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Blockade by fenspiride of endotoxin-induced neutrophil migration in the rat

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Fenspiride, an antiinflammatory drug with low anti-cyclooxygenase activity, administered orally at 60–200 mg/kg inhibited neutrophil migration into peritoneal and air pouches cavities as well as exudation into peritoneal cavities induced by endotoxin but not induced by carrageenin. Up to 100 μM, fenspiride failed to inhibit the in vitro release of a neutrophil chemotactic activity by endotoxin-stimulated macrophages and the in vivo migration into the peritoneal cavities induced by the supernatant of those macrophages. The release of tumour necrosis factor by stimulated macrophages was inhibited by fenspiride in a dose-dependent manner. These results suggest that the antiinflammatory effects of fenspiride are associated with the inhibition of the tumour necrosis factor release by resident macrophages.

Fenspiride; Tumour necrosis factor; Carrageenin; Endotoxin; Neutrophil migration

1. Introduction

Fenspiride is an antiinflammatory drug targeted for the upper and lower respiratory tract (Le Douarec et al., 1969). Since fenspiride prevents neutrophil migration and plasma exudation induced by intrapleural injections of zymosan, Lima et al. (1988) suggested that it interferes with the cross-talk between vessels and inflammatory cells. Inhibition or antagonism of chemotactic factors released by resident cells are alternative explanations for these antiinflammatory effects (Ferreira, 1980; Cunha and Ferreira, 1986), which were now investigated.

Neutrophil recruitment during early inflammation is mediated by leucotriene B_4, complement fragments (Higgs et al., 1981; Vogt, 1985; Till, 1988) and by cytokines. The recognized cytokines released during the inflammatory reaction, and which display chemotactic activities, are interleukins 1 and 8, interferon γ and tumour necrosis factor (Matsushima et al., 1988; Rampart and Williams, 1988; Faccioli et al., 1990; Harmsen and Havel, 1990; Ribeiro et al., 1990). Several laboratories, including ours, have shown that in vivo neutrophil migration induced by these cytokines is not due to direct chemoattractant activity but is mediated by the release of secondary chemotactic factors (Faccioli et al., 1990; Harmsen and Havel, 1990; Ribeiro et al., 1990, 1991). We have recently shown that macrophages stimulated by interleukin 1, tumour necrosis factor, interferon γ or endotoxin release a chemotactic substance named macrophage neutrophil chemotactic factor (MNCF) (Cunha and Ferreira, 1986; Faccioli et al., 1990; Ribeiro et al., 1990). This factor differs from the other cytokines generated by macrophages (i.e. tumour necrosis factor, interleukin 1 and 8) by its ability to cause in vivo neutrophil migration in glucocorticosteroid-treated animals (Cunha and Ferreira, 1986; Facciolli et al., 1990; Ribeiro et al., 1991).

In order to further investigate the mode of action of fenspiride, we studied its effect on neutrophil migration into the peritoneal cavity of rats injected with native MNCF, or with pro-inflammatory substances, carrageenin and endotoxin. In addition, we have also investigated the ability of fenspiride to inhibit the release of MNCF, or of tumour necrosis factor by macrophage monolayers stimulated by endotoxin, as possible explanations for its effectiveness.

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2. Materials and methods

2.1. Animals

Male Wistar rats weighing 180–200 g and male BALB/c mice weighing 20–25 g, housed in temperature-controlled rooms, received water and food ad libitum until the time of experiments.

2.2. Modulation of in vivo neutrophil migration induced by carrageenin and endotoxin

2.2.1. Peritonitis

Carrageenin (300 μg) or endotoxin (200 ng) was diluted in 3 ml of phosphate-buffered saline (PBS) and injected intraperitoneally into control or fenspiride-treated rats. Fenspiride hydrochloride was administrated orally at 60, 100 and 200 mg/kg 1 h before the intraperitoneal injection of the stimuli. Control animals received 3 ml of PBS.

