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Effect of rolipram in a murine model of acute inflammation: comparison with the corticoid dexamethasone

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Abstract

Treatment of mice with rolipram, a phosphodiesterase type 4 inhibitor, selectively modified the acute inflammatory reaction elicited by zymosan administration in 6-day-old mouse air-pouches. Rolipram (1–10 mg kg^{-1}, i.p.) prevented the rise of endogenous tumor necrosis factor-α (TNF-α) in the lavage fluids ($\sim$ 60% inhibition) induced by zymosan, with no effect upon interleukin-1α levels. This action was not accompanied by changes in neutrophil accumulation, but the amount of elastase released in the lavage fluids was significantly reduced ($\sim$ 50%). Dexamethasone (1.5 mg kg^{-1}, i.v.), used for comparative purposes, significantly reduced the release of TNF-α (> 50%), interleukin-1α (> 70%) and cellular infiltration ($\sim$ 50%), but had only a marginal effect on the release of elastase activity. In conclusion, in this murine model of acute inflammation induced by zymosan, rolipram inhibited the endogenous TNF-α production at a local site of inflammation, such as the subcutaneous air-pouch, and prevented the full activation of migrated cells.

Keywords: Cytokine; TNF (tumor necrosis factor); Interleukin-1; Elastase; Neutrophil

1. Introduction

Endogenous tumor necrosis factor-α (TNF-α) and interleukin-1α mediate the recruitment of polymorphonuclear leukocytes to tissue sites during the inflammatory host response (Goto et al., 1984; Cybulsky et al., 1988; Ulich et al., 1991; Perretti et al., 1992). Since recognition of this important role in the development of acute and chronic inflammatory diseases (Arend and Dayer, 1990; Arai et al., 1990), attempts have been made either to neutralize the activity of cytokines, both circulating (Beutler et al., 1985; Ohlsson et al., 1990) and locally formed (Meyers et al., 1993), or to suppress their biosynthesis/production (Schade, 1990; Mohler et al., 1994; Miller et al., 1993).

Phosphodiesterase inhibitors elevate intracellular cyclic AMP levels and, amongst other actions, inhibit the biosynthesis of certain cytokines (Endres et al., 1991; Katakami et al., 1988; Semmler et al., 1993a). Rolipram, which has a phenylpyrrolidinone structure, is a specific inhibitor of the cyclic AMP-selective family of phosphodiesterase (Beavo and Reifsnyder, 1990; Nicholson et al., 1991), now referred to as phosphodiesterase 4 family (Beavo et al., 1994), which is the predominant phosphodiesterase isoenzyme in monocytes (Torphy and Undem, 1991). Interestingly, addition of rolipram to human monocytes selectively inhibits the release of TNF-α stimulated by endotoxin (Molnar-Kimber et al., 1992; Semmler et al., 1993b). In vivo, treatment of mice with rolipram protects against endotoxin-induced liver injury, which is mediated by endogenous TNF-α (Fischer et al., 1993).

A marked release of pro-inflammatory cytokines occurs when zymosan, a non-specific inflammogen which acts as a phagocytic stimulus for monomyelocytic cells, is added to cells in vitro (Rankin et al., 1990; Roberge et al., 1991) or injected into specific tissue sites in vivo (Collins et al., 1990; Perretti et al., 1992;

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Erdö et al., 1994). To evaluate the potential effect of rolipram on TNF-α production at the local site of acute inflammation, we have used the mouse air-pouch inflamed with zymosan. In particular, we have assessed the effect of systemic administration of rolipram on the local production of TNF-α in vivo, and on other parameters of the acute inflammatory response. For comparative purposes, we have also examined the effect of the potent anti-inflammatory corticosteroid, dexamethasone.

