression we compared the inhibitory effect of doubling the concentration of interleukin-10 or of rolipram with the combination of 0.1 ng/ml interleukin-10 and 100 nM rolipram. Maximal TNF synthesis was 4.0 ± 0.7 ng/ml. Interleukin-10 at 0.1 ng/ml inhibited TNF synthesis to 3.0 ± 0.3 ng/ml TNF, and slightly more, to 2.6 ± 0.1 ng/ml TNF at 0.2 ng/ml interleukin-10. Similarly, rolipram suppressed TNF synthesis to 2.4 ± 0.2 ng/ml and to 2.2 ± 0.1 ng/ml at 100 and 200 nM, respectively. Finally, the combination of 0.1 ng/ml interleukin-10 and 100 nM rolipram confirmed the additive inhibitory effect with a suppression to 2.0 ± 0.1 ng/ml TNF.

3.3. Kinetic analysis of tumor necrosis factor-α production in the presence or absence of interleukin-10 and rolipram

Human peripheral blood mononuclear cells were incubated with 10 ng/ml lipopolysaccharide in the presence or absence of 1.0 ng/ml interleukin-10 or 1 μM rolipram for different time intervals (Fig. 3). The appearance of TNF was detected 2 h after lipopolysaccharide stimulation. Maximal levels of TNF occurred between 10 and 20 h. When lipopolysaccharide stimulation was performed in the presence of 0.1 ng/ml interleukin-10 or 1 μM rolipram TNF production was inhibited at all time points tested. In the presence of interleukin-10 a plateau of TNF production was reached at about 2.5 ng/ml and in the presence of rolipram at about 1.5 ng/ml TNF. Thus, each of the compounds – and their combination – inhibited TNF synthesis beginning from the earliest time point, when TNF synthesis was detected.

3.4. Suppression of tumor necrosis factor-α synthesis by the combination of interleukin-10 and cicaprost

TNF accumulation in human peripheral blood mononuclear cells stimulated with lipopolysaccharide (10 ng/ml) was markedly inhibited by cicaprost, an activator of adenylate cyclase. This effect was enhanced in the presence of 0.1 ng/ml interleukin-10 (Fig. 4). The IC_{50} (i.e., concentration that inhibits TNF synthesis to 50% of control) of cicaprost was determined at 3 nM in accordance with

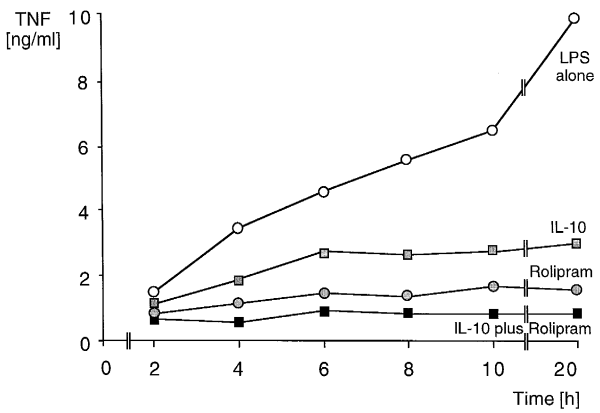

Fig. 3. Kinetic analysis of TNF production in the presence or absence of interleukin-10 and rolipram. Human peripheral blood mononuclear cells were stimulated with 10 ng/ml lipopolysaccharide in the presence of interleukin-10 (1.0 ng/ml) or rolipram (1 μM) or a combination of both. Results are shown as means of two different donors. For clarity, the range of interindividual variation is not illustrated by bars.

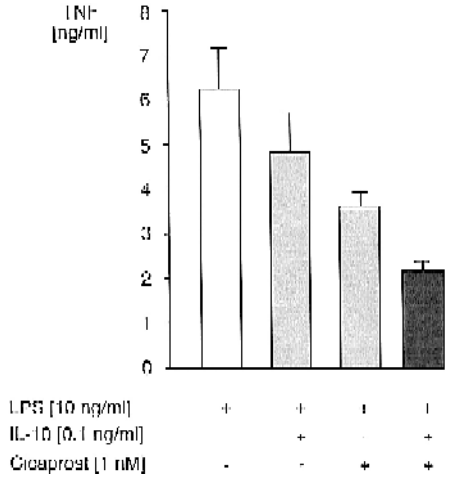

Fig. 4. Combined suppressive effect of interleukin-10 and cicaprost on TNF synthesis. Human peripheral blood mononuclear cells were stimulated with 10 ng/ml lipopolysaccharide for 20 h in the presence of interleukin-10 (0.1 ng/ml) or cicaprost (1 nM) or a combination of both. Bars represent means ± S.E.M. of six different donors. Asterisk (*) indicates statistically significant difference at $P = 0.040$ for comparison of the combination (interleukin-10 plus cicaprost) with cicaprost alone and at $P = 0.002$ for comparison of the combination (interleukin-10 plus cicaprost) with interleukin-10 alone.