2.2.2. Air pouch

Rat skin air pouches were produced as previously described (Edwards et al., 1981). The backs of the rats were shaved and 20 ml of sterile air was injected subcutaneously. Three days later, 10 ml of sterile air was again injected to maintain pouch patency. Six days after the first injection of air, carrageenin (300 μg/ml) or endotoxin (200 ng/ml) was injected into the air pouch of normal or fenspiride-treated rats. Fenspiride was administered by oral route in doses of 100 and 200 mg/kg, 1 h before the injection of the inflammatory stimuli. Control animals received 1 ml of PBS into the air pouch.

2.3. Collection of exudates

Four hours after the injection of PBS or of the stimuli the animals were exsanguinated and the peritoneal or air pouch cells were harvested by injecting 5 ml of PBS containing 5 IU/ml of heparin. Total and differential cell counts were performed by standard methods (Souza and Ferreira, 1985). The results are reported as the number of cells per ml of wash.

2.4. Modulation of the increase in vascular permeability induced by endotoxin and carrageenin

The increase in vascular permeability was evaluated according to the colorimetric determination of the amounts of Evans blue extravasated into the peritoneal cavities of the rats. Normal and fenspiride-treated rats received 25 mg/kg of Evans blue i.v. 10 min after the i.p. injection of PBS or stimuli. Two hours later, the animals were exsanguinated and the peritoneal cavities were washed with 5 ml of heparinized PBS. The washes were then centrifuged at $300 \times g$ (10 min, at room temperature) and the optical density at 600 nm in the supernatant was determined. The results are expressed as percentages of increase of absorbance in relation to the control (PBS injection).

2.5. Effect of fenspiride on the release of tumour necrosis factor from macrophage monolayer stimulated with endotoxin plus interferon

Mouse peritoneal macrophages were harvested with RPMI with 10% of fetal calf serum from peritoneal cavities previously stimulated with thioglycollate (2 ml of 3% thioglycollate in water i.p., 4 days earlier) and were cultured at 37°C in 24-well plastic tissue culture plates at 10^6 cells/ml of culture medium, for 1 h in an atmosphere of air mixed with 5% CO_2. Non-adherent cells were removed and the adherent cells were washed 3 times with pre-warmed medium. Fenspiride (30, 100 and 300 μM) was added to the macrophage monolayer 30 min before stimulation with endotoxin (0.2 and 2 μg/ml) or with endotoxin (10 ng/ml) plus interferon γ. Twelve hours after the addition of the stimuli, the concentration of tumour necrosis factor in the supernatants and the viability of the macrophages were determined by a sandwich type enzyme-linked immunosorbent assay (ELISA) and by trypan blue exclusion, respectively. Briefly, microtiter plates were coated overnight at 4°C with a monoclonal antibody against tumour necrosis factor (10 μg/ml). After blocking plates, the samples and standard in various dilutions were added in duplicate and incubated at room temperature for 2 h. Rabbit anti-tumour necrosis factor polyclonal antibody was added at 10 μg/ml followed by incubation at room temperature for 1 h. An anti-rabbit IgG alkaline phosphate conjugate diluted 1000 times was added following incubation for 1 h at room temperature. Finally, p-nitrophenyl phosphate substrate was added and the plates were read at 405 nm. The results were expressed as pg/ml of tumour necrosis factor by comparing the optical density with standard curves made with recombinant purified murine tumour necrosis factor.

2.6. Effect of fenspiride on the release of MNCF from macrophage monolayer stimulated with endotoxin

Briefly, rat peritoneal macrophages were harvested with RPMI from peritoneal cavities previously stimulated with thioglycollate (10 ml of 3% thioglycollate in water i.p., 4 days earlier) and incubated in plastic tissue culture dishes for 1 h at 37°C in an atmosphere of air mixed with 5% CO_2. The adherent monolayer (95% of macrophages) was then washed 3 times with RPMI medium (pH 7.4). After a 30-min incubation at 37°C with 5 μg/ml of endotoxin, the supernatant was dis-

carded and the cells were washed 3 times with RPMI. This was followed by a final incubation with 4 ml of RPMI medium without endotoxin for 1 h at 37°C for MNCF release. To test the effect of fenspiride (100 μM) and dexamethasone (3 μM) upon MNCF release, the drugs were added to the cell suspension 1 h before endotoxin, as well as during endotoxin stimulation and during MNCF release. The cell-free incubation fluids were sterilized (Millipore filters, 0.22 μm) and injected into the peritoneal cavities of dexamethasone-pretreated rats.

