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Infiltration of neutrophils by intrapleural injection of tumour necrosis factor, interleukin-1, and interleukin-8 in rats, and its modification by actinomycin D

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1 To assess *in vivo* chemotactic activity of tumour necrosis factor (TNF), interleukin-1 (IL-1), IL-8, and cytokine-induced neutrophil chemoattractant (CINC), we injected these cytokines into the pleural cavity of rats.

2 CINC (0.1-1 μg) and recombinant human IL-8 (rhIL-8, 0.2-5 μg) caused neutrophil infiltration into the rat pleural cavity in a dose-dependent fashion, peaking at 3 h. The number of leukocytes in the peripheral blood did not change significantly.

3 RhTNFα and rhIL-1α also induced neutrophil accumulation. The dose response curves of rhTNFα (0.67 ng-6.7 μg) and rhIL-1α (0.45 ng-4.5 μg) at 3 h were bell shaped. On the other hand, unlike CINC and rhIL-8, rhTNFα and rhIL-1α caused transient marked leukopenia at 3 h in a simple dose-dependent fashion.

4 Concomitant injection of actinomycin D dose-dependently and completely at 10 μg inhibited neutrophil infiltration induced by rhTNFα (0.67 μg) and rhIL-1α (0.45 μg) at 3 h. However, that induced by CINC or rhIL-8 was not affected by actinomycin D.

5 Peaking at 1 h, CINC production in the pleural cavity was found after intrapleural injection of rhTNFα (0.67 μg) or rhIL-1α (0.45 μg), but not after that of rhIL-8 (5 μg). The CINC production induced by rhTNFα or rhIL-1α and the neutrophil infiltration was suppressed by concomitant injection of actinomycin D (1 and 10 μg).

6 These results indicate that CINC and IL-8 themselves are direct chemoattractants for neutrophils, whereas TNF and IL-1 induce neutrophil infiltration indirectly via newly synthesized mRNA for chemotactic protein including CINC, which may be involved in neutrophil emigration at local inflammatory sites in rats.

Keywords: Neutrophil infiltration; cytokine-induced neutrophil chemoattractant; interleukin-8; interleukin-1; tumour necrosis factor; actinomycin D

Introduction

Neutrophil accumulation at local inflammatory sites is the hallmark of acute inflammation and is mediated in part by locally produced chemotactic factors, such as C5a, platelet activating factor (PAF), and leukotriene B4 (LTB_4) (Movat, 1985). Besides them, proinflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), also induced neutrophil accumulation *in vivo* (Le & Vilček, 1987). However, the action of TNF and IL-1 could be indirect (Yoshimura *et al.*, 1987a; Mrowietz *et al.*, 1988) and protein synthesis dependent (Rampart & Williams, 1988; Cybulsky *et al.*, 1989). In this context, human IL-8 was purified (Yoshimura *et al.*, 1987b), cDNA encoding it was cloned (Matsushima *et al.*, 1988), and IL-8 was reported to be a direct chemoattractant for neutrophils induced by TNF and IL-1. Involvement of IL-8 in neutrophil infiltration in inflammatory models in rabbits was also demonstrated (Harada *et al.*, 1993; Sekido *et al.*, 1993; Wada *et al.*, 1994).

On the other hand, a family of various polypeptide chemotactic cytokines (chemokines) was discovered following IL-8 (Oppenheim *et al.*, 1991). In rats, cytokine-induced neutrophil chemoattractant (CINC) was purified from the culture supernatant of a rat kidney epitheloid cell line stimulated with IL-1β (Watanabe *et al.*, 1989a, b) and was shown to have higher homology to human MGSA/gro, which is a member of the chemokine family and also a neutrophil chemoattractant (Derynck *et al.*, 1990), than to IL-8. Although several studies demonstrated that IL-8 (Larsen *et al.*, 1989; Ribeiro *et al.*, 1991; Rot, 1991) and CINC (Watanabe *et al.*, 1991; Hirasawa *et al.*, 1992) induced neutrophil infiltration in rats *in vivo*, there is, to our knowledge, no report comparing the neutrophil chemotactic activity of CINC and IL-8 in the same inflammatory model in rats. Furthermore, comprehensive study including TNF and IL-1 is required for elucidation of the mechanism of neutrophil infiltration at local inflammatory sites evoked by cooperation of these cytokines, especially the induction of IL-8 by TNF and IL-1 *in vivo*.