2. Materials and methods

2.1. Mouse air-pouch model

Male Swiss albino mice (24–26 g; Tuck, Essex, UK) were used for all experiments. Dorsal s.c. air-pouches were prepared by injection of 2.5 ml of air on day 0 and day 3 as recently described (Perretti and Flower, 1993). Six days after the initial injection of air, mice received zymosan (5 mg in 0.5 ml sterile NaCl 0.9%) or vehicle alone directly into the pouch. This dose was chosen on the basis of preliminary experiments and confirmed the findings of a previous study (Erdö et al., 1994). At various times (2, 4 and 16 h) after zymosan injection, animals were killed by exposure to CO_2 and the pouches washed thoroughly with 1–1.5 ml of phosphate-buffered solution containg 50 U ml^{-1} heparin. Lavage fluids were centrifuged at $220 \times g$ for 12 min at 4° C. Aliquots of the supernatants were stored at −20° C for no longer than 1 week prior to determination of cytokine and leukocyte elastase activity (see below). Cell pellets were resuspended in 2 ml of phosphate-buffered solution containing heparin and differential cell counts performed after staining (1:10 dilution) in Turk's solution (crystal violet 0.01% in acetic acid 3%) in an improved Neubauer hemocytometer. Data are reported as 10^6 polymorphonuclear leukocytes recovered from each mouse. To investigate the drug effects mice received either vehicle, rolipram (1–10 mg kg^{-1}, i.p., −30 min) or dexamethasone (1.5 mg kg^{-1}, i.v., −60 min) prior to the local injection of zymosan.

The doses of rolipram were selected on the basis of preliminary experiments when the effect of this drug on TNF-α release during endotoxemia was evaluated (data not shown). The dose of dexamethasone has previously been reported to inhibit both interleukin-1α and interleukin-8-induced neutrophil accumulation into a murine air-pouch (Perretti and Flower, 1993; Perretti et al., 1994).

2.2. Assay of TNF-α and interleukin-1α levels in the lavage fluid

The concentration of immunoreactive TNF-α and interleukin-1α in 50 and 100 μl aliquots, respectively, of lavage fluids were determined by ELISA in 96-well plates. Binding was detected by a peroxidase-conjugated polyclonal anti-mouse TNF-α or anti-mouse interleukin-1α using tetramethylbenzidine as a substrate. Following acidification (sulphuric acid, 0.5 M final concentration) the absorbance of each well was measured at 450 nm (Anthos Labtec Instruments). The ELISA specific for TNF-α does not cross react with murine interleukin-1α or interleukin-1β up to 1 μg ml^{-1} and 100 ng ml^{-1}, respectively, or with murine TNF-β (up to 1 μg ml^{-1}). The ELISA specific for interleukin-1α does not cross react with murine interleukin-1β or murine TNF-α up to 0.5–1.0 mg ml^{-1}. Intra-assay and inter-assay reproducibilities were 2.0–2.8% and 8.3–8.7%, or 2.9–3.5% and 5.9–7.3% for the TNF-α or interleukin-1α ELISA, respectively (data furnished by the manufacturer).

2.3. Assay of leukocyte elastase activity released in lavage fluids

Leukocyte elastase activity released from polymorphonuclear leukocytes (serpin type enzyme) in lavage fluids was measured using the modified methodology described by Iwamura et al. (1993). Briefly, 50 μl of sample or standard human leukocyte elastase (EC 3.4.21.37; Calbiochem Novabiochem, UK) was pipetted in duplicate to a 96-well plate with 50 μl TBS (0.2 M Tris HCl, 0.15 M NaCl, pH 8.0) and 10 μl of triton X-100 (0.2% in TBS) added. After mixing for 30 s at room temperature, 10 μl of the specific elastase substrate, methoxy-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (17.8 mM in dimethylsulfoxyde (15% v/v in 0.2 M Tris-HCl pH 8.0) were added. Incubations were carried out for a further 120 min. The concentration of *p*-nitroaniline released was measured spectrophotometrically at 405 nm. Leukocyte elastase activity is reported as ng leukocyte elastase equivalent per ml of lavage fluid or normalized per 10^6 of polymorphonuclear leukocytes.

2.4. Materials

Dexamethasone-21-phosphate was obtained from David Ball Laboratories (Warwick, UK) as a sterile 4 mg ml^{-1} solution. Zymosan type A and all other chemicals were obtained from Sigma Chemical Company (Poole, UK). The specific ELISA for murine TNF-α and interleukin-1α were purchased from Genzyme

(MA, USA. Rolipram was a generous gift from Dr. H. Wachtel (Schering AG, Berlin, Germany).

