ELSEVIER

European Journal of Pharmacology 299 (1996) 229–233

ejp

Short communication

Cicaprost and the type IV phosphodiesterase inhibitor, rolipram, synergize in suppression of tumor necrosis factor-α synthesis

Tim F. Greten, Bhanu Sinha, Christiane Haslberger, Andreas Eigler, Stefan Endres *

Medizinische Klinik, Klinikum Innenstadt der Ludwig-Maximilians-Universität, München, Germany

Received 20 November 1995; revised 18 December 1995; accepted 19 December 1995

Abstract

Suppression of tumor necrosis factor-α (TNF) synthesis is one major target in pharmacological immunomodulation. We now showed the synergistic suppressive effect of the specific type IV phosphodiesterase inhibitor, rolipram, and of the stable prostacyclin analogue, cicaprost, on TNF synthesis. This effect was seen with lipopolysaccharide and *Staphylococcus epidermidis* as stimuli in human peripheral blood mononuclear cells and in whole blood. Lipopolysaccharide-induced TNF synthesis by mononuclear cells decreased from 3.4 ng/ml to 1.5 ng/ml in the presence of 100 nM rolipram and to 0.7 ng/ml in the presence of 10 nM cicaprost. The combination of both agents suppressed TNF synthesis more than 10-fold, to 0.3 ng/ml. Synergistic suppression was also demonstrated for TNF mRNA.

Keywords: TNF (tumor necrosis factor-α); Rolipram; Cicaprost; Prostaglandin E_2

1. Introduction

Tumor necrosis factor-α (TNF) is a cytokine with pleiotropic functions in the immune response. As a proinflammatory mediator, it plays a central role in the pathogenesis of severe disease states such as septic shock (Tracey and Cerami, 1994). In several disease models suppression of excessive TNF synthesis has proved to be a remarkable benefit in vivo (Tracey and Cerami, 1994). Recently, the first successful therapy of a human autoimmune disease with anti-TNF antibody has been reported (Elliott et al., 1994). A limitation of this study was the possibly antibody-related side-effects, which increased with repeated application of the antibody. The need for alternative pharmacological strategies to suppress TNF formation or TNF action is thus emphasized. We and others have previously studied suppression of TNF synthesis by pharmacological elevation of intracellular cAMP (Endres et al., 1991; Prabhakar et al., 1994; Sinha et al., 1995).

Phosphodiesterase inhibitors elevate cAMP levels by impeding its degradation. Six different types of phosphodiesterases are known in human tissue. Type IV phosphodiesterase is predominant in monocytes, the main source of TNF (Thompson, 1991). Therefore, we have investigated the effect of the specific type IV phosphodiesterase inhibitor, rolipram, on TNF suppression. In a previous study we found a 500-fold stronger suppression of TNF synthesis by rolipram, on a molar basis, as compared to that by the non-specific phosphodiesterase inhibitor, pentoxifylline (Semmler et al., 1993).

Prostanoids such as prostaglandin E_2 and prostacyclin, act via stimulation of adenylyl cyclase, and thereby enhance cAMP formation. Like others we were able to demonstrate a suppressing effect on lipopolysaccharide-induced TNF synthesis by two stable prostacyclin analogues, cicaprost and iloprost. This was accompanied by accumulation of cAMP in human peripheral blood mononuclear cells (Eisenhut et al., 1993). Similar results have been observed with cicaprost in the human monocytic cell line THP-1 (Crutchley et al., 1994).

In the present study we investigated (i) whether there is a synergistic suppressive effect on lipopolysaccharide-induced TNF synthesis by the simultaneous action of the specific type IV phosphodiesterase inhibitor, rolipram, and the stable prostacyclin analogue, cicaprost. (ii) Secondly, we examined whether prostaglandin E_2 shows effects similar to those of cicaprost on TNF suppression alone, and in combination with rolipram. (iii) Thirdly, we investigated the effect of rolipram and cicaprost on heat-killed *Staphylococcus epidermidis* (*S. epidermidis*)-induced TNF syn-

* Corresponding author. Medizinische Klinik, Klinikum Innenstadt, Ziemssenstraße 1, 80336 München, Germany. Tel.: 89-5160-2291; fax: 89-5160-4576; e-mail: u7g11an@sunmail.lrz-muenchen.de.

