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# The effect of a novel, non-steroidal anti-inflammatory compound, nabumetone (BRL 14777), on cellular infiltration into 24-hour polyvinyl sponge implants in the rat, compared with some steroidal and non-steroidal anti-inflammatory drugs

ELIZABETH A. BOYLE AND F. R. MANGAN*

*Beecham Pharmaceuticals Research Division, Medicinal Research Centre, Coldharbour Road, The Pinnacles, Harlow, Essex, CM19 5AD, U.K.*

The time-course of cell migration into saline-soaked sponge implants over 5 days showed peak polymorphonuclear leucocyte (PMNL) infiltration at 24 h. In common with the corticosteroids dexamethasone and hydrocortisone, and the non-steroidal anti-inflammatory drugs indomethacin, (+)-naproxen, BW 755C and benoxaprofen, nabumetone decreased cell migration into the sponges. PMNLs and mononuclear cells were reduced at 24 h, and there was a parallel decrease in exudate levels of the lysosomal acid hydrolase β-*N*-acetyl glucosaminidase [NAG ECB, 3, 2, 1, 30]. Impregnation of sponges with λ-carrageenan (1%) caused a 2–3 fold increase in cell numbers, with a relatively greater proportion of PMNLs; drug effects were more marked in these implants.

One of the actions of the glucocorticoids affects cell accumulation in inflammation. Although effects on chemotaxis are controversial, the decreased migration of PMNLs and monocytes to the site of inflammation is well-documented (Rinehart et al 1974; Fauci et al 1976; Katler & Weissmann 1977).

Recently, attention has been focused on the effects of the non-steroidal anti-inflammatory drugs (NSAIDs), particularly indomethacin, on cell migration and leucocyte prostaglandin production during the acute phase of the inflammatory response (McCall & Youlten 1973, 1974; Blackham & Owen 1975; Walker et al 1976; Ford-Hutchinson et al 1977; Brown & Collins 1978; Higgs et al 1979).

In view of the interesting pharmacological profile of nabumetone (Boyle et al 1978; Mangan et al 1981), with good activity in the cotton pellet granuloma test without associated toxicity, and lack of gastric irritancy, we have compared it with both steroidal and NSAIDs using cellular infiltration into polyvinyl sponge implants over the course of 5 days, and the effect of the drugs on the 24 h PMNL peak. We have also compared the cell contents of 0·9% NaCl(saline)-soaked and carrageenan-impregnated sponges at 24 h in control and drug-treated animals.

## MATERIALS AND METHODS

Hydrocortisone, dexamethasone, bovine serum albumin, calf thymus DNA and *p*-nitrophenyl β-D-*N*-acetylglucosaminidine (NAG) were obtained from Sigma Chemicals. Indomethacin (Merck, Sharpe and Dohme), benoxaprofen (Lilly), BW 755C (Wellcome Research Laboratories) and (+)-naproxen (Syntex) were generous gifts. Trypsin, Hanks buffered saline (HBS) and Eagles medium 199 were from Flow Laboratories; Valium (Roche) and Hypnorm (Janssen) were used for anaesthesia. Carrageenan was prepared from a sample of Marine Colloids carrageenan by Mr. Verrall (B.P.R.D., Brockham Park). Polyvinyl sponges were cut from Declon 49 (Declon Plastics Ltd.) and prepared as previously described (Boyle & Mangan 1980). Carrageenan-impregnated sponges were prepared by soaking the sponges in 1% carrageenan, drying overnight at 60 °C, then sterilizing and soaking them in sterile saline before implantation.

Wistar strain rats (Charles River or OLAC), 140–180 g were housed 5 per cage, with free access to food (FFGM Rat and Mouse Diet, Dixons of Ware) and water. Females were used for the initial time-course and males for subsequent experiments (unpublished observations had shown no sex difference in the time-course of cellular infiltration into the implanted sponge).

Compounds were suspended in methyl cellulose (0·7%) and dosed orally to groups of between 6 and 10 rats; controls received vehicle alone. One hour after dosing on day 0, rats were anaesthetized, and pairs of sterile sponges were implanted ventrolaterally as described by (Freeman et al 1979).

* Correspondence.

*Methods*

In experiments to determine DNA and NAG levels, animals were killed by cervical dislocation the sponges removed, placed on ice and within 30 min transferred to plastic cones and centrifuged for 20 min at 4 °C (1500 rev $min^{-1}$ in an MSE Mistral 4L). After the volume had been recorded, the exudate was kept at 0 °C before NAG determination. Ice-cold 0·5% perchloric acid was added to the residual sponge and cell pellet, and DNA levels determined in duplicate by the diphenylamine method of Burton (1956) after extraction at 95 °C for 15 min. Extinction was measured at 595 nm and results recorded as μg DNA per two sponges. Duplicate NAG levels were assayed manually by the method of Beck & Tappel (1968), and extinction measured at 405 nm. Results were recorded as units (nmol of NAG hydrolysed $h^{-1}$) per exudate and units per exudate $\mu g^{-1}$ DNA.