values in earlier experiments (Eisenhut et al., 1993). Cells stimulated with lipopolysaccharide alone reached a maximal TNF concentration of 6.2 ± 0.9 ng/ml. Addition of 0.1 ng/ml interleukin-10 led to a suppression to 4.9 ± 0.9 ng/ml TNF ($P = 0.031$). Cicaprost, 1 nM, led to an inhibition of TNF synthesis to 3.6 ± 0.3 ng/ml ($P = 0.026$). In the presence of both 0.1 ng/ml interleukin-10 and 1 nM cicaprost an enhanced inhibitory effect was observed with a TNF concentration of 1.9 ± 0.2 ng/ml ($P = 0.049$ in comparison with interleukin-10, and $P = 0.046$ in comparison with cicaprost alone).

3.5. Suppressive effect of interleukin-10 and rolipram on TNF synthesis induced by S. epidermidis-stimulated peripheral blood mononuclear cells

In contrast to the above sections human peripheral blood mononuclear cells were stimulated with *S. epidermidis* (optical density at 570 nm = 1.0×10^{-3}/cm). We compared TNF synthesis induced by *S. epidermidis* with the results obtained above (Fig. 5A). Stimulation of peripheral blood mononuclear cells with *S. epidermidis* led to a TNF synthesis of 24.7 ± 3.8 ng/ml which was suppressed by 1 ng/ml interleukin-10 to 5.2 ± 0.8 ng/ml ($P = 0.009$) and by 100 nM of rolipram to 20 ± 3.6 ng/ml ($P = 0.003$). Simultaneous addition of 1 ng/ml interleukin-10 and 100 nM rolipram enhanced inhibition of TNF (3.8 ± 0.5 ng/ml; $P = 0.046$ in comparison to interleukin-10 and $P = 0.013$ in comparison to rolipram alone). Thus the additive suppression of TNF synthesis by rolipram and interleukin-10 could also be shown for the phagocytic stimulus *S. epidermidis*.

