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Effects of the non-steroidal anti-inflammatory drug benoxaprofen on leucocyte migration

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Possible modes of action of the anti-inflammatory drug, benoxaprofen, have been explored. The drug caused inhibition of leucocyte migration, principally of mononuclear cells into the pleural cavity of rats undergoing carrageenan-induced pleurisy. Evidence was obtained from in vitro leucocyte migration and chemotaxis models that the drug acted directly on the mononuclear cells rather than by inhibition of chemotactic factors.

Benoxaprofen is a new long acting anti-inflammatory antipyretic drug with some analgesic activity (Cashin et al 1977). Unlike many other non-steroidal acidic anti-inflammatory agents it is only a weak inhibitor of prostaglandin synthesis and therefore is an exception to Vane's (1971) hypothesis that acidic anti-inflammatory compounds owe their activity to inhibition of prostaglandin synthetase. In searching further for a mode of action we have studied the effects of benoxaprofen on the movement of inflammatory cells in both in vivo and in vitro models. Our results indicate that benoxaprofen inhibits cell migration into an inflammatory site and that it appears to act directly on the migrating cells rather than by inhibition of the action of chemotactic factors.

MATERIALS AND METHODS

Animals

Male Lilly Lodge Moor Wistar rats (130–170 g) and male Dunkin Hartley guinea-pigs (500 g) were used.

Benoxaprofen preparations

For administration by mouth, homogenized suspensions of benoxaprofen in 0·5% sodium carboxymethylcellulose with 0·05% Tween 80 were used. In in vitro experiments the drug was dissolved in the minimum dilute alkali at room temperature (22 °C) and quickly adjusted with dilute acid to pH 7·3 before addition to cell cultures.

Cell migration tests

Rat pleurisy. Pleurisy was induced by injecting 1 ml of 6% dextran (Dextran 250, Pharmacia, Uppsala, Sweden) or 0·5 ml of a 1% saline solution of Seakem carrageenan (Lot No. 312505, kindly donated by Marine Colloids Inc., Springfield, U.S.A.) into the pleural cavity as previously described (Meacock & Kitchen 1976) except that ethylenediamine tetracetic acid was replaced by heparin (5 units ml^{-1}) in the saline used to wash the exudate from the pleural cavity. Groups of 8–10 rats were used.

Leucocyte migration in vitro. Details of the glass capillary tube method used, based on that of George & Vaughan (1962), have already been described (Meacock & Kitchen 1976). To examine effects on mononuclear cells, rats were injected i.p. with 2 ml of 1% oyster glycogen (Koch-Light) and peritoneal exudate cells were collected 24 h later. Routine differential cell counts indicated that most were mononuclear cells (60–65%) and the remainder were mainly neutrophils with some eosinophils. Guinea-pig peritoneal cells were used from animals which had been injected i.p. with 15 ml of 1% glycogen 72 h previously. Most of these cells were mononuclear (>85%). In those experiments in which benoxaprofen was tested in vivo, the drug was administered 4 h before collecting the rat or guinea-pig peritoneal cells. Cells were pooled from groups of 4 rats or 3 guinea-pigs before use. In examining effects on polymorphonuclear cells, pleural exudate cells were used from rats which had been injected intrapleurally 4 h previously with 1 ml of rat serum.

Chemotaxis in vitro. The method used was based on that of Wilkinson (1974) using glass tubes (i.d. 0·7 cm) with machined ends to which polycarbonate filters, pore size 5 μm (Nuclepore Corporation) were glued. Peritoneal cells from glycogen stimulated rats were collected in Gey's solution, washed by light centrifugation and resuspended at 5×10^6 leucocytes ml^{-1} in Gey's solution containing benoxaprofen, or in Gey's solution alone. 0·2 ml of each cell suspension was placed in the glass tubes above the

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nuclepore filters and the cells were allowed to migrate towards zymosan-activated serum (Thompson & Rowe 1968) diluted to 5% in Gey's solution or towards Gey's solution alone. In some experiments bovine serum albumin (RIA grade, Sigma) was added to the Gey's solution. In experiments designed to assess the effect of benoxaprofen on chemokinesis (stimulated random movement) alone, zymosan-actived serum was placed in both upper and lower chambers. After 2 h the glass tubes were inverted to remove excess fluid and the filters were fixed in 95% ethanol. After staining the filters with haematoxylin, cells which had migrated to the lower surface of each filter were counted. Counts of both mononuclear and polymorphonuclear cells were made at 630X magnification in 10 fields per filter, using 4 filters per group.