We previously reported that TNF, IL-1 and IL-6 appeared sequentially in the pleural exudate of rats during carrageenin-induced pleurisy, indicating these cytokines may participate in acute inflammatory reactions (Utsunomiya *et al.*, 1991a,b; 1994). In this study we examined the neutrophil chemotactic activity *in vivo* of TNF, IL-1, IL-8 and CINC by injecting them into the pleural cavity of rats. We describe the profiles of the neutrophil accumulation into the pleural space and changes in the number of blood leukocytes caused by these cytokines, examine whether the chemotaxis is protein synthesis-dependent or independent, and discuss the possible involvement of endogenous CINC in TNF- and IL-1-induced neutrophil infiltration.

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Methods

Experimental protocol

Male 6-week-old Sprague-Dawley rats (200–250 g) were purchased from Japan SLC (Hamamatsu, Japan) and used after 1 week.

Recombinant human TNFα (rhTNFα, 6.7 ng ml^{-1}–67 μg ml^{-1}), rhIL-1α (4.5 ng ml^{-1}–45 μg ml^{-1}), rhIL-8 (2–50 μg ml^{-1}), and chemically synthesized CINC (1–10 μg ml^{-1}) were dissolved in sterile saline. Rats were anaesthetized with ether and received an intrapleural injection of 0.1 ml of each cytokine. In separate experiments, actinomycin D (1 or 10 μg) was injected concomitantly with rhTNFα (0.67 μg) or rhIL-1α (0.45 μg).

The precise procedure for sample collection was described previously (Kikuchi & Oh-ishi, 1985). The rats were killed at selected times; and blood and pleural cavity washings, the latter obtained with two 1-ml volumes of sterile saline, were collected in polyethylene tubes containing 1/10 volume of 3.8% sodium citrate. The number of cells in the collected sample was counted by a Sysmex microcellcounter F-800 (Toa Electric Co., Kobe, Japan). Films of pleural cavity washing and peripheral blood were stained with Giemsa, and a differential cell count was performed under a light microscope. Pleural cavity washings were centrifuged, and the supernatant was frozen at −70°C until used for the CINC assay.

CINC assay

CINC levels were measured with an enzyme immunoassay (EIA) kit according the manufacturer's instruction. RhIL-8 showed no cross reactivity, even at 5 μg ml^{-1}, in this EIA. The limit of detection in the assay was approximately 0.08 ng ml^{-1}.

Materials

RhTNFα (sp. act. 3×10^6 u mg^{-1}), rhIL-1α (sp. act. 2.26×10^7 u mg^{-1}), and rhIL-8 (Furuta *et al.*, 1989) were generously provided by Dainippon Pharmaceutical Co. (Osaka, Japan). CINC was synthesized chemically (Nishiuchi *et al.*, 1992). Kits for EIA of CINC and actinomycin D were purchased from Immuno-Biological Laboratories Co. (Fujioka, Japan) and Sigma (St Louis, U.S.A.), respectively.

Statistics

The values were expressed as means ± s.e. The statistical significance of differences between the amounts of CINC with and without treatment with actinomycin D was analyzed by a non-parametric test (Kruskal-Wallis multiple comparison (Colquhoun, 1971)). The other data were evaluated for statistical significance by one-way analysis of variance using Dunnett's test for multiple comparison. P values of less than 0.05 were considered as significant.

Results

Leukocyte infiltration induced by CINC and rhIL-8

At the time of injection (0 h) resident leukocytes in the pleural cavity consisted chiefly of monocytes (approximately 80%). There were also some mast cells but no neutrophils, as seen in Figure 1c. By intrapleural injection of even 0.1 μg of CINC, leukocytes that comprised mostly neutrophils had already infiltrated into the pleural cavity by 1 h, and the numbers peaked at 3–5 h, and then declined after 8 h (Figure 1a and 1c). The neutrophil and leukocyte accumulation was dose-dependent (0.1–1 μg). Similarly, rhIL-8 (0.2–5 μg) caused neutrophil infiltration into the pleural cavity, which was already evident by 1 h. This invasion peaked at 3–5 h, and the number was still high at 8 h when the total leukocyte number was maximum (Figure 1b and 1d). The leukocyte accumulation induced by CINC and rhIL-8 was still evident at 24 h.