2.7. Effect of fenspiride on neutrophil migration into peritoneal cavities induced by MNCF

Rats were pretreated with fenspiride (100 or 200 mg/kg, p.o.) or dexamethasone (0.5 mg/kg) and fenspiride (200 mg/kg) 1 h before the i.p. challenge with 3 ml of MNCF (equivalent to the material released by 3×10^6 macrophages) or of PBS. Neutrophil migration was evaluated 4 h after i.p. injection of the stimuli, as described above.

2.8. Drugs and solutions

Carrageenin (Marine Colloids, USA), endotoxin (lipopolysaccharide from *Escherichia coli* 0111 B_4, control number 635050), thioglycollate and RPMI medium, No. 1640 (Difco, USA), anti-rabbit IgG alkaline phosphate conjugate, p-nitrophenyl phosphate substrate and Evans blue (Sigma), fenspiride hydrochloride (Servier, France), dexamethasone (MerckSharp Dohme, Brazil).

3. Results

3.1. Interference of fenspiride with neutrophil migration in vivo

Endotoxin-induced neutrophil migration into the peritoneal cavity was significantly inhibited by fenspiride at 100 and 200 mg/kg. Doses of and below 60 mg/kg were ineffective. In contrast, migration induced by carrageenin was not modified by fenspiride at doses up to 200 mg/kg (fig. 1). Similarly, fenspiride failed to prevent neutrophil migration into carrageenin-injected air pouches, but was effective against migration induced by endotoxin (fig. 2).

3.2. Interference of fenspiride with increased vascular permeability

In agreement with its selective ability to interfere with neutrophil chemoattraction, fenspiride reduced the increased vascular permeability induced by endotoxin, but not by carrageenin (fig. 3).

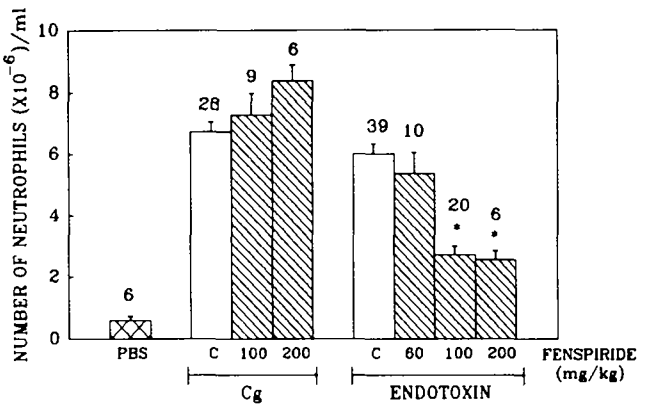

Fig. 1. Interference of fenspiride with neutrophil migration into peritoneal cavities induced by carrageenin (Cg; 300 μg) or endotoxin (200 ng). The open and hatched bars indicate, respectively, neutrophil migration into peritoneal cavities in normal or in fenspiride-treated rats, at the doses indicated. The first column represents neutrophil migration induced by the injection of 3 ml of PBS (control). The bars represent the mean ± S.E.M. and the number of animals tested per group is indicated above each column. Asterisks indicate significant differences when compared with the respective control (untreated rats; $P < 0.05$; unpaired Student t-test).