2.5. Data and statistics

Data are reported as means ± S.E.M. of n separate determinations. Statistical analysis was performed using one-way analysis of variance followed by Bonferroni test for intergroup differences or unpaired Student's t-test when only two groups were compared. A threshold P value < 0.05 was taken as significant.

3. Results

3.1. Time course of zymosan-induced inflammation

In vehicle-treated pouches, immunoreactive TNF-α and interleukin-1α levels were negligible and only marginally exceeded the limit of detection at the 4 h time-point (Fig. 1A). In contrast, considerable amounts of TNF-α and interleukin-1α were found in zymosan-treated pouches throughout the entire time course examined (Fig. 1 A). Both TNF-α and interleukin-1α peaked at 2 h after zymosan injection. TNF-α content steadily declined within 16 h whereas interleukin-1α levels remained elevated over the entire duration of the experiments (Fig. 1A). Local injection of zymosan caused a rapid influx of polymorphonuclear leukocytes which was maximal at the 4 h time-point and had declined significantly by 16 h. Saline injection caused negligible polymorphonuclear leukocyte infiltration (Fig. 1B). Similarly, a low influx of mononuclear cells was observed in zymosan-treated air-pouches at 16 h only, this effect being negligible at all other time-points. Indeed, $0.18 \pm 0.05 \times 10^6$ mononuclear cells were counted in the lavage fluids of untreated air-pouches ($n = 4$); $0.29 \pm 0.07 \times 10^6$ and $0.54 \pm 0.13 \times 10^6$ mononuclear cells were counted at the 4 h time-point in saline- and zymosan-treated air-pouches, respectively ($n = 10$ in both cases, not significant). At the 16 h time-point, $0.30 \pm 0.05 \times 10^6$ mononuclear cells were collected from saline-treated air-pouches ($n = 5$), whereas $1.28 \pm 0.31 \times 10^6$ mononuclear cells were found in the air-pouches which had been challenged with 5 mg zymosan ($n = 16$, $P < 0.05$ vs. saline-treated group).

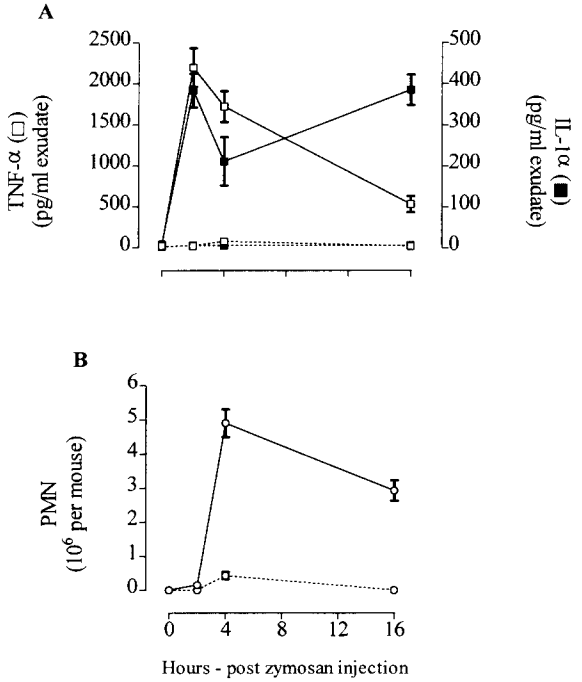

Fig. 1. Time course of zymosan-induced inflammation. Animals were treated with zymosan (5 mg in 0.5 sterile saline) or saline at time 0 and air-pouches washed at different times. (A) Time course of TNF-α ($n = 12$–15) and interleukin-1α (IL-1α; $n = 8$–13) release in the lavage fluids after local injection of zymosan (solid lines) or saline (dotted lines). (B) Polymorphonuclear leukocyte (PMN) influx into the air-pouches measured 2, 4 and 16 h after local injection of zymosan (solid lines, $n = 8$) or saline (dotted lines, $n = 8$). Values are means ± S.E.M. of n mice per group.