SSDI 0014-2999(95)00867-5

thesis in human peripheral mononuclear cells and in whole blood. (iv) Finally, we quantified *S. epidermidis*-induced TNF mRNA in the presence or absence of rolipram and cicaprost.

2. Materials and methods

2.1. Stimulation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells were prepared from blood samples from healthy volunteers as described (Sinha et al., 1995). Rolipram, cicaprost (kindly provived by Dr. Wachtel and Dr. Kapp, Schering, Berlin, Germany) or prostaglandin E_2 (Sigma, Munich, Germany) were added. The cells were stimulated with 10 ng/ml lipopolysaccharide (Sigma, Munich, Germany) or heat-killed *S. epidermidis* (final concentration equal to optical density $[O.D.]_{570\,nm}/cm = 0.4 \times 10^{-3}$) for 20 h. *S. epidermidis* had been prepared as described (Wakabayashi et al., 1991). Total TNF, i.e. cell-associated and secreted TNF, was determined by specific radioimmunoassay as described (Sinha et al., 1995) using a polyclonal anti-TNF antiserum.

2.2. Whole blood stimulation

Heparinized (final concentration of 25 I.E./ml) blood was drawn from healthy volunteers and was diluted with an equal volume of RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM Hepes (all from Sigma, Munich, Germany). A final volume of 0.6 ml diluted blood was stimulated with heat-killed *S. epidermidis* (final concentration of $[O.D.]_{570\,nm}/cm = 0.4 \times 10^{-3}$) in the presence or

