The effect of α-(3,5-di-t-butyl-4-hydroxybenzylidene)-γ-butyro-lactone (KME-4), a new anti-inflammatory drug, on leucocyte migration in rat carrageenan pleurisy

Takayoshi Hidaka*, Kazunori Hosoe, Ikuo Katsumi, Toshiaki Yamashita, Kiyoshi Watanabe, *Biochemical Research Laboratories, Kanegafuchi Chemical Industry Co., Ltd., Takasago, Hyogo 676, Japan*

KME-4,α-(3,5-di-t-butyl-4-hydroxybenzylidene)-γ-butyro-lactone was found to reduce the accumulation of leucocytes and exudate volume in the rat carrageenan pleurisy model. When administered orally 1 h before carrageenan, KME-4 (3–10 mg kg^{-1}) induced a degree of inhibition of leucocyte migration almost equal to that of indomethacin (3–10 mg kg^{-1}) in both 5 h and 24 h pleurisies. Furthermore, KME-4, when administered orally 5 h after the carrageenan, inhibited both monocyte numbers and exudate volume in a 24 h pleurisy and was more effective than indomethacin and BW755c which inhibited only monocyte migration. These results suggest that KME-4 has a differential anti-inflammatory activity. Dexamethasone (0·25 mg kg^{-1}) showed strong inhibition of total cell numbers and exudate volume.

KME-4   (α-(3,5-di-t-butyl-4-hydroxybenzylidene)-γ-butyrolactone) is a non-steroidal anti-inflammatory drug with a new chemical structure (Hidaka et al 1984). Unlike most NSAIDs such as aspirin and indomethacin, which are selective cyclooxygenase inhibitors, this drug has been shown to possess a dual inhibition of prosta-glandin synthetase (cyclooxygenase) and 5-lipoxyge-nase (Hidaka et al 1984, 1985). It has been suggested that the drugs which inhibit both enzymes have a more favourable anti-inflammatory action than selective cyclooxygenase inhibitors (Higgs et al 1981) since the lipoxygenase products (leukotrienes and HETEs) of arachidonic acid are thought to be putative inflam-matory mediators (Samuelsson 1983) as well as prosta-glandins.

To evaluate the anti-inflammatory property of KME-4, we have examined its effect on rat carrageenan pleurisy, a typical inflammatory model with the accu-mulation of leucocytes and exudate (Vinegar et al 1973; Almeida et al 1980).

Materials and methods
Drugs and reagents. KME-4 and BW755c (3-amino-1-(m-(trifluoromethyl)-phenyl]-2-pyrazoline) were syn-thesized in our laboratories. Indomethacin was obtained from Hachidai Pharmaceutical Co.; dexa-methasone from Sigma and lambda (λ)-carrageenan (Picnin A) was from Zushi Chemical Laboratory. All other reagents were of the highest grade commercially available.

Carrageenan pleurisy in rats. Male Wistar rats (Shizuoka Laboratory Animal Center) 10 weeks old and in groups

* Correspondence.

of 6–10 animals were injected intrapleurally with 0·2 ml of sterile 1% λ-carrageenan in 0·9% NaCl (saline) under light ether anaesthesia. All the drugs were suspended in 2·5% arabic gum containing 0·2% polysorbate 80 and administered orally in a volume of 5 ml kg^{-1}, 1 h before or 5 h after the injection of carrageenan. Control animals received vehicle alone similarly.

The pleural exudate was collected at the indicated times and the pleural cavity was washed with 2 ml of phosphate buffered saline/foetal calf serum (1:1 v/v) (Ackerman et al 1980). The exudate volume was recorded and total cell numbers were determined with an automatic cell counter (TOA-CC108). Exudative cells on glass slides were stained with Wright-Giemsa, and differential counts of at least 400 cells per prepara-tion were determined. Animals having an exudate contaminated with red cells were omitted from the experiments.

Statistical analyses were by Student's t-test.