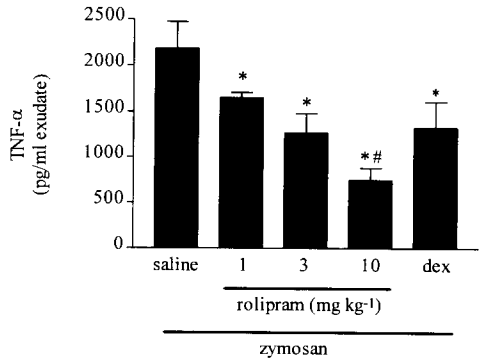

Fig. 2. Effect of rolipram on TNF-α production during zymosan-induced inflammation. Mice received either saline (10 ml kg^{-1}, −30 min, i.p.), rolipram (1–10 mg kg^{-1}, −30 min, i.p.) or dexamethasone (1.5 mg kg^{-1}, −60 min, i.v.) prior to local challenge with zymosan (5 mg in 0.5 ml saline). Air-pouches were washed 2 h later and TNF-α levels measured in the lavage fluids by ELISA. Values are means ± S.E.M. of five mice per group. * $P < 0.05$ vs. saline-treated group. $^\#$ $P < 0.05$ vs. rolipram 1 mg kg^{-1} group.

3.2. Dose-dependent effect of rolipram on TNF-α production

The efficacy of rolipram in inhibiting TNF-α production was assessed at the 2 h time-point, which corresponded to the peak release of this cytokine. Rolipram significantly reduced the levels of TNF-α measured in the lavage fluids in a dose-dependent manner (Fig. 2). A maximal inhibition of ~ 60% was

obtained at the highest dose tested of 10 mg kg^{-1}. A significant reduction was also achieved by treatment of mice with dexamethasone (1.5 mg kg^{-1}) (Fig. 2).

3.3. Effects of rolipram and dexamethasone on TNF-α, interleukin-1α levels and leukocyte infiltration

Drug effects on leukocyte infiltration were assessed at 4 h, a time-point at which TNF-α and interleukin-1α could also be reliably measured. Rolipram (10 mg kg^{-1}) inhibited TNF-α production with no effect on interleukin-1α levels (Fig. 3A,B). On the other hand, dexamethasone produced a marked reduction of both TNF-α and interleukin-1α production (Fig. 3A,B). Rolipram did not modify the number of polymorphonuclear leukocytes recovered from the air-pouches whereas dexamethasone substantially affected this parameter of cell infiltration (Fig. 3C).