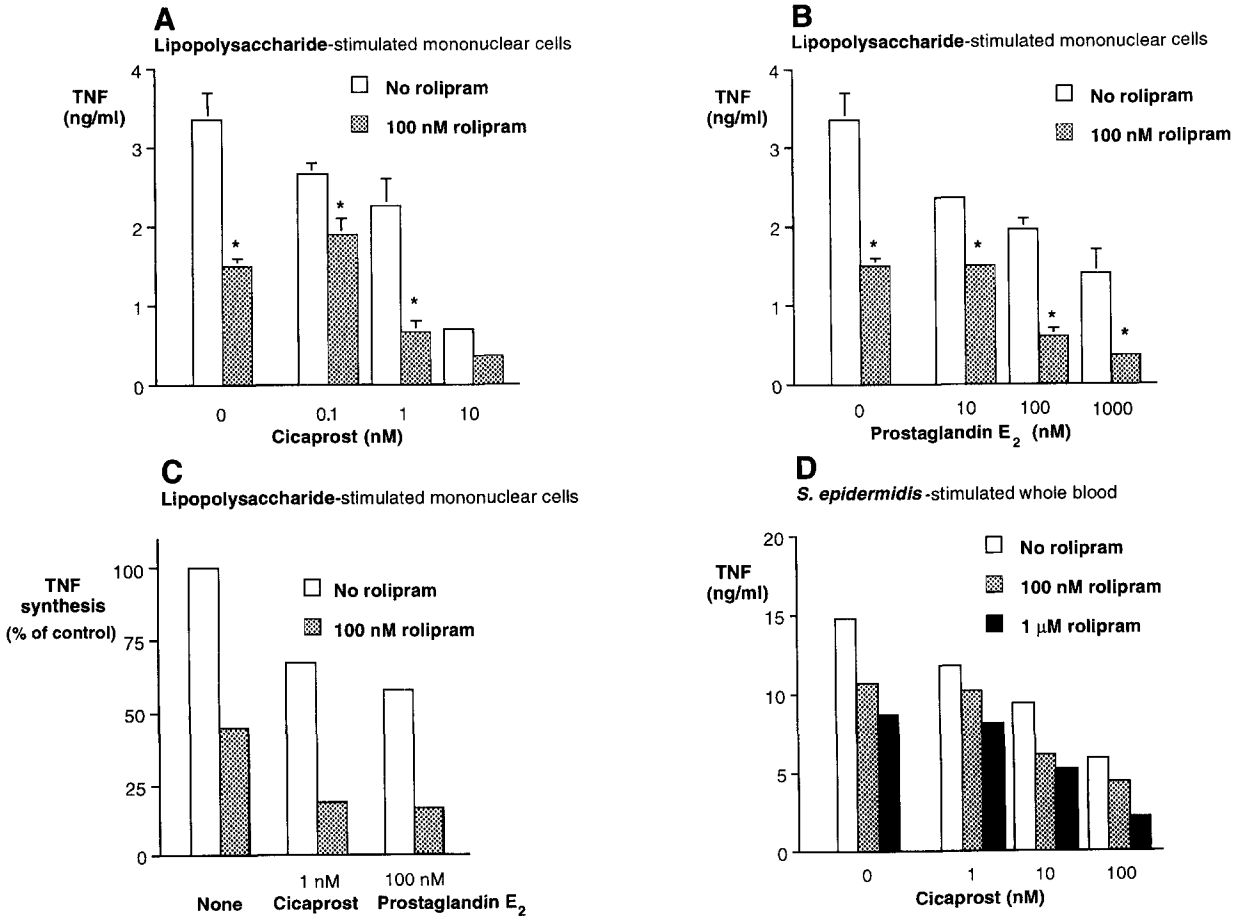

Fig. 1. Synergistic inhibition of TNF synthesis by rolipram, cicaprost and prostaglandin E_2. (A) Rolipram plus cicaprost or (B) rolipram plus prostaglandin E_2 dose dependently suppressed lipopolysaccharide-induced TNF synthesis in mononuclear cells incubated for 20 h. 100 nM rolipram in combination with 10 nM cicaprost and 100 nM rolipram in combination with 1 μM prostaglandin E_2 decreased TNF synthesis to 0.4 ± 0.1 and 0.4 ± 0.1 ng/ml, respectively (means ± S.E.M. of two donors, each assayed in duplicate, asterisks (*) indicate significant difference from TNF concentrations induced without rolipram [white bars]). (C) The synergistic suppressive effect is illustrated by the plot of the percentage suppression achieved with each of the substances or their combinations. Rolipram alone, at 100 nM, suppressed lipopolysaccharide-induced TNF formation to 45%, while cicaprost (1 nM) and prostaglandin E_2 (100 nM) suppressed TNF synthesis to 67 and 58% respectively. When either of the two drugs was combined with rolipram a suppression to 19% and 18% could be achieved. (D) Supernatants from *S. epidermidis*-stimulated whole blood cells yielded similar results after a 7-h incubation period (means ± S.E.M. of two different donors, each assayed in duplicate).

absence of different concentrations of cicaprost and rolipram for 9 h. Supernatants were stored at −20°C and TNF was determined by specific radioimmunoassay.

2.3. Northern blot analysis

Human peripheral blood mononuclear cells (10 ml) were prepared as described above and stimulated with *S. epidermidis* ($[O.D.]_{570\,nm}/cm = 1 \times 10^{-3}$) at a concentration of 4×10^6 cells/ml for 4 h in the presence of 0.5 μM rolipram, 5 nM cicaprost or a combination of both in 50 ml polypropylene tubes (Greiner, Germany). Cells were harvested by centrifugation at $200 \times g$ for 5 min and total RNA was extracted. Aliquots, 12 μg, of total RNA were run on a 1.2% agarose glyoxal gel and fixed onto nylon membranes as described (Sambrook et al., 1989). The membranes were then hybridized with a ^{33}P-labeled, random primed cDNA probe for TNF (#53165, American Type Culture Collection, Rockville, USA). The hybridized blots were exposed to a β-ray sensitive film (Hyperfilm-βmax, Amersham Life Science, Braunschweig, Germany) at room temperature for 2 days. The film was scanned (ImageMaster DTS, Pharmacia LKB) and optical density was analyzed using standard software (Image Master, Pharmacia LKB).