On the other hand, the numbers of peripheral blood leukocytes and neutrophils were not much changed by CINC and rhIL-8 injection, being around the normal level of 9×10^6 ml^{-1}.

Leukocyte infiltration and leukopenia induced by rhTNFα and rhIL-1α

RhTNFα (6.7 ng–0.67 μg) and rhIL-1α (0.45–45 ng) caused leukocyte as well as neutrophil migration into the rat pleural cavity at 3 h, and their dose-response curves displayed similar bell shapes (Figure 2a and 2b). These effects were considered as statistically significant when compared with the zero dose group by multiple comparison of Dunnett's test as indicated by star marks. In addition, rhIL-1α at a dose of 0.45 μg ($n=9$) even caused significant neutrophil infiltration when compared with zero dose group by Student's t test ($P<0.01$). Leukocyte numbers in the peripheral blood were not much changed up to 67 ng of rhTFNα or 45 ng of rhIL-1α (Figure 2c and 2d), doses which even caused peak infiltration into the cavity. Doses of 0.67 and 6.7 μg of rhTNFα and 0.45 and 4.5 μg of rhIL-1α, caused significant leukopenia in rats (Figure 2c and 2d). On the other hand, peripheral neutrophil numbers were slightly but significantly increased at the doses of 6.7 and 67 ng of rhTNFα and 0.45–45 ng of rhIL-1α.

Effect of actinomycin D on neutrophil infiltration induced by cytokines

To examine whether the neutrophil infiltration induced by each cytokine was dependent on RNA synthesis, actinomycin D (1 or 10 μg) was injected intrapleurally concomitantly with each cytokine. The increase in total number of leukocytes as well as the neutrophil infiltration at 3 h induced by rhTNFα (0.67 μg) or rhIL-1α (0.45 μg) was inhibited by actinomycin D dose-dependently, and at 10 μg was almost completely abolished (Figure 3a and 3b). In contrast, actinomycin D was without effect on both leukocyte and neutrophil infiltration induced by rhIL-8 and CINC (Figure 3c and 3d).

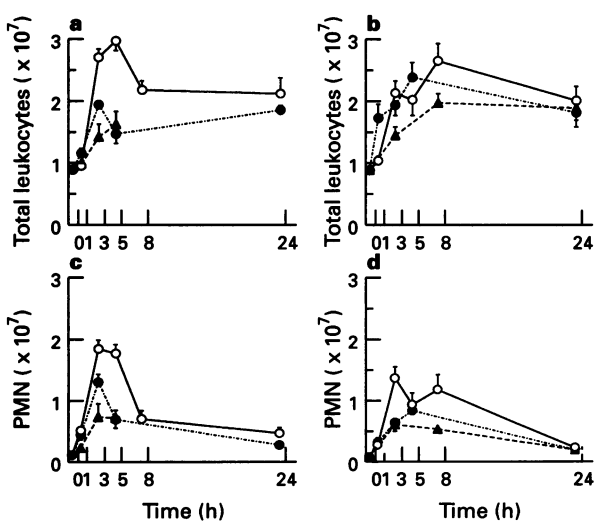

Figure 1 Time courses of the number of leukocytes (a, b) and neutrophils (c, d) in the pleural cavity elicited by intrapleural injection of CINC (a, c) at doses of 0.1 μg (▲), 0.3 μg (●), and 1 μg (○) and rhIL-8 (b, d) at doses of 0.2 μg (▲), 1 μg (●), and 5 μg (○). The values are expressed as mean ± s.e. of 4–5 rats except rhIL-8 5 μg ($n=11$–14), rhIL-8 1 μg ($n=3$) and 0 h control ($n=8$).