Results
The time course of carrageenan pleurisy in rats. Exudate volume and total and differential counts of leucocytes in the pleural cavity were determined between 5 and 48 h after the intrapleural injection of carrageenan (Fig. 1). Total cell numbers reached a maximum at about 24 h. The exudate volume and neutrophil numbers showed peaks at about 15 and 24 h, respectively, and thereafter decreased, whereas monocytes steadily increased and
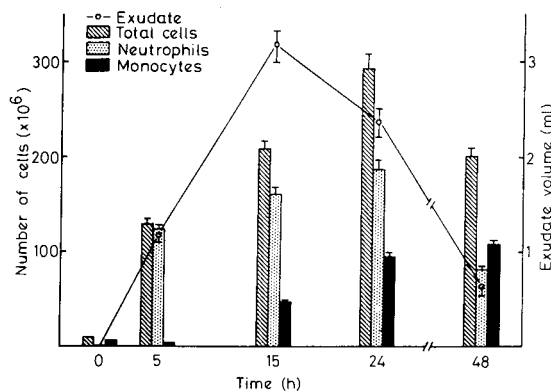

Fig. 1. Time course of accumulation of pleural exudate and leucocytes after the intrapleural injection of carrageenan in rats. The results show the mean ± s.e.m. of values from 9–10 animals.
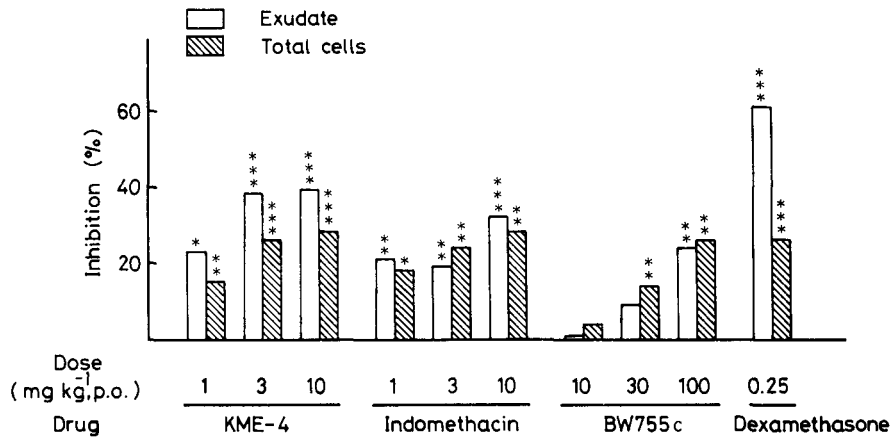
FIG. 2. The effect of KME-4, indomethacin, BW755c and dexamethasone, given orally 1 h before carrageenan, on exudate volume and leucocyte numbers in a 5 h rat carrageenan pleurisy test. Four separate experiments were conducted using 6–10 animals and the results are expressed as percent inhibition of the respective control value. Values (mean ± s.e.m.) for total cell numbers and exudate volume in control groups were from $118.7 \pm 4.0 \times 10^6$ cells (neutrophils 94·4%, monocytes 3·5%) and 0.94 ± 0.05 ml to $152.7 \pm 5.5 \times 10^6$ cells (neutrophils 94·8%, monocytes 3·3%) and 1.30 ± 0.10 ml. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$: Statistically significant difference from the respective control.

became the predominant cells (about 53%) by 48 h. This fluctuation in cellular components and exudation was essentially similar to that reported by Ackerman et al (1980). In the early stage (5 h) of pleurisy, total cell numbers were $129.3 \pm 5.4 \times 10^6$ cells (mean ± s.e.m., n = 10) of which 95·4% were neutrophils, 2·8% were monocytes, 1·8% the remainders (lymphocytes etc), and exudate volume was 1.16 ± 0.06 ml. However, in the late phase (24 h), total cell numbers were $292.1 \pm 16.6 \times 10^6$ cells (mean ± s.e.m., n = 10) of which 64·1% were neutrophils, 32·3% were monocytes, and 3·6%

were remainders, and exudate volume was 2.36 ± 0.15 ml. Under these experimental conditions, the effect of KME-4 on 5 and 24 h carrageenan pleurisies was compared with indomethacin, BW755c and dexamethasone.