2.4. Statistical analysis

The results, expressed as means ± S.E.M., were analyzed by paired, two-tailed Student *t*-test (Statview II, Abacus Concepts, Berkeley, USA). Differences were considered statistically significant for P values < 0.05 (indicated with an asterisk in the figures). IC_{50} values were determined graphically.

3. Results

3.1. Suppression of TNF synthesis in lipopolysaccharide-stimulated human peripheral blood mononuclear cells

Lipopolysaccharide, 10 ng/ml, stimulated peripheral blood mononuclear cells to produce 3.4 ± 0.34 ng/ml TNF after 20 h. TNF synthesis was suppressed by rolipram, and dose dependently by cicaprost and prostaglandin E_2 with IC_{50} values of 90 nM for rolipram, 3 nM for cicaprost and 500 nM for prostaglandin E_2 (Fig. 1A and 1B). Next, combinations of 100 nM rolipram, which alone suppressed TNF synthesis to 1.5 ± 0.1 ng/ml, a suppression to 45%, with either cicaprost or prostaglandin E_2 were tested for their suppressive effect on TNF formation. Alone, 1 nM cicaprost reduced TNF synthesis to 2.3 ± 0.4 ng/ml, equal to a 67% suppression. A more than additive effect was seen with 1 nM cicaprost plus 100 nM rolipram, which led to a TNF concentration of 0.7 ± 0.2 ng/ml, a suppression to 19% of control (Fig. 1C). Synergistic effects were also achieved with rolipram plus prostaglandin E_2. Here 2.0 ± 0.2 ng/ml TNF was formed with 100 nM prostaglandin E_2, a suppression to 58%, and 0.6 ± 0.1 ng/ml with prostaglandin E_2, plus 100 nM rolipram, suppression to 18% (Fig. 1B and 1C).

3.2. Suppression of TNF synthesis and of TNF mRNA in S. epidermidis-stimulated human peripheral blood mononuclear cells

Next, we studied whether this synergistic suppressive effect could be confirmed using another stimulus for TNF