Where total and differential cell counts were required, sponges were excised and placed in 1 ml aliquots of ice-cold 0·5% trypsin in HBS at pH 7·4, as described by Ford-Hutchinson et al (1978). They were incubated at 37 °C for 20 min, gently squeezed, and the medium transferred to plastic centrifuge tubes. The sponges were washed in HBS (0·5 ml), and the wash added to the medium. The sponges were placed in plastic cones and centrifuged for 10 min at 1500 rev $min^{-1}$.

The cell pellets were resuspended by vortexing in appropriate volumes (100 or 200 μl) of 30% bovine serum albumin (BSA), and aliquots (50 μl) were diluted 1/10 in Eagles: HBS containing 0·1% Trypan Blue for counting in a standard Neubaeur haemocytometer. Viability was always >99%.

Cell smears were made from the BSA suspension, immediately air-dried and fixed in methanol. They were stained with either May-Grunwald: Giemsa or Wrights Stain, and 200 cells counted to obtain the percentage of PMNL's and mononuclear cells in each sample.

## RESULTS

*Time course of cellular infiltration into the implanted polyvinyl sponge*

In an initial experiment, saline-soaked sponges were implanted in groups of 6 female rats on day 0, then removed on days 1, 2, 3, 4 and 5. Sponges were trypsinized, and the subsequent cell pellet assayed for DNA content and total cell counts. DNA levels were determined on the residual sponges to obtain total DNA and hence the percentage of cells recovered by trypsinization.

The results (Fig. 1) showed that cell infiltration peaked on day 1, and fell off steadily until day 5; cell recovery was between 70 and 90% during this period.

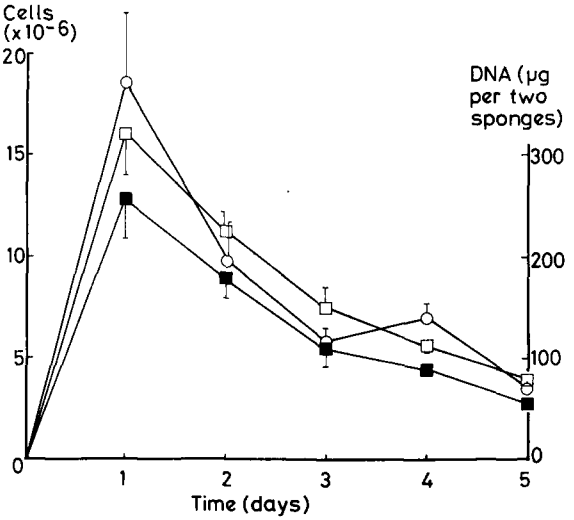


FIG. 1. The DNA content and total cell counts from trypsinized polyvinyl sponges 1–5 days after implantation, compared with total sponge DNA (means ± s.e.m.). ○ Total cell counts (× $10^{-6}$). □ Total sponge DNA (μg/2 sponges). ■ DNA content of cell suspension.

In a more detailed experiment, sponges were implanted in groups of six female rats on day 0, and removed at 6, 12, 18, 24, 30, 36 and 48 h, then daily until day 8. Sponges were trypsinized, and total and differential cell counts obtained. In addition, DNA levels were measured in sponges from a further two animals on days 4 to 8, to confirm our previous findings of a steep increase in DNA from days 5 to 9, during the proliferative phase of granuloma development (Boyle & Mangan 1980). The results are in Fig. 2.

The classical PMNL peak occurred at 24 h, and the mononuclear cells at this stage had the morphological appearance of monocytes, with small cytoplasmic: nuclear ratios; there were few lymphocytes.

By day 3 in this experiment, the predominant cells were mononuclear, and had the appearance of macrophages, with increased cytoplasmic: nuclear ratios; often there were phagocytosed erythrocytes. Some binucleate cells were present, as well as the occasional strongly basophilic spindle-shaped cell, presumably a fibroblast. There were very few eosinophils and mast cells.