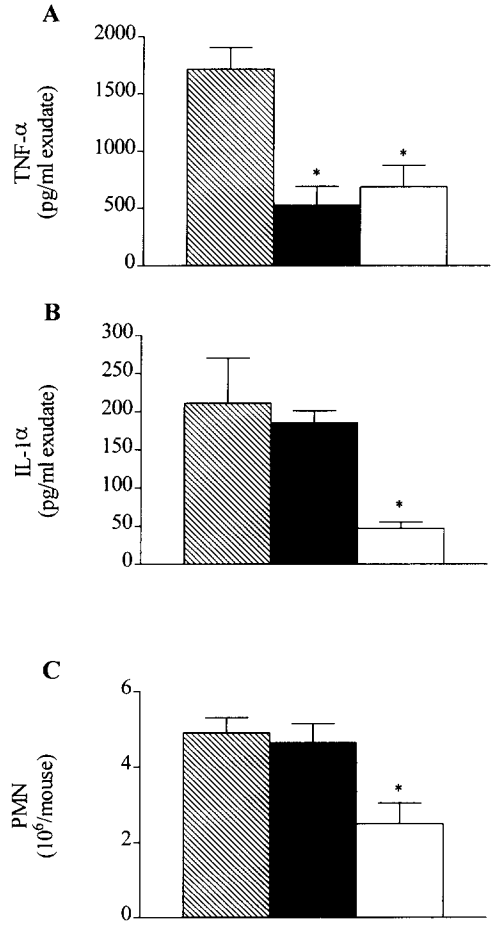

Fig. 3. Effects of systemic administration of rolipram and dexamethasone on TNF-α (A), interleukin-1α (IL-1α) (B) and polymorphonuclear leukocyte (PMN) infiltration (C) in zymosan-induced inflammation. Mice received either saline (hatched columns, 10 ml kg^{-1}, −30 min, i.p., $n = 8$) rolipram (solid columns, 10 mg kg^{-1}, −30 min, i.p., $n = 6$–10) or dexamethasone (open columns, 1.5 mg kg^{-1}, −60 min, i.v., $n = 4$–6) prior to the local injection of zymosan (5 mg in 0.5 ml saline). Pouches were washed 4 h later. Values are means ± S.E.M. of n mice per group. * $P < 0.05$ vs. saline-treated group.

Table 1
Drug effect on leukocyte elastase activity released in inflammatory lavage fluids

Pretreatment	Treatment	Leukocyte elastase activity	
		ng ml^{-1} fluid	ng per 10^6 cells
Vehicle	Saline	n.d.	n.d.
Vehicle	Zymosan	103 ± 11	22 ± 2
Rolipram	Zymosan	48 ± 10 [a]	10 ± 2 [a]
Dexamethasone	Zymosan	37 ± 11 [a]	17 ± 6

Mice ($n = 6$ per group) were treated with saline (10 ml kg^{-1}, −30 min, i.p.), rolipram (10 mg kg^{-1}, −30 min, i.p.), or dexamethasone (1.5 mg kg^{-1}, −60 min, i.v.) prior to local challenge with saline (0.5 ml) or zymosan (5 mg in 0.5 ml saline). Air-pouches were washed 4 h later and leukocyte elastase activity measured as described in Methods. Values (means ± S.E.M.) are reported either as leukocyte elastase activity per ml of lavage fluid or as leukocyte elastase activity per 10^6 of polymorphonuclear leukocytes counted in the same air-pouches. n.d. = not detectable. [a] $P < 0.05$ vs. zymosan in vehicle-treated mice.

3.4. Effects of rolipram and dexamethasone on leukocyte elastase

Leukocyte elastase activity was easily detected in 4 h lavage fluids following zymosan administration (Table 1). A significant reduction (~ 50%) in leukocyte elastase release was observed following treatment of mice with rolipram (10 mg kg^{-1}). A similar reduction was also observed after dexamethasone, although the effect of the steroid was not significant any longer if data were normalized in relation to the accumulation of polymorphonuclear leukocytes (Table 1).

4. Discussion

Rolipram is a selective inhibitor of the phosphodiesterase 4 family (Torphy et al., 1992) and causes a rise in intracellular cyclic AMP after addition to cells in vitro (Schudt et al., 1991; Endres et al., 1991). It has been suggested that as a direct consequence of this (Torphy and Undem, 1991; Molnar-Kimber et al., 1992), rolipram inhibited TNF-α production by mononuclear leukocytes with no effect on interleukin-1 synthesis (Semmler et al., 1993b). In line with these studies we have examined rolipram as a putative selective inhibitor of TNF-α production in a model of acute inflammation in vivo.

Several characteristics of the acute inflammatory response are reproduced following administration of zymosan into specific tissue sites. The recruitment of