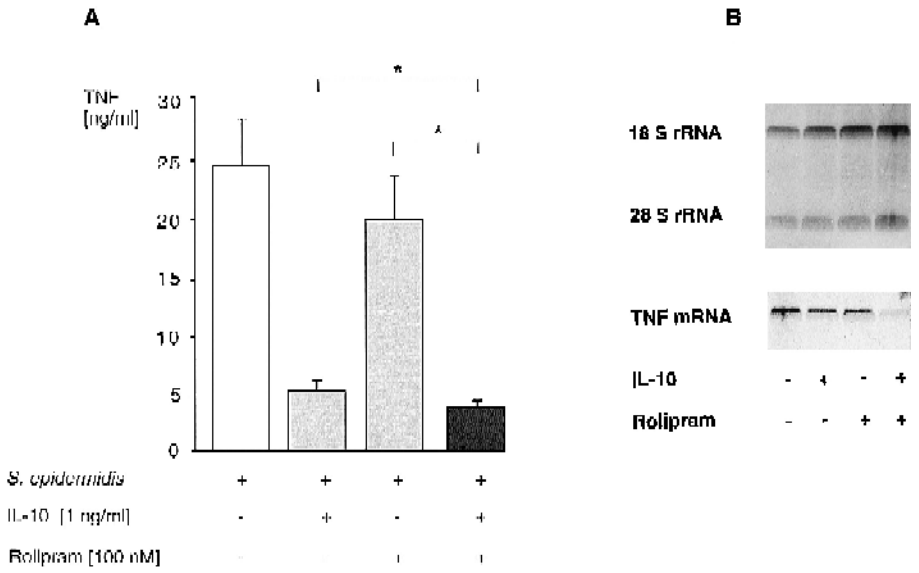

Fig. 5. (A) Suppression of *S. epidermidis*-induced TNF production by interleukin-10 and rolipram. Human peripheral blood mononuclear cells (5.7×10^6/ml) were stimulated with a suspension of heat-killed *S. epidermidis* (optical density at 570 nm = 1.0×10^{-3}/cm) in the presence of interleukin-10 (1 ng/ml), rolipram (100 nM) or a combination of both. Total, i.e. secreted and cell-associated TNF synthesis, was measured by radioimmunoassay. Values represent means ± S.E.M. of five experiments. Asterisk (*) indicates statistically significant difference at $P = 0.046$ for comparison of the combination (interleukin-10 plus rolipram) with interleukin-10 alone and at $P = 0.013$ for comparison of the combination (interleukin-10 plus rolipram) with rolipram alone. (B) Suppression of TNF mRNA synthesis by interleukin-10, rolipram or a combination of both. Human peripheral blood mononuclear cells were stimulated with a suspension of heat-killed *S. epidermidis* (optical density at 570 nm = 1.0×10^{-3}/cm) in the presence or absence of interleukin-10 (1 ng/ml) or rolipram (100 nM). Northern blot analysis was performed as described in Section 2. A photograph of the methylene blue staining for total RNA of the membrane is shown in the top half.

3.6. Additive effect of interleukin-10 and rolipram on the level of TNF mRNA

To achieve larger amounts of TNF mRNA a high number of cells (4×10^7 peripheral blood mononuclear cells) were stimulated with *S. epidermidis* (optical density at 570 nm = 0.4×10^{-3}/cm), a stronger stimulus compared to lipopolysaccharide.

Northern blot analysis was performed to elucidate whether the suppression observed on the protein level takes place on a pretranslational level. Cells were stimulated for 4 h in the presence or absence of 0.1 ng/ml interleukin-10 and 100 nM rolipram (Fig. 5B). A strong induction of TNF mRNA synthesis by *S. epidermidis* was detected and set to 1.0 standardized relative TNF mRNA. The amount of TNF mRNA was reduced by 100 nM rolipram to 0.84 standardized relative TNF mRNA; this did not correspond to an extent matching the inhibition of TNF production at the protein level. TNF mRNA was also reduced by 1 ng/ml interleukin-10 to 0.75 standardized relative TNF mRNA. The highest extent could be detected by the combination of interleukin-10 and rolipram to 0.48 standardized relative TNF mRNA.

3.7. Inhibiton of TNF synthesis by interleukin-10 is more effective after stimulation with S. epidermidis than after stimulation with lipopolysaccharide

Peripheral blood mononuclear cells were stimulated either by lipopolysaccharide or by *S. epidermidis* for 20 h.

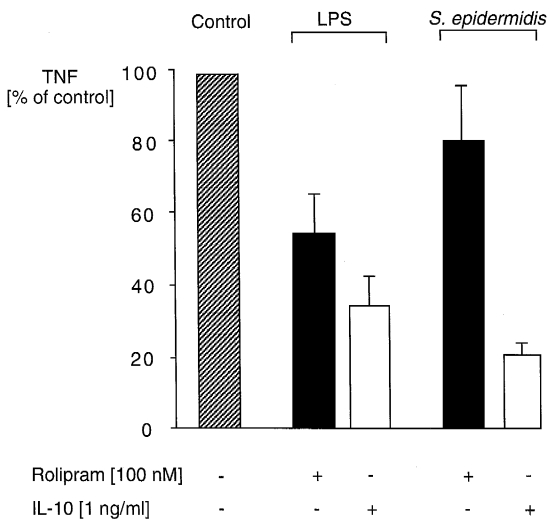

Fig. 6. Different inhibitory effects of interleukin-10 and rolipram after stimulation with lipopolysaccharide or *S. epidermidis*. Control represents the maximal TNF production of the lipopolysaccharide (10 ng/ml)- or *S. epidermidis* (optical density at 570 nm = 1×10^{-3}/cm)-stimulated human peripheral blood mononuclear cells after a 20 h incubation period. After stimulation with lipopolysaccharide, TNF synthesis was reduced by rolipram to $54 \pm 11\%$ and by interleukin-10 to $35 \pm 8\%$ of control. After stimulation with *S. epidermidis*, rolipram reduced TNF production to $81 \pm 15\%$ and interleukin-10 to $21 \pm 3\%$ of control. Columns represent means of control ± S.E.M. of six different donors.