3.3. Interference of fenspiride with tumour necrosis factor release

The release of tumour necrosis factor by macrophages stimulated by endotoxin plus interferon γ was inhibited in a dose-dependent manner by fenspiride

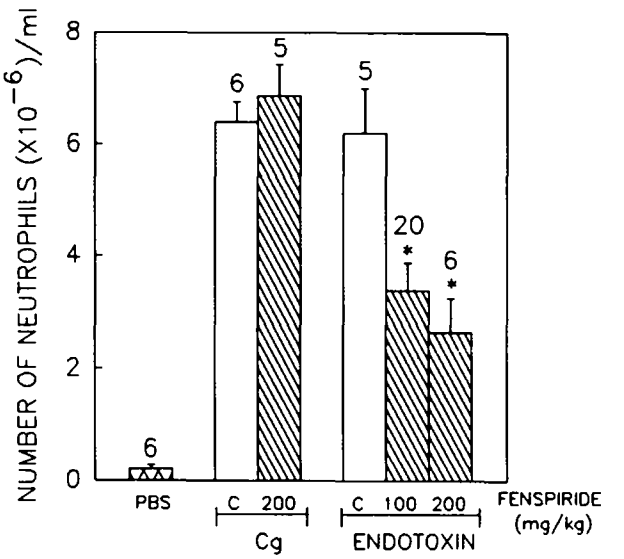

Fig. 2. Interference of fenspiride with neutrophil migration into air pouch induced by carrageenin (Cg) and endotoxin. The open and hatched bars indicate, respectively, neutrophil migration into air pouch induced by Cg (300 μg) and endotoxin (200 ng) in normal and fenspiride-treated rats at the doses indicated. The first column represents neutrophil migration induced by the injection of 1 ml of PBS (control). The bars represent the mean ± S.E.M. and the number of animals tested per group is indicated above each column. Asterisks indicate significant differences when compared with the respective control (untreated rats; $P < 0.05$; unpaired Student t-test).

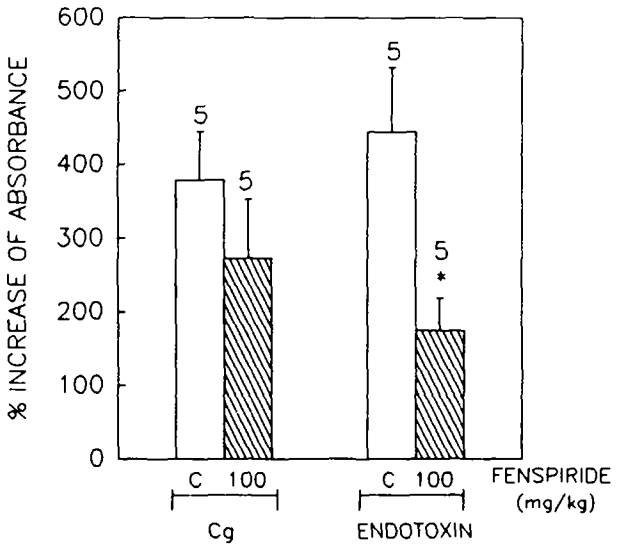

Fig. 3. Interference of fenspiride (100 mg/kg) with protein exudation into peritoneal cavities induced by carrageenin (Cg) and endotoxin. The open and hatched bars indicate, respectively, the percentage increase of protein exudation in relation to control (PBS injection), induced by carrageenin (300 μg) and endotoxin (200 ng) in normal and fenspiride-treated rats. The increase of vascular permeability was evaluated according to the extravasation of Evans blue. The bars represent the mean ± S.E.M. and the number of animals tested per group is indicated above each column. Asterisks indicate significant differences when compared with the respective control (untreated rats; $P < 0.05$; unpaired Student t-test).