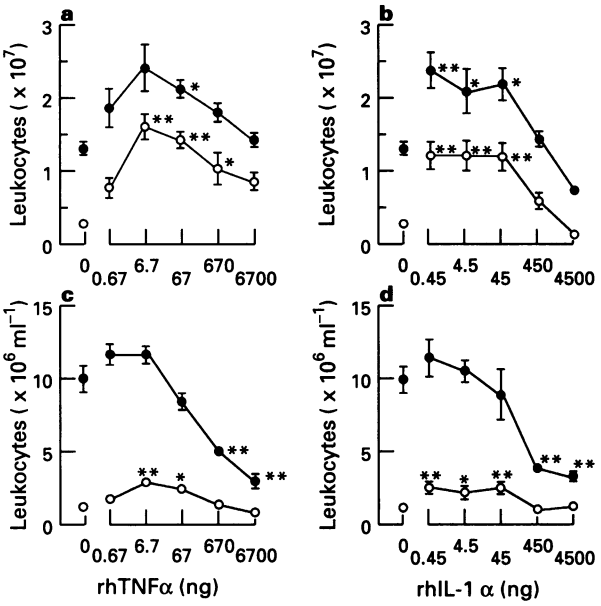

Figure 2 Dose-response of the infiltration of leukocytes (●) and neutophils (○) into the pleural cavity (a, b) and leukopenia (c, d) by intrapleural injection of rhTNFα (a,c) and rhIL-1α (b, d) at 3 h. The group shown as dose 0 recieved intrapleural injection of saline. The values are expressed as means±s.e. of 4 rats. $*P<0.05$ and $**P<0.01$ *vs* dose 0 group by multiple comparison of Dunnett's *t* test.

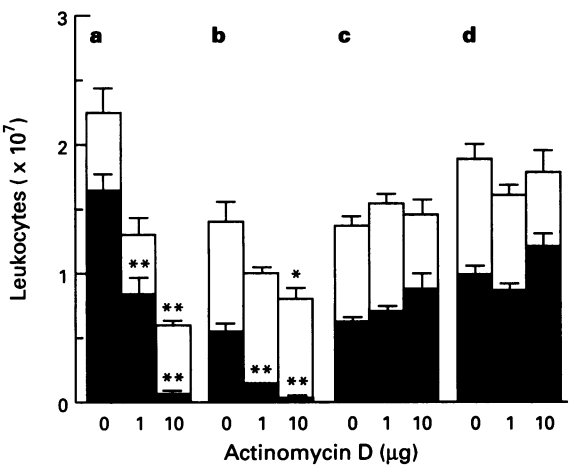

Figure 3 Effect of actinomycin D on cytokine-induced neutrophil accumulation in the pleural cavity at 3 h. Actinomycin D at a dose of 1 or 10 μg was injected concomitantly with rhTNFα at 0.67 μg (a), rhIL-1α at 0.45 μg (b), rhIL-8 at 5 μg (c) or CINC at 1 μg (d). The number of leukocytes (open + solid column) and neutrophils (solid column) are shown. The values are expressed as means±s.e. of 4 rats. $*P<0.05$ and $**P<0.01$ *vs* cytokine alone (actinomycin D dose 0) by multiple comparison with Dunnett's *t* test.

CINC production induced by rhTNFα and rhIL-1α and effect of actinomycin D on this production

The basal level of CINC in the pleural cavity washing (at 0 h) was below the limit of detection (< 0.08 ng). After intrapleural injection of rhTNFα (0.67 μg) or rhIL-1α (0.45 μg), the endogenous CINC level increased rapidly, peaking at 1 h, and then decreased at 3 h (Figure 4). The levels at 1 h were markedly suppressed by actinomycin D (1 and 10 μg) dose-dependently.

rhIL-8 induced an amount of CINC in this model (0.78±0.34 ng at 5 μg at 1 h) near the lower limit of detection.

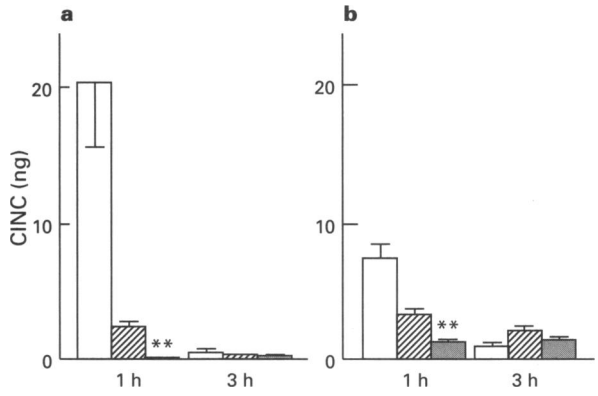

Figure 4 Effect of actinomycin D on rhTNFα- and rhIL-1α-induced CINC production in the pleural cavity. Actinomycin D at a dose of 1 μg (hatched column) or 10 μg (stippled column) was injected concomitantly with rhTNFα at 0.67 μg (a) or rhIL-1α at 0.45 μg (b). The values are expressed as means±s.e. of 4 rats. $**P<0.01$ *vs* cytokine alone (actinomycin D dose 0, open column) by non-parametric multiple comparison of Kruskal-Wallis test.