To compare the different inhibitory effects of interleukin-10 and rolipram maximal TNF synthesis was set at 100% (Fig. 6). Rolipram suppresses TNF synthesis induced by lipopolysaccharide to a higher degree (to $54 \pm 11\%$ of control) than TNF synthesis induced by *S. epidermidis* (to $81 \pm 15\%$; Fig. 6, black columns). In contrast, interleukin-10 was more effective in suppressing TNF synthesis induced by *S. epidermidis* (to $21 \pm 3.2\%$ of control; Fig. 6, white columns). This indicates that, under identical conditions, the extent of TNF suppression by different classes of inhibitors depends on the stimulus used.

To specify the role of monocytes in TNF production we investigated the effect of different stimuli in isolated monocytes. We found the same pattern of stimulus-dependent rolipram action as in peripheral blood mononuclear cells: lipopolysaccharide-induced TNF synthesis in monocytes was reduced by rolipram (100 nM) from 8.1 ng/ml to 4.5 ng/ml; in contrast, *S. epidermidis*-induced TNF synthesis was not affected by rolipram (data not shown as figure).

4. Discussion

In the present study we could demonstrate an additive effect of interleukin-10 and cAMP-elevating agents in the suppression of lipopolysaccharide-stimulated TNF synthesis. Dose-response analyses were performed for interleukin-10 and the cAMP-elevating substances alone and the IC_{50} for each agent was determined. Among the cAMP-elevating agents studied in combination with interleukin-10, rolipram achieved the most extensive suppression of TNF production. The additive effect of this combination could be confirmed after stimulation with *S. epidermidis*, both on the level of TNF protein and on the level of TNF mRNA. The inhibitory potency of rolipram was less effective in *S. epidermidis*-stimulated compared to lipopolysaccharide-induced TNF synthesis. Quantification of TNF mRNA revealed a suppressive activity of interleukin-10 and rolipram on a pretranslational level.

The mechanism for suppression of TNF synthesis by interleukin-10 has not been completely elucidated. A pathway mediated only by elevated cAMP concentration as described by Platzer et al. (1995) does not seem probable since the synthesis of other pro-inflammatory cytokines such as interleukin-1, which is not inhibited by cAMP elevation, is suppressed by interleukin-10 as well. We therefore assumed partially different pathways of TNF suppression by interleukin-10 and cAMP-elevating agents and postulated enhanced inhibition of TNF synthesis in the presence of both agents.

To investigate this hypothesis we tested two cAMP-elevating agents in combination with interleukin-10. Rolipram is a specific inhibitor of phosphodiesterase inhibitor type IV. Phosphodiesterase inhibitor type IV, one

of the seven different types of phosphodiesterase inhibitors known in human tissues, is predominant in monocytes, the main source of TNF (Beavo, 1995). Because of its high specificity and the resulting 500-fold stronger inhibition of TNF synthesis compared to non-specific inhibitors of phosphodiesterase inhibitor we chose rolipram for the present study (Semmler et al., 1992). The suppressive effect of phosphodiesterase inhibitors on TNF synthesis is assumed to be mediated by cAMP (Eisenhut et al., 1993; Sinha et al., 1995). As illustrated in Fig. 2 the combination of rolipram and interleukin-10 achieved an additive effect in suppression of TNF synthesis. The combination of interleukin-10 and rolipram was the most effective on the protein level. We therefore studied its effect on TNF mRNA in Northern blot analysis. Interleukin-10 showed a pronounced inhibitory effect on the level of TNF mRNA. In contrast, the pronounced TNF suppressive effect of rolipram on the protein level was only accompanied by a small reduction of the TNF mRNA amount. The marked discrepancy between the efficacy of rolipram in LPS- (marked TNF suppression) and *S. epidermidis*- (little TNF suppression) stimulated peripheral blood mononuclear cells is not yet fully understood. One might speculate that T cells contribute to *S. epidermidis*-induced TNF synthesis in peripheral blood mononuclear cells. However, in additional experiments we found that adherent-isolated monocytes stimulated with lipopolysaccharide or *S. epidermidis* produce two- to three-fold higher TNF concentrations than the same number of monocytes contained in a peripheral blood mononuclear cell preparation. This suggests that T cells do not contribute appreciable amounts to the TNF formation in lipopolysaccharide- or *S. epidermidis*-stimulated peripheral blood mononuclear cells. Additionally, in isolated monocytes we found the same pattern of stimulus-dependent rolipram action as in peripheral blood mononuclear cells. It therefore appears probable that TNF stimulation with *S. epidermidis* engages a not cAMP-suppressable signal transduction pathway. The exact role of rolipram in TNF suppression in peripheral blood mononuclear cells warrants further investigations.