RESULTS

Rat pleurisy

Benoxaprofen was administered orally 1 h before and 6 h after the carrageenan injection and pleural exudates were collected at 24 h. The results of two experiments are given in Table 1. In this and each other Table, significance values were calculated using the means and standard errors of the absolute data before calculating percentage change from control. Infiltration of mononuclear cells into the pleural cavity was reduced in a dose-related manner but the numbers of polymorphonuclear cells or the volumes of total exudate were virtually unaffected. The effect of benoxaprofen on the inflammatory response at an earlier time was also measured. Benoxaprofen (25 and 50 mg kg^{-1}) was administered orally 1 h before injection of either carrageenan or dextran solutions and pleural exudates were collected at 4 h when the cellular exudate consists predominantly of polymorphonuclear cells. Again benoxaprofen had no effect on the volume of exudate in either pleurisy model. The only significant ($P < 0.01$) effect on cell migration was a small reduction in the numbers of polymorphonuclear cells in the dextran pleural exudate at 50 mg kg^{-1} (control 39·2 ± 1·9; drug 29·3 ± 2·6 polymorphs/cavity × 10^6).

Table 1. Anti-inflammatory activity of benoxaprofen in carrageenan-induced rat pleurisy at 24 h.

Dose (mg kg^{-1})	% change in absolute numbers of cells				Av. Vol. of exudate	
	Monos.	P*	Polys	P*	ml	P*
—**					2·9	
2 × 10	−35	<0·01	−5	N.S.	3·2	N.S.
2 × 25	−48	<0·01	−10	N.S.	3·0	N.S.
—***					2·4	
2 × 10	−24	N.S.	+7	N.S.	3·2	N.S.
2 × 25	−40	<0·001	−22	<0·01	2·6	N.S.
2 × 50	−50	<0·001	−5	N.S.	2·2	N.S.

* Student's *t*-test; N.S. = Not significant.
** Mean absolute number/cavity, monos 76·03 ± 6·98 × 10^6, polys 141·73 ± 13·7 × 10^6
*** Mean absolute number/cavity, monos 121·45 ± 9·37 × 10^6, polys 150·56 ± 8·7 × 10^6.

Leucocyte migration in vitro

The results in Tables 2 and 3 indicate that benoxaprofen can reduce the migration of mononuclear cells from capillary tubes in vitro either by administration in vivo before collecting the cells or by addition in vitro before cell migration. Since the rat peritoneal exudate cells also contained some polymorphonuclear cells, the effects of benoxaprofen were evaluated with leucocytes from a 4 h rat serum pleural exudate. This exudate was composed predominantly of polymorphonuclear cells (>90%) and cell viability, as measured by standard dye exclusion techniques, was always >95% at the end of the 18 h migration period. The results in Table 3 show that benoxaprofen suppressed polymorphonuclear cell migration, though it appeared to be less active than against mononuclear cells.

Table 2. Effect of benoxaprofen administered in vivo on the migration of rat and guinea-pig leucocytes in vitro.

Dose (mg kg^{-1})	% reduction* in area of migrating cells	
	Rat cells	Guinea-pig cells
50	36	38
25	29	36
10	18	—

* All results $P < 0.01$.

Chemotaxis

Preliminary experiments, in which benoxaprofen at 30 and 60 μg ml^{-1} was placed in the lower compartment of the chemotaxis chamber without activated serum, established that the drug itself was not chemo-

Table 3. Effect of benoxaprofen on the in vitro migration of rat and guinea-pig leucocytes.

Cell source	Drug (μg ml^{-1})	Rat cells: % reduction in area of migrated cells	Rat cells: P	Guinea-pig cells: % reduction in area of migrated cells	Guinea-pig cells: P
Peritoneal	50	41	<0·001	60	<0·001
	25	33	<0·001	49	<0·001
Pleural	60	32	<0·01	—	
	40	28	<0·01	—	
	30	11	N.S.	—	

tactic for rat peritoneal leucocytes. Furthermore, when benoxaprofen was added to both compartments the numbers of cells found on the lower surface of the filters in the absence of activated serum did not change, indicating that random migration of cells was not significantly affected by the drug during the 2 h experiment. Finally, when benoxaprofen at 30 μg ml^{-1} was added to 5% or 25% activated serum in the lower compartment the chemotactic activity of the serum was not inhibited.