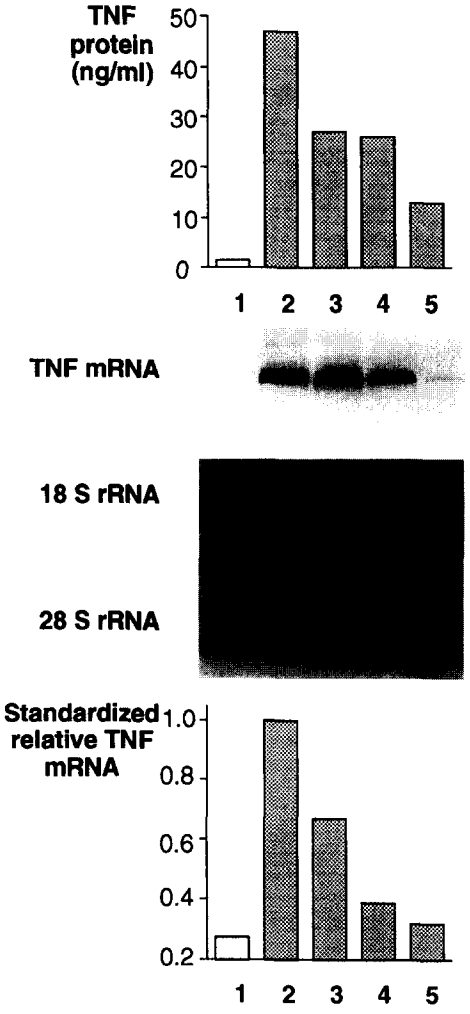

Fig. 2. Suppression of TNF mRNA in mononuclear cells by rolipram, cicaprost or both. Four hours after stimulation of mononuclear cells with *S. epidermidis*, RNA was harvested and Northern blot analysis was performed using a human ^{33}P-dCTP-labeled TNF-cDNA probe. The conditions of the five lanes were (1) non-stimulated cells, (2) cells stimulated with *S. epidermidis*, (3) stimulated cells plus 0.5 μM rolipram, (4) stimulated cells plus 5 nM cicaprost, and (5) stimulated cells plus 0.5 μM rolipram and 5 nM cicaprost. (A, top panel) shows TNF protein synthesized after 20 h under identical conditions. (B, center panel) Northern blot analysis of TNF mRNA with a strong signal after stimulation (2), a weaker signal with either rolipram or cicaprost (3, 4) and the lowest signal with rolipram plus cicaprost (5). (C, bottom panel) TNF mRNA optical density of (B) standardized to 18 S rRNA.

synthesis in mononuclear cells. Heat-killed *S. epidermidis* stimulates mononuclear cells by phagocytosis, a mechanism principally different from the activation by lipopolysaccharide (Wakabayashi et al., 1991). This stimulus induced one order of magnitude higher concentrations of TNF as compared to lipopolysaccharide, i.e. 42 ng/ml TNF after a 20-h incubation period of mononuclear cells with *S. epidermidis* ($[O.D.]_{570\,nm}/cm = 0.4 \times 10^{-3}$). TNF synthesis was reduced to 27 ng/ml with 0.5 μM rolipram and to 26 ng/ml with 5 nM cicaprost. A combination of both suppressed TNF synthesis to 13 ng/ml (Fig. 2 top panel). A Northern blot analysis was performed to elucidate whether this suppression takes place on a pretranslational level. Mononuclear cells, 4×10^7, were stimulated with *S. epidermidis* ($[O.D.]_{570\,nm}/cm = 1 \times 10^{-3}$) for 4 h in the presence of different TNF-suppressing agents (Fig. 2). We found a strong induction of TNF mRNA synthesis by *S. epidermidis*. The amount of TNF mRNA was reduced by 0.5 μM rolipram, by 5 nM cicaprost and to a higher extent by a combination of both.

3.3. Suppression of TNF synthesis in supernatants of S. epidermidis-stimulated whole blood

Finally, we chose whole blood stimulation to examine the effects of cicaprost and rolipram on TNF synthesis in a model more comparable to in vivo conditions. In whole blood we observed effects similar to those in peripheral mononuclear cells. In supernatants of whole blood 14.8 ± 0.5 ng/ml TNF was measured after a 7-h incubation period with *S. epidermidis* ($[O.D.]_{570\,nm}/cm = 0.4 \times 10^{-3}$). TNF synthesis was dose dependently suppressed to 8.7 ± 0.2 and 9.4 ± 0.1 ng/ml TNF by 1 μM rolipram and 10 nM cicaprost, respectively. A combination of both enhanced TNF suppression to 5.2 ± 0.04 ng/ml (Fig. 1D).

4. Discussion

We have demonstrated the potency of rolipram and cicaprost to suppress lipopolysaccharide- and *S. epidermidis*-induced TNF synthesis in human peripheral blood mononuclear cells and in whole blood. The simultaneous action of both substances suppressed TNF synthesis to a higher degree than could be expected from mere addition of the effects of the single substances. The combination of rolipram and cicaprost was not synergistic at all concentrations tested. Synergy was most prominent with drug concentrations close to the IC_{50} of each substance. The suppression of TNF protein synthesis could also be demonstrated for TNF mRNA. Most in vitro studies investigating the pharmacological suppression of TNF synthesis use concentrations in the micromolar range. Applying a combination of cAMP-increasing substances, we identified conditions under which 10-fold suppression of TNF synthesis was obtained at nanomolar drug concentrations, i.e. 100 nM rolipram and 10 nM cicaprost.