Rolipram and interleukin-10 not only exert additive effects on TNF suppression, endogenous interleukin-10 may also be induced by rolipram itself. Kambayashi et al. (1995) recently reported that the addition of rolipram to lipopolysaccharide-stimulated murine macrophages results in a significant augmentation of interleukin-10 release while TNF synthesis was suppressed. Furthermore, addition of neutralizing monoclonal antibody anti-interleukin-10 significantly but incompletely reversed the inhibitory effect of rolipram on TNF synthesis (Kambayashi et al., 1995). We have confirmed the induction of interleukin-10 by rolipram in human peripheral blood mononuclear cells (Eigler et al., manuscript in preparation). Platzer et al. (1995) described the identification of a cAMP-responsive element in the interleukin-10 gene sequence. Furthermore, they demonstrated an increase in interleukin-10 synthesis induced by several cAMP-elevating agents. This mechanism, induction of endogenous interleukin-10 by rolipram, could contribute to the suppression of TNF synthesis by rolipram. In addition to rolipram, we studied cicaprost, a stable prostacyclin analogue which increases cAMP formation by activation of the adenylate cyclase. The combination of interleukin-10 and cicaprost inhibited TNF production more than each of the two substances alone. On a molar basis, cicaprost is 5-fold more potent in suppressing TNF synthesis compared to iloprost (Sinha et al., 1995). Iloprost – a prostacyclin analogue which is already in clinical application – has the advantage of bioavailability after oral application and a higher potency.

Finally, we observed a different inhibitory capacity of interleukin-10 and rolipram on TNF synthesis, depending on the stimulus. In vivo, a protective effect of interleukin-10 has been shown both for lipopolysaccharide- and for *S. epidermidis*-induced sepsis (Howard et al., 1993; Smith et al., 1994). We found a significantly higher inhibitory effect of interleukin-10 in peripheral blood mononuclear cells stimulated by *S. epidermidis* than in cells stimulated with lipopolysaccharide. The two stimuli activate monocytes by different pathways: soluble lipopolysaccharide by signal transduction via the membrane receptor CD14, the particulate stimulus heat-killed *S. epidermidis* by phagocytosis. This could form the basis of further studies to investigate the differences in regulation of TNF synthesis by rolipram and interleukin-10.

The clinical relevance of TNF inhibition in human inflammatory diseases has recently been demonstrated. Elliott et al. (1994) showed a marked improvement of subjective and objective parameters in patients with rheumatoid arthritis treated with a single dose of monoclonal anti-TNF antibody. However, the repeated application of anti-TNF antibodies resulted in increased antibody-related side-effects. This underlines the need for alternative strategies to attenuate TNF activity or production. Rolipram has been demonstrated to suppress TNF synthesis in vivo accompanied by a protective effect in animal models (Turner et al., 1993). Rolipram reduced acute and chronic inflammatory responses to antigen and prevented the development of airway hyperresponsiveness. Sommer et al. (1995) showed treatment with rolipram to be effective in an animal model of multiple sclerosis associated with decreased production of TNF, lymphotoxin-α and interferon-γ. Rolipram has been studied in clinical trials when it was originally developed as an antidepressant (Wachtel, 1983). The pharmacokinetics of interleukin-10 are known as well: Chernoff et al. (1995) described no side-effects after application of interleukin-10 to healthy volunteers. Most in vitro studies investigating the pharmacological suppression of TNF synthesis used concentrations in the micromolar or microgram/ml ranges. Applying a combination of a cAMP-increasing substance and interleukin-10, we identified conditions under which a marked suppression of TNF synthesis can be obtained at

nanomolar or nanogram/ml drug conditions, i.e., 100 nM rolipram, 1 nM cicaprost and 0.1 ng/ml interleukin-10.

The results of the present study *contribute* to the prerequisites necessary to study the combination of interleukin-10 and cAMP-elevating agents in TNF-mediated diseases.

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