Effect on 5 h carrageenan pleurisy. KME-4 (1, 3 and 10 mg kg^{-1}), given orally, 1 h before carrageenan, significantly reduced both exudate volume and total cell counts (about 95% were neutrophils) as did indomethacin (1, 3 and 10 mg kg^{-1}). BW755c at 100 mg kg^{-1}
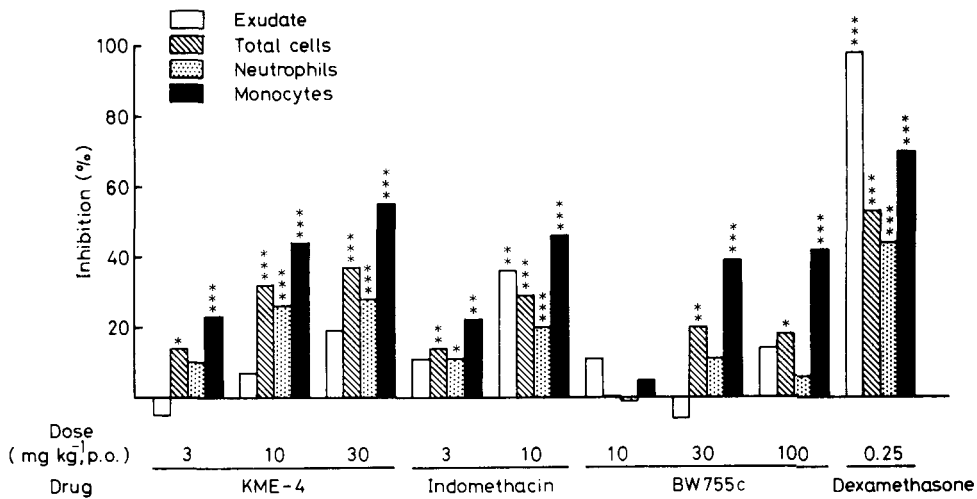

FIG. 3. The effect of KME-4, indomethacin, BW755c and dexamethasone, given orally 1 h before carrageenan, on exudate volume, total and differential leucocyte numbers in a 24 h rat carrageenan pleurisy test. Three separate experiments were conducted using 7–8 animals and the results are expressed as percent inhibition of the respective control value. Values (mean ± s.e.m.) for total cell numbers and exudate volume in control groups were from $216.7 \pm 7.5 \times 10^6$ cells (neutrophils 62·6%, monocytes 35·0%) and 2.44 ± 0.23 ml to $287.2 \pm 10.8 \times 10^6$ cells (neutrophils 64·3%, monocytes 33·7%) and 2.90 ± 0.23 ml. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$: Statistically significant difference from the respective control.

was required to inhibit both exudate volume and total cell counts. Dexamethasone (0·25 mg kg^{-1}) inhibited them, particularly reducing exudate volume by about 60% (Fig. 2).

Effect on 24 h carrageenan pleurisy. KME-4 (3 to 30 mg kg^{-1}) caused a dose-related inhibition of the total number of cells (neutrophils and monocytes), preferentially reducing the monocytes by about 60%, but it had no significant effect on exudate volume at any doses used (Fig. 3). Indomethacin (3 and 10 mg kg^{-1}) reduced the number of neutrophils and monocytes, and exudate volume was inhibited by 10 but not by 3 mg kg^{-1}. BW755c (30 and 100 mg kg^{-1}) caused a significant reduction in monocyte migration but had no effect on either neutrophil numbers or exudate volume. Dexamethasone (0·25 mg kg^{-1}) strongly inhibited differential total cell numbers and exudate volume. As KME-4 showed a relatively strong effect we examined whether it had an inhibitory effect on 24 h pleurisy when administered orally 5 h after carrageenan injection, at which time the inflammatory response is in progress.

KME-4 (10 and 30 mg kg^{-1}) also significantly reduced total cell numbers, monocyte numbers and exudate volume but had little effect on neutrophil numbers. Indomethacin (10 mg kg^{-1}) and BW755c (100 mg kg^{-1}) showed only a weak inhibition of monocyte numbers. Dexamethasone (0·25 mg kg^{-1}) showed strong inhibition of all the parameters in this treatment schedule (Fig. 4).
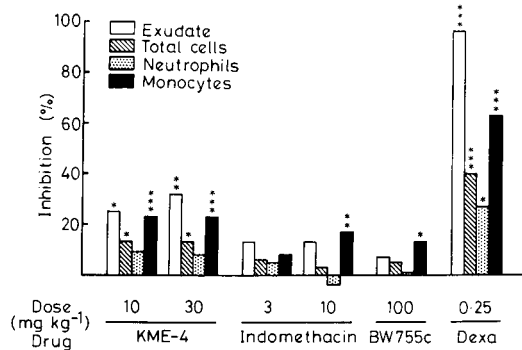

FIG. 4. The effect of KME-4, indomethacin, BW755c and dexamethasone on exudate volume, total and differential leucocyte numbers in a 24 h rat carrageenan pleurisy when given orally 5 h after carrageenan injection. Two separate experiments were conducted using 8–10 animals and the results are expressed as percent inhibition of the respective control value. Values (mean ± s.e.m.) for total leucocyte cell numbers and exudate volume in control groups were 212·5 ± 6·8 × 10^6 cells (neutrophils 63·2%, monocytes 33·4%) and 2·47 ± 0·02 ml, and 237·9 ± 9·5 × 10^6 cells (neutrophils 63·9%, monocytes 32·5%) and 2·88 ± 0·20 ml. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$: Statistically significant difference from the respective control.