Monocytes are one major source of TNF, and type IV phosphodiesterase is predominant in these cells (Thompson, 1991). Therefore it might be advantageous to use specific type IV phosphodiesterase inhibitors in studies investigating the pharmacological inhibition of TNF synthesis (Semmler et al., 1993). We (Sinha et al., 1995) and others (Crutchley et al., 1994) have previously studied the combination of cicaprost with non-specific phosphodiesterase inhibitors for their TNF-suppressing activity. We now extend these studies using the specific type IV phosphodiesterase inhibitor, rolipram, which is 500-fold more potent with regard to TNF suppression, than the non-specific phosphodiesterase inhibitors.

Wakabayashi et al. (1991) compared the effect of Gram-negative bacteria and of Gram-positive *S. epidermidis* on TNF synthesis in vivo and in mononuclear cells. They found measurable TNF induction with either stimulus. These results explain why septic shock, even caused by Gram-positive organisms, that do not contain lipopolysaccharide, is accompanied by elevated TNF levels. Thus, when studying the TNF-suppressive capacity of substances, the experimental model should not be limited to the use of lipopolysaccharide as a stimulus. In the present study we obtained comparable results with both stimuli, heat-killed *S. epidermidis* and lipopolysaccharide.

Furthermore, we extended our studies of rolipram and cicaprost to stimulation in whole blood. Whole blood represents a more physiologic environment for examining cytokine production, since the cellular interactions are better preserved as compared to those of isolated mononuclear cells. This model has been used with either lipopolysaccharide or *S. epidermidis* as a stimulus to approximate in vivo conditions (Nerad et al., 1992).

The suppressing effect of both phosphodiesterase inhibitors and prostacyclin analogues is assumed to be mediated by cAMP (Eisenhut et al., 1993; Sinha et al., 1995). In previous studies we had found a small but significant cAMP accumulation in murine macrophages (Greten et al., 1995) with rolipram, whereas the TNF-suppressing effect was strong. This might be explained by compartmentalization of intracellular phosphodiesterases (Thompson, 1991). In accordance with this we found only a small cAMP accumulation with 1 nM cicaprost and 90 nM rolipram in peripheral blood mononuclear cells compared to a strong elevation of cAMP with the high dose of 1 μM cicaprost alone (Sinha et al., 1995).

The rationale for combining cicaprost with rolipram stems from several lines of evidence. The prostacyclin analogue, cicaprost, increases cAMP formation via stimulation of adenylyl cyclase. In contrast phosphodiesterase inhibitors lead to an increase of cAMP accumulation by impeding degradation. Thus both substances act on cAMP metabolism, but at sequential, mutually enhancing sites. Indeed, we observed a synergistic effect with rolipram plus

cicaprost not only on the TNF protein but also on the TNF mRNA. Finally, this effect was seen using two different stimuli in mononuclear cells and in a second cell system, human whole blood.

Clinical studies have been performed with cicaprost and rolipram. Thus the pharmacokinetics of both substances are known (Hildebrand et al., 1990; Krause et al., 1990). Rolipram was developed originally as an antidepressant. Its efficacy and toxicity have been studied in large clinical trials. While rolipram has never been marketed for the original indication, there is now resurging interest in this compound because of its specific phosphodiesterase type IV-inhibiting activity. Combining a prostacyclin analogue and a phosphodiesterase inhibitor has several advantages. Only low concentrations of either substance are required. Thus dose-related side-effects of these drugs may be reduced, since lower plasma levels of either substance will be needed to obtain an optimal effect. Finally, both drugs can be given orally.

Our results would support in vivo studies in animal models using a combination of the synergistically acting rolipram and cicaprost. If the synergistic suppression of TNF can be confirmed in vivo and can be linked to protection, e.g. from experimental septic shock, this could form the basis for clinical studies.