When benoxaprofen at 30 μg ml^{-1} was added to the cells in the upper compartment without prior incubation, the chemotactic response of those cells to 5% activated serum was not changed. However, when the cells were incubated previously with benoxaprofen for 1 h at 37 °C, followed by washing and resuspension in benoxaprofen solution, and with zymosan activated serum in the lower chamber, the chemotactic response of the mononuclear cells to activated serum was significantly inhibited by 56% (Table 4). Under these same experimental conditions, chemokinesis of mononuclear cells was significantly reduced although to a lesser extent (31%) (Table 5). This suggests that the effect of benoxaprofen on the chemotactic response of mononuclear cells is not solely attributable to a reduction in chemokinesis. If the washed cells were resuspended in Gey's solution alone, their chemotactic response was normal, indicating that the drug effect was reversible. Polymorphonuclear cell migration was not always inhibited; it was less than 30% when positive and as with the mononuclear cells was also reversible by washing.

Table 4. Chemotactic response of rat mononuclear cells after incubation with benoxaprofen at 30 μg ml^{-1} for 1 h.

Solutions in upper chamber		Solution in lower chamber	Mean no. of mononuclear cells migrating per field ± s.e.m.	% reduction	P <
Incubated in	Resuspended in				
Gey's	Gey's	Gey's	0·57 ± 0·17		
Benox.	Gey's	Gey's	0.66 ± 0·28	—	N.S.
Benox.	Benox.	Gey's	0·53 ± 0·18	—	N.S.
Gey's	Gey's	ZAS	39·6 ± 2·53		
Benox.	Gey's	ZAS	30·8 ± 3·76	—	N.S.
Benox.	Benox.	ZAS	17·4 ± 1·12	56	0·001

Table 5. Effect of benoxaprofen on the chemotaxis and chemokinesis of mononuclear cells.

Contents of:		Mean no. of mononuclear cells migrating per field ± s.e.m.	% reduction	P <
upper chamber	lower chamber			
Gey's	ZAS	27·8 ± 3·6		
Benox.*	ZAS	13·2 ± 1·2	52	0·01
ZAS	ZAS	15·0 ± 0·7		
Benox.* + ZAS	ZAS	10·3 ± 0·6	31	0·01

*Benoxaprofen concentration 30 μg ml^{-1}

Rivkin (1977) has previously reported that the addition of albumin to leucocytes used to investigate chemotaxis has a major effect in removing the apparent inhibition due to the addition of anti-inflammatory drugs. Rat peritoneal exudate leucocytes were therefore incubated with benoxaprofen as above in the presence of different concentrations of bovine serum albumin and their chemotactic response to activated serum was measured. The results obtained are illustrated in Fig. 1. Increasing the concentration of albumin to 2% was found to increase the chemotactic response of the mononuclear cells but the effects on the polymorphonuclear cells were not statistically significant. Benoxaprofen inhibited the chemotaxis of the mononuclear cells in the presence of 0·1–2% serum albumin but inhibition was reduced from 36 to 14% with increasing concentrations of the albumin; the chemotaxis of polymorphonuclear cells by benoxaprofen was reduced only in 1% (29·5%) and 2% albumin (16%).

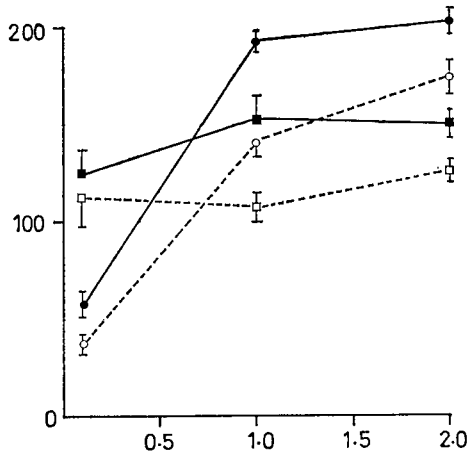

FIG. 1. Effect of benoxaprofen (30 μg ml^{-1}) on the chemotactic response of rat leucocytes to activated serum in the presence of bovine serum albumin. ● mononuclear cells, albumin; ○ mononuclear cells, albumin + drug; ■ polymorphonuclear cells, albumin; □ polymorphonuclear cells, albumin + drug. Vertical bars, ± s.e.m. Abscissa: concentration of bovine serum albumin (%). Ordinate: number of leucocytes per field.

Blood leucocyte counts

The results in Table 6 show that the absolute counts of the different blood leucocytes remained unaffected

Table 6. Blood leucocyte counts (10^3 per mm^3) of male Wistar rats after treatment by mouth with benoxaprofen for 1 month at 40 mg kg^{-1} day^{-1}.