It proved impossible to remove and identify more than a small percentage of the cells after day 5 where total DNA levels increased steeply, but the cell

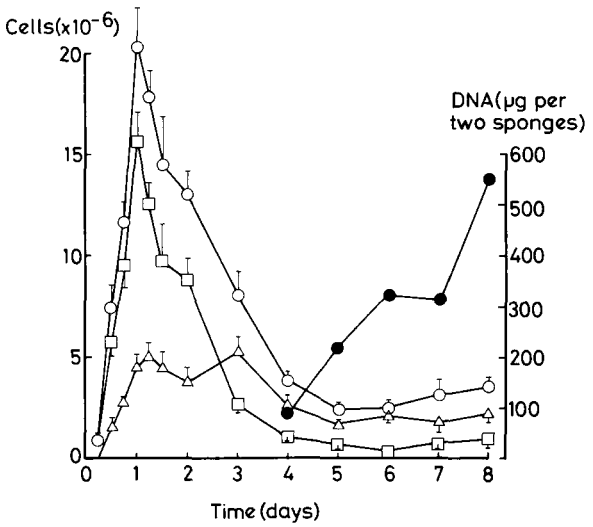


FIG. 2. The time-course of cellular infiltration into implanted polyvinyl sponges over 8 days: total and differential cell counts (means ± s.e.m.). Total DNA levels from days 4–8 are included (means from 2 rats day$^{-1}$). ○ Total cell counts (× 10$^{-6}$). □ PMNL (× 10$^{-6}$). △ Mononuclear cells (× 10$^{-6}$). ● μg DNA (per 2 sponges).

counts remained low. Of these cells, the macrophages had greatly increased cytoplasmic:nuclear ratios, were often strongly basophilic, and multinucleate giant cells were present.

*The effect of nabumetone at 24 h in the saline-soaked sponge, compared with hydrocortisone, dexamethasone and some NSAID's*

Groups of 6, 7 or 8 male rats were dosed orally with dexamethasone (45 μg kg$^{-1}$), hydrocortisone (10 mg kg$^{-1}$), indomethacin (3 mg kg$^{-1}$), (+)-naproxen (20 mg kg$^{-1}$), benoxaprofen (40 or 50 mg kg$^{-1}$), nabumetone (50 mg kg$^{-1}$), or vehicle alone (0·7% methylcellulose) 1 h before implantation. They received a further dose 6–8 h later, and were killed exactly 24 h after implantation.

DNA levels were determined in the sponge, and NAG levels in the exudates; the results are in Table 1. Cell migration was depressed in all the drug-treated groups, although the decrease in DNA was not always significant because of the wide variation in control values. Decreased exudate NAG levels paralleled the reduction in cell numbers, and the NAG release μg$^{-1}$ DNA did not change significantly in any group.

Where total and differential cell counts were done, experiments were split into three parts, each with a control and two test compounds, dosed as above. The results are in Table 2. PMNL's were significantly decreased in all the drug-treated groups, but effects on mononuclear cells were more marginal. There was some variation in the percentage of PMNL's in the three experiments.

*Dose-response to nabumetone: effects on cell migration into the saline-soaked sponge at 24 h*

Groups of 16 rats were dosed with nabumetone at 150, 50, or 16·5 mg kg$^{-1}$, or vehicle alone, 1 h before implantation; they received a further dose 6–8 h later, and were killed exactly 24 h after implantation.

DNA levels were measured in sponges from eight animals in each group, and total and differential counts determined in sponges from the remaining 8 animals. The results (Fig. 3) show a dose-related decrease in DNA (top slope) and in total cell counts (bottom slope). There did not appear to be a preferential effect on PMNLs, confirming the results in Table 2.

*Comparison of the cell content of saline-soaked and carrageenan-impregnated sponges 24 h after implantation*

Groups of 10 Olac Wistar rats were implanted with either saline-soaked or carrageenan-impregnated saline-soaked sponges, and were killed after exactly 24 h. Total and differential cell counts were obtained after trypsinization. Table 3 shows that there was a 2–3 fold increase in total cell counts, and in the

Table 1. The effect of nabumetone on DNA/NAG content of 24 h sponge implants compared with steroidal and non-steroidal anti-inflammatory drugs.

| | Compound | Dose | DNA μg/2 sponges | NAG units/ exudate | NAG units μg$^{-1}$ DNA |
|---|---|---|---|---|---|
| I | Methylcellulose | 0·7% | 252·0 ±44·95 | 368·2 ±35·7 | 1·60 ±0·19 |
| | Dexamethasone | 45 μg kg$^{-1}$ | **107·6 ±17·00 | ****169·2 ±16·8 | 1·69 ±0·19 |
| | Hydrocortisone | 10 mg kg$^{-1}$ | **103·0 ±25·6 | *221·4 ±40·5 | 2·40 ±0·38 |
| II | Methylcellulose | 0·7% | 271·6 ±21·4 | 484·4 ±25·5 | 