inflammatory cells (polymorphonuclear leukocytes) induced by zymosan is driven by complement-derived factors and leukotriene B_4 during the initial phases (up to 2 h) (Forrest et al., 1986; Collins et al., 1990), whereas at later time-points their role is sustained by pro-inflammatory cytokines such as interleukin-1 and interleukin-8 (Collins et al., 1990; Perretti et al., 1992; Erdö et al., 1994). In particular, treatment of mice with anti-interleukin-1 monoclonal antibody significantly attenuated the accumulation of polymorphonuclear leukocytes into mouse peritoneal cavities 4 h after challenge with this inflammogen (Perretti et al., 1992). We have used the mouse air-pouch model because detectable quantities of TNF-α were found in the lavage fluids after injection with zymosan. Therefore this model appeared to satisfy the major aim of the study, i.e. to evaluate the effect of rolipram on TNF-α production at a local site of inflammation. Using the zymosan air-pouch model we could reliably measure both humoral and cellular parameters, with the only exception of the influx of mononuclear leukocytes which was particularly modest. This lack of monocyte infiltration may be related to the site injected (air-pouch vs. peritoneal cavity), or to the time-points investigated, i.e. possibly these cells could have appeared at later time-points, as reported by Erdö et al. (1994). However, intense accumulation of highly specialized cells, such as polymorphonuclear leukocytes, was easily detected in the lavage fluids after challenge with zymosan. The state of activation of migrated cells could also be assessed by measuring a detectable elastase activity released in these fluids. It is noteworthy that the peak of polymorphonuclear leukocyte accumulation coincided with high levels of pro-inflammatory cytokines, such as TNF-α and interleukin-1. For all these reasons, the effect of rolipram was then investigated at these time-points (2 h and 4 h). Systemic treatment of mice with rolipram resulted in a dose-dependent reduction of TNF-α levels without significant changes in polymorphonuclear leukocyte infiltration. In contrast, dexamethasone markedly reduced TNF-α and interleukin-1α levels and this was accompanied by a significant reduction in cell migration. Therefore we suggest that endogenous interleukin-1 plays a more important role than TNF-α for cell recruitment to the inflammatory site after zymosan administration, this essentially being in agreement with the studies cited above. It should also be noted that systemic treatment with rolipram has been reported to inhibit the modest (less than 0.5×10^6 cells) recruitment of polymorphonuclear leukocytes induced by leukotriene B_4 in the mouse peritoneal cavity (Griswold et al., 1993), whereas we were unable to see any effect on the much greater cell accumulation induced by zymosan. However, our data are in agreement with those of Teixeira et al. (1994), who reported that systemic treatment with rolipram did not modify neutrophil accumulation in the guinea pig skin, though inhibiting the accumulation of eosinophils.

Since cells must be recruited to the site of inflammation and became activated in order to effectively remove the inflammatory stimulus, we used the measurement of leukocyte elastase released in the lavage fluid as an indicator of polymorphonuclear leukocyte activation. By using a selective substrate for leukocyte elastase (Nakajima et al., 1979) we measured a reliable release of this enzyme in the inflammatory fluid at the peak of cell infiltration. This type of elastase is released from the azurophil granules of neutrophils and is a serpin type of enzyme, different from that found and released by monocytes (Nakajima et al., 1979; Sandborg and Smolen, 1988). Lower leukocyte elastase activity levels were measured not only in the lavage fluids collected from dexamethasone-treated mice but also in those harvested from rolipram-treated animals. This is indicative of an anti-inflammatory action of rolipram, especially if considering that significant reduction in enzymatic activity was maintained when the data were expressed per cell as well as per ml of lavage fluid, i.e. differentially from dexamethasone, rolipram may interfere with the neutrophil degranulation process.

From a mechanistic point of view, our data do not distinguish between a direct effect of rolipram on polymorphonuclear leukocyte degranulation or an indirect effect via reduction of the release of TNF-α. However, increased levels of intracellular cyclic AMP impair the degranulation process of leukocytes (Sandborg and Smolen, 1988) and indeed, rolipram is able to reduce leukocyte activation in vitro (Schudt et al., 1991). Although these observations would suggest a direct effect upon the polymorphonuclear leukocyte rather than an indirect inhibition of leukocyte elastase activity secondary to inhibition of TNF-α release, further investigations are necessary to confirm whether this also applies to the inflammatory model described here.

Based upon the data presented in this study, we propose that rolipram may have a beneficial anti-inflammatory action not only in those conditions where a pronounced systemic release of endogenous TNF-α occurs (e.g. endotoxemia), but also in cases where the production of TNF-α is more localized (e.g. the joint during acute episodes of rheumatoid arthritis) or where release of proteolytic enzymes appears to play a pivotal role (e.g. adult respiratory distress syndrome and joint erosion).

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