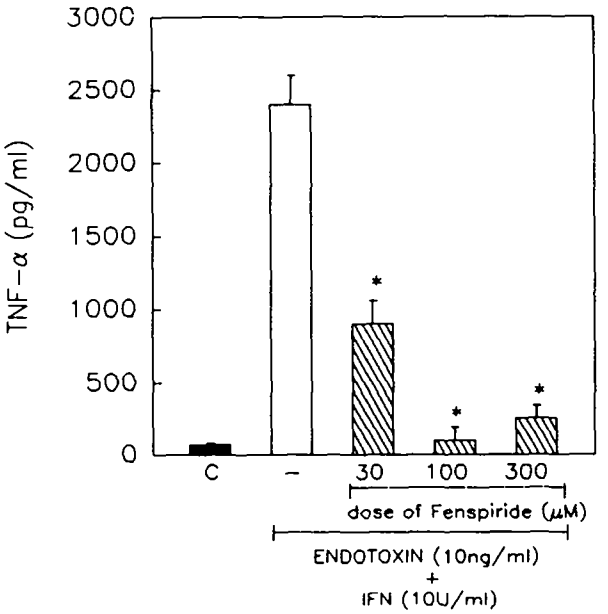

Fig. 4. Interference of fenspiride with tumour necrosis factor release by mice macrophages exposed to endotoxin and interferon γ. The macrophages were pretreated with fenspiride in the doses indicated 30 min before addition of endotoxin plus interferon γ. The first bar represents the spontaneous tumour necrosis factor released (control). The viability of the macrophages determined by trypan blue exclusion was over 85%. The bars represent the mean ± S.E.M. The assay was done in quadruplicate and was repeated twice. Asterisks indicate significant differences when compared with the respective control (untreated macrophages; $P < 0.05$; unpaired Student t-test).

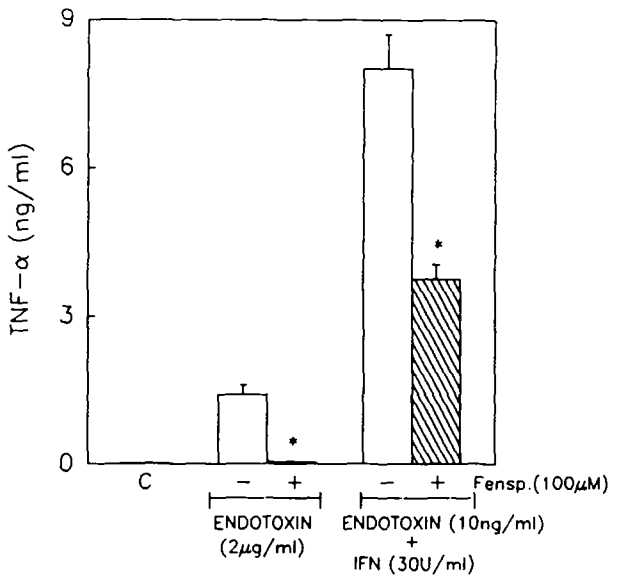

Fig. 5. Interference of fenspiride with tumour necrosis factor release by mice macrophages. Legend as for fig. 4, except that endotoxin alone, 2 μg/ml, induced the release of tumour necrosis factor which was also blocked by fenspiride.

(fig. 4). The release of tumour necrosis factor by macrophages stimulated with endotoxin alone (0.2 or 2 μg/ml) also was inhibited by 90% by fenspiride at concentration of 100 μM (fig. 5)