Discussion

All four cytokines, TNF, IL-1, IL-8, and CINC, tested in this study induced almost neutrophil-specific infiltration into the pleural cavity of rats as expected. By these characteristic profiles of the leukocyte migration, these cytokines can be divided into two groups. The most prominent difference between a group of CINC and rhIL-8, and the other, rhTNFα and rhIL-1α is their sensitivity to actinomycin D on neutrophil infiltration. The selective inhibition of rhTNFα- and rhIL-1α-induced neutrophil infiltration by actinomycin D indicates that the process included *de novo* synthesis of RNA. This result agrees well with previous reports (Cybulsky *et al.*, 1989; Rampart & Williams, 1988). Futhermore, the induction of endogenous CINC production by rhTNFα and rhIL-1α, but not rhIL-8, suggests that TNF and IL-1 can induce neutrophil chemotaxis indirectly via the synthesis of chemoattractant protein including CINC, whereas CINC and IL-8 themselves seem to be direct chemoattractants for neutrophils.

It is difficult to determine which cytokine was the most potent chemoattractant in this series of experiments since the injections of much higher doses of CINC and rhIL-8 were not examined because of the limited supply of these cytokines, although the ranges of effective doses of rhTNFα and rhIL-1α were found to be wide, i.e., over 10^3 fold (Figure 2a and 2b). However, at the same range of $10^{-11}-10^{-10}$ mol, CINC and rhIL-8 increased the numbers of leukocytes and neutrophils in the pleural cavity (Figure 1), whereas rhTNFα and rhIL-1α decreased them (Figure 2a and 2b), suggesting sensitivity to these cytokines were different. Furthermore, rhTNFα and rhIL-1α, but not CINC and rhIL-8, induced marked leukopenia at 3 h (Figure 2c and 2d), as was also seen in rabbits (Okusawa *et al.*, 1988). Vascular adhesion molecules induced by TNF and IL-1 (Carlos & Harlan, 1994) may be partly involved in this phenomenon. These results may also support the difference in actions of TNF and IL-1 *vs* IL-8 and CINC.

CINC induced in the pleural cavity by rhTNFα and rhIL-1α at 1 h (Figure 4) could possibly account for the neutrophil infiltration by them after 3 h (Figure 3a and 3b) when the CINC level declined (Figure 4), because exogenous CINC required 3–5 h to attract the maximal number of neutrophils (Figure 1c). Furthermore, actinomycin D markedly inhibited the preceding CINC production induced by the cytokines at 1 h and the neutrophil infiltration at 3 h. These results suggest that endogenous CINC may be involved, at least in part, in TNF- and IL-1-induced neutrophil infiltration into the rat pleural cavity.

When we compare the potency of CINC with that of IL-8 on a molar basis for their neutrophil infiltration, CINC seemed

to be a more effective chemoattractant than rhIL-8 in this model (Figure 1c and 1d). This may be due to the difference of responsiveness of rat neutrophils to human and rat cytokines, because it was reported that the chemotactic activity of CINC for rat neutrophils *in vitro* was stronger than that of rhIL-8 (Watanabe *et al.*, 1991). Furthermore, the response of rat neutrophils to human IL-8 was reported to be much weaker than that of human neutrophils (Rot, 1991). Therefore, it is reasonable that local injection of exogenous CINC into rats resulted in a more remarkable neutrophil infiltration than that achieved with rhIL-8.

In conclusion, this study identifies the rat pleurisy model as a useful experimental system for the study of cytokine-induced neutrophil infiltration and suggests that, at least in rats, TNF- and IL-1-induced neutrophil accumulation *in vivo* may require endogenous CINC.

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