Lymphocytes	Monocytes	Neutrophils	Eosinophils
Control			
7·36 ± 0·77	0·41 ± 0·07	1·61 ± 0·09	0·08 ± 0·03
Benoxaprofen			
6·19 ± 0·26	0·42 ± 0·06	1·73 ± 0·28	0·09 ± 0·04

after daily treatment with benoxaprofen for 1 month. It was concluded that inhibition of leucocyte migration into inflammatory lesions by benoxaprofen was not due to inhibition of leucocyte synthesis or inhibition of migration from the bone marrow.

DISCUSSION

Anti-inflammatory activity has at various times been associated with lysosomal membrane stabilization (Ignarro 1971), lysosomal enzyme inhibition (Anderson 1968), inhibition of mononuclear leucocyte migration (Di Rosa et al 1971) and inhibition of prostaglandin synthetase (Vane 1971). The results of the present study indicate that the anti-inflammatory drug benoxaprofen, which is only a weak inhibitor of prostaglandin synthetase, is able to inhibit the movement of leucocytes in vivo and in vitro. In carrageenan pleurisy models benoxaprofen inhibited the migration of mononuclear cells into the pleural cavity but had little or no effect on the numbers of migrating polymorphonuclear cells and no effect on the volume of oedema fluid. In the milder dextran pleurisy model inhibition of migration of polymorphonuclear cells was only apparent at 50 mg kg^{-1}. The numbers of migrating mononuclear cells were too few in this model for drug effects to be meaningful. More potent inhibitors of prostaglandin synthetase, such as aspirin, fenoprofen and naproxen, had no significant effect on mononuclear cell migration (Meacock & Kitchen 1976), and therefore it is unlikely that ability to inhibit prostaglandin synthesis and inhibition of leucocyte migration are directly related. A similar conclusion was reached by Ford-Hutchinson et al (1977) who found no relationship between the effects of non-steroidal anti-inflammatory drugs on leucocyte migration and prostaglandin accumulation in implanted sponges. The latter workers also observed inhibition of leucocyte migration in benoxaprofen-treated animals but at the lower dose level of 9 mg kg^{-1}. Their model of inflammation was clearly different and presumably milder than our carrageenan pleurisy model, since some 40-fold fewer leucocytes were collected in their sponges, and it is likely, therefore, that a lower dose was able to control the inflammatory reaction more readily, as was apparent to some extent in our dextran pleurisy model.

Evidence that benoxaprofen was acting directly on the migrating cells came from studies of leucocyte migration from capillary tubes in vitro and from experiments which measured leucocyte chemotaxis through nuclepore membranes. Using peritoneal exudate cells from rats or guinea-pigs which had received benoxaprofen, leucocyte migration from capillary tubes was significantly inhibited (Table 2). Addition of the drug to the culture medium at concentrations which are achieved in plasma after oral administration (Chatfield & Green 1978) also inhibited mononuclear cell migration (Table 3). Inhibition of polymorphonuclear cell migration however, was distinctly less, which was in agreement with the pleurisy results.

Benoxaprofen inhibited the chemotaxis of rat macrophages towards zymosan-activated serum but had little or no effect on the movement of polymorphonuclear cells. Specific studies have shown that this inhibition of chemotaxis is not solely attributable to a reduction in chemokinesis. The effect on macrophages was only seen if the cells were incubated for 1 h before the chemotaxis experiments and normal migratory behaviour returned if the drug was removed before exposure to the chemotaxin (Table 4). Incubation of zymosan-activated serum with benoxaprofen before exposure to untreated leucocytes did not affect its chemotactic activity.

The reduction in benoxaprofen-induced changes in the chemotaxis of mononuclear cells in the presence of serum albumin raises questions about the relevance of observations made in vitro with regard to the mode of action of benoxaprofen in vivo. Much of the apparent loss in drug activity may be attributed indirectly to the large stimulation of the chemotactic response of the mononuclear cells in the in vitro system. To what extent this may also occur in vivo is not known. In addition, as Rivkin (1977) has pointed out with respect to other anti-inflammatory drugs, binding by benoxaprofen to the serum albumin may render the drug less accessible to the cells during the short period of the in vitro experiments. On the other hand exposure of leucocytes for a longer period in the presence of 10% horse serum in the migration studies from capillary tubes did not prevent drug inhibition and therefore the roles of protein and drug binding in the regulation of cell movement have not yet been resolved.

In conclusion, these studies show that the inhibition of mononuclear cell migration into sites of

inflammation may be an important contribution to the anti-inflammatory action of benoxaprofen in chronic inflammation. In addition, the maintenance of blood leucocyte counts after long-term dosing and the minimal effects seen on polymorphonuclear cell migration suggest that both leucocyte synthesis and host defence to microbial attack will remain unimpaired during treatment.

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