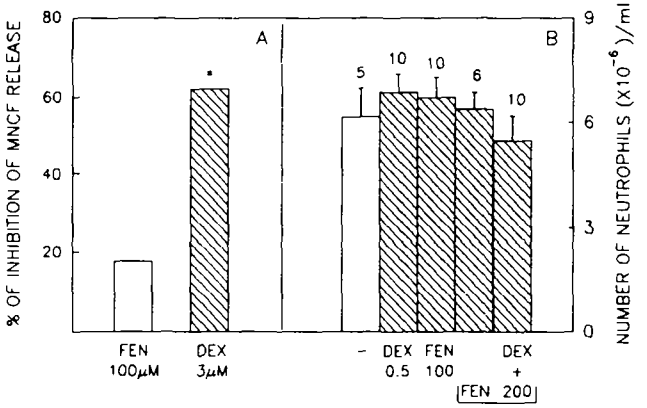

Fig. 6. Interference of fenspiride and dexamethasone with MNCF release by endotoxin-stimulated macrophage monolayer and with neutrophil migration induced by MNCF. Left panel: Percentage of the inhibition by fenspiride (FEN, 100 μM) or dexamethasone (DEX, 3 μM) of the MNCF release. The percentage was obtained by comparison with the neutrophil migration induced by 3 ml of the supernatant obtained by incubating the macrophage monolayer with endotoxin (5 μg/ml). Neutrophil migration was studied in dexamethasone-pretreated rats (0.5 mg/kg, s.c., 1 h earlier). Right panel: Neutrophil migration induced by MNCF in normal rats (open bar) or animals pretreated with dexamethasone (0.5 mg/kg), fenspiride (100 and 200 mg/kg) or fenspiride (200 mg/kg) plus dexamethasone (0.5 mg/kg). The results are means ± S.E.M. and the number of animals tested per group is indicated above each column. Asterisks indicate significant differences when compared with the respective control (untreated rats; $P < 0.05$; unpaired Student t-test).

3.4. Failure of fenspiride to inhibit the effect or the generation of MNCF

In contrast with dexamethasone, which inhibited the MNCF release by endotoxin-stimulated macrophages, fenspiride failed to block MNCF generation in vitro (fig. 6, panel A). However, pretreatment of the rats with dexamethasone with fenspiride or both, did not inhibit neutrophil migration into peritoneal cavities induced by MNCF itself (fig. 6, panel B).

4. Discussion

In the present study we have confirmed that fenspiride inhibits neutrophil migration and exudation into the peritoneal and air pouches cavities as previously described for zymosan and the pleural cavity (Lima et al., 1988). In addition, we showed that fenspiride also inhibits migration and exudation induced by endotoxin, but not by carrageenin. Our results suggest that the antiinflammatory effects of fenspiride on neutrophil migration and protein exudation are due at least in part to the inhibition of tumour necrosis factor release by resident cells.

Indeed, besides that of interleukins 1 and 8, leucotriene B_4 and complement fragments, the participation of tumour necrosis factor in the neutrophil recruitment to the inflammatory site induced by stimuli such as endotoxin and zymosan has been described (Higgs et al., 1981; Adamson and Bowden, 1982; Movat, 1987; Yoshimura et al., 1987).

We previously showed that macrophages stimulated by interleukin 1, tumour necrosis factor-α, interferon γ or endotoxin release a new chemotactic substance, MNCF, into the supernatant (Cunha and Ferreira, 1986; Faccioli et al., 1990; Ribeiro et. at.,1990). In contrast to that of other cytokines derived from macrophages such as interleukins 1 and 8 or tumour necrosis factor (Faccioli et al., 1990; Ribeiro et al., 1991), the ability of MNCF to cause in vivo neutrophil migration in animals is not inhibited by glucocorticosteroids (Cunha and Ferreira, 1986). For this reason, we hypothesized that the chemotactic effect of these cytokines is mediated by the release of the secondary chemotactic factor, as suggested by others (Nakagawa et al., 1989; Harmsen and Havel, 1990). In this study, we also showed that fenspiride does not interfere with MNCF release, ruling out the possibility that it acts via the inhibition of the release of MNCF by endotoxin-stimulated macrophages or via the expression of MNCF chemoattractant activity when injected into the peritoneal cavity. As a positive control, we confirmed that the release of MNCF by endotoxin-stimulated macrophages is blocked by dexamethasone.

The ineffectiveness of fenspiride against carrageenin-induced exudation and neutrophil migration may result from the fact that other inflammatory mediators are released by this stimulus and overshadow the participation of tumour necrosis factor. It is recognized that a distinct array of inflammatory mediators are involved in different inflammatory models or inflammatory diseases. This fact may explain why the various antiinflammatory drugs do not have similar effects on the distinct inflammatory diseases. Fenspiride, for example, seems to be particularly effective in the respiratory tract (Le Douarec et al., 1969).

In conclusion, our results show that the antiinflammatory effects of fenspiride are associated with the antagonism of tumour necrosis factor release by resident macrophages and stress the importance of investigating the potential clinical usefulness of this drug in diseases such as septic shock in which tumour necrosis factor plays an important role.

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