Discussion

KME-4 significantly inhibited the accumulation of leucocytes and exudate in rat carrageenan pleurisy whether it was administered orally 1 h before or 5 h after

the injection of carrageenan. NSAIDs and steroidal anti-inflammatory drugs have been reported to have inhibitory effects on leucocyte migration in rat carrageenan pleurisy, although the results with NSAIDs are contradictory (Ammendola et al 1975; Meacock & Kitchen 1976; Almeida et al 1980; Ackerman et al 1980; Miyasaka & Mikami 1982; Bradshaw et al 1984). However, in the present study, no drug (KME-4, indomethacin or BW755c) tested potentiated either exudate volume or leucocyte migration in the three experimental schedules.

While the inhibitory effect of KME-4 on carrageenan pleurisy was similar to that of indomethacin given 1 h before carrageenan, when the drugs were given 5 h after carrageenan, KME-4 was the more effective in inhibiting accumulation of leucocytes and exudate in 24 h pleurisy, although its anti-inflammatory potency is weaker in the rat carrageenan paw oedema and granuloma tests (Hidaka et al 1984). These results suggest that there is a difference in the actions of KME-4 and indomethacin on carrageenan pleurisy. KME-4 is a dual inhibitor of cyclooxygenase and 5-lipoxygenase whereas indomethacin is a selective cyclooxygenase inhibitor. However, the difference in this effect cannot be explained solely by the inhibition of the lipoxygenase pathway of arachidonic acid because we found that indomethacin, to some extent, inhibited the accumulation of monocytes and a similar result was reported by Bradshaw et al (1984).

Higgs et al (1980) and Higgs & Mugridge (1983) have reported that BW755c, which is known as a dual inhibitor of arachidonic acid metabolism, inhibits leucocyte migration in the rat carrageenan sponge model, but indomethacin increases cell accumulation at low doses which inhibit prostaglandin biosynthesis. They therefore suggested that this effect of BW755c is due to the inhibition of lipoxygenase. In the present study, BW755c caused preferential reduction of monocyte numbers in 24 h pleurisy whether administered orally 1 h before or 5 h after the carrageenan although our model is different from that of Higgs et al (1980). A similar result with benoxaprofen has been shown in the same model as ours (Meacock & Kitchen 1979; Yonemoto 1983). Benoxaprofen is also a lipoxygenase inhibitor with a weaker inhibition of prostaglandin synthetase (Cashin et al 1977; Walker & Dawson 1979).

We did not prove the correlation of lipoxygenase products with the induction of leucocyte migration and exudation in this model. However, it has been reported that leukotriene B4, prostaglandins and thromboxane are detected in inflammatory exudate induced by carrageenan (Harada et al 1982; Simmons et al 1983) and it was suggested that these mediators contributed to the inflammatory response. It is therefore possible that the inhibition of leucocyte migration by such drugs may be related, at least in part, to their ability to inhibit not only the cyclooxygenase pathway but also the lipoxygenase pathway.

In the present study dexamethasone showed marked reduction of both exudate volume and leucocyte numbers in all three experimental schedules. This is in agreement with the results of Miyasaka & Mikami (1982) but they did not determine its effect on differential cell counts. It has been postulated that anti-inflammatory corticosteroids exert their anti-inflammatory action through producing phospholipase A2 inhibitory protein in terms of macrocortin or lipomodulin (Flower et al 1984).

We wish to thank Dr H. Fujimura, the President of Kyoto Pharmaceutical University, for valuable advice. We also thank Mr Y. Ariki for his excellent technical assistance.