Acknowledgements

The authors thank Dr. Jochen Moeller for most valuable discussions and providing *S. epidermidis*. This work was supported by grant En 169-2/3 from the Deutsche Forschungsgemeinschaft and by the Ernst und Berta Grimmke-Stiftung.

References

Crutchley, D.J., L.B. Conanan and B.G. Que, 1994, Effects of prostacyclin analogs on the synthesis of tissue factor, tumor necrosis factor-α and interleukin-1β in human monocytic THP-1 cells, J. Pharmacol. Exp. Ther. 271, 446.

Eisenhut, T., B. Sinha, E. Gröttrup-Wolfers, J. Semmler, W. Siess and S. Endres, 1993, Prostacyclin analogs suppress the synthesis of tumor necrosis factor-α in LPS-stimulated human peripheral blood mononuclear cells, Immunopharmacology 26, 259.

Elliott, M.J., R.N. Maini, M. Feldmann, J.R. Kalden, C. Antoni, J.S. Smolen, B. Leeb, F.C. Breedveld, J.D. Macfarlane, H. Bijl and J.N. Woody, 1994, Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor α (cA2) versus placebo in rheumatoid arthritis, Lancet 344, 1105.

Endres, S., H.J. Fülle, B. Sinha, D. Stoll, C.A. Dinarello, R. Gerzer and P.C. Weber, 1991, Cyclic nucleotides differentially regulate the synthesis of tumour necrosis factor-α and interleukin-1β by human mononuclear cells, Immunology 72, 56.

Greten, T.F., A. Eigler, B. Sinha, J. Moeller and S. Endres, 1995, The specific type IV phosphodiesterase inhibitor rolipram differentially regulates the proinflammatory mediators TNF-α and nitric oxide, Int. J. Immunopharmacol. 17, 605.

Hildebrand, M., T. Staks and B. Nieuweboer, 1990, Pharmakokinetics and pharmacodynamics of cicaprost in healthy volunteers after oral administration of 5 to 20 μg, Eur. J. Clin. Pharmacol. 39, 149.

Krause, W., G. Kühne and N. Sauerbrey, 1990, Pharmacokinetics of (+)-rolipram and (−)-rolipram in healthy volunteers, Eur. J. Clin. Pharmacol. 38, 71.

Nerad, L.D., J.K. Griffiths, J.W.M. Van der Meer, S. Endres, D.D. Poutsiaka, G.T. Keusch, M. Bennish, M.A. Salam, C.A. Dinarello and J.G. Cannon, 1992, Interleukin-1β (IL-1β), IL-1 receptor antagonist, and TNF-α production in whole blood, J. Leukocyte Biol. 52, 200.

Prabhakar, U., D. Lipshutz, J. O'Leary Bartus, M.J. Slivjak, E.F. Smith, J.C. Lee and K. Esser, 1994, Characterization of cAMP-dependent inhibition of LPS-induced TNF-α production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor, Int. J. Immunopharmacol. 16, 805.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989, Molecular Cloning (Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

Semmler, J., H. Wachtel and S. Endres, 1993, The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-α production by human mononuclear cells, Int. J. Immunopharmacol. 15, 409.

Sinha, B., J. Semmler, T. Eisenhut, A. Eigler and S. Endres, 1995, Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by combination of phosphodiesterase inhibitors and prostanoids, Eur. J. Immunol. 25, 147.

Thompson, W.J., 1991, Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function, Pharmacol. Ther. 51, 13.

Tracey, K.J. and A. Cerami, 1994, Tumor necrosis factor: a pleiotropic cytokine and therapeutic target, Annu. Rev. Med. 45, 491.

Wakabayashi, G., J.A. Gelfand, W.K. Jung, R.J. Connolly, J.F. Burke and C.A. Dinarello, 1991, *Staphylococcus epidermidis* induces complement activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. Comparison to *Escherichia coli*, J. Clin. Invest. 87, 1925.