REFERENCES

Ackerman, N., Tomolonis, A., Miram, L., Kheifets, J., Martinez, S., Carter, A. (1980) J. Pharmacol. Exp. Ther. 215: 588–595

Almeida, A. P., Bayer, B. M., Horakova, Z., Beaven, M. A. (1980) Ibid. 214: 74–79

Ammendola, G., Di Rosa, M., Sorrentino, L. (1975) Agents Actions 5: 250–255

Bradshaw, D., Franz, P. H., Greenham, S. J. (1984) Ibid. 14: 667–672

Cashin, C. H., Dawson, W., Kitchen, E. A. (1977) J. Pharm. Pharmacol. 29: 330–336

Flower, R. J., Wood, J. N., Parente, L. (1984) in: Otterness, I., Capetola, R., Wong, S. (eds) Adv. Inflam. Res. Vol. 7, Raven Press, New York, pp 61–70

Harada, Y., Tanaka, K., Uchida, Y., Ueno, A., Oh-Ishi, S., Yamashita, K., Ishibashi, M., Miyazaki, H., Katori, M. (1982) Prostaglandins 23: 881–895

Hidaka, T., Hosoe, K., Ariki, Y., Takeo, K., Yamashita, T., Katsumi, I., Kondo, H., Yamashita, K., Watanabe, K. (1984) Jap. J. Pharmacol. 36: 77–85

Hidaka, T., Takeo, K., Hosoe, K., Katsumi, I., Yamashita, T., Watanabe, K. (1985) Ibid. 38: 267–272

Higgs, G. A., Mugridge, K. G. (1983) in: Samuelsson, B., Paoletti, R., Ramwell, P. (eds) Advances in Prostaglandin, Thromboxane and Leukotriene Research Vol. 12, Raven Press, New York, pp 19–23

Higgs, G. A., Eakins, K. E., Mugridge, K. G., Moncada, S., Vane, J. R. (1980) Eur. J. Pharmacol. 66: 81–86

Higgs, G. A., Palmer, R. M. J., Eakins, K. E., Moncada, S. (1981) Molec. Aspects Med. 4: 275–301

Meacock, S. C. R., Kitchen, E. A. (1976) Agents Actions 6: 320–325

Meacock, S. C. R., Kitchen, E. A. (1979) J. Pharm. Pharmacol. 31: 366–370

Miyasaka, K., Mikami, T. (1982) Eur. J. Pharmacol. 77: 229–236

Samuelsson, B. (1983) Science 220: 568–575

Simmons, P. M., Salmon, J. A., Moncada, S. (1983) Biochem. Pharmacol. 32: 1353–1359

Vinegar, R., Truax, J. F., Selph, J. L. (1973) Proc. Soc. Exp. Biol. Med. 143: 711–714

Walker, J. R., Dawson, W. (1979) J. Pharm. Pharmacol. 31: 778–780

Yonemoto, K. (1983) Jap. J. Inflam. 3: 51–62

Letters to the Editor

Chronic clenbuterol treatment modulates a 5-hydroxytryptaminergic system

H. FRANCÈS*, P. SIMON, Département de Pharmacologie, Faculté de Médecine Pitié-Salpêtrière, 91, Boulevard de l'Hôpital—F75634 Paris Cedex 13, France

After prolonged or repeated treatment with antidepressants or β-adrenoceptor agonists, resistance to the drugs develops due to hyposensitivity of β_1- (Banerjee et al 1977) and β_2-receptors (Dooley & Hauser 1983). However, we have observed, that antagonism of reserpine-induced hypothermia in mice by clenbuterol (a liposoluble β-adrenoceptor agonist) is, on the contrary, facilitated by chronic treatment (Francès et al 1985), although under the same experimental conditions resistance develops to clenbuterol-induced hypomotility. This observation may be of importance since reserpine-induced hypothermia in mice is generally

considered to be predictive of antidepressant activity in man, and β-adrenoceptor agonists have been successfully used to treat depression (Widlöcher et al 1978). Since 5-hydroxytryptamine (5-HT) is thought to play an important role in depression, we have sought to determine whether a 5-HT system is implicated in the facilitatory effect of chronic clenbuterol treatment on the antagonism of reserpine-induced hypothermia by the β-agonist. 5-HT neurons were lesioned in male, Swiss, NMRI mice (20–24 g) by intracerebroventricular injections of 5,7-dihydroxytryptamine (5,7-DHT) (0·2 mg per mouse, dissolved in 0·01% ascorbic acid), 30 min after i.p. injection of the highly potent inhibitor of noradrenaline reuptake, nisoxetine (20 mg kg^{-1}).

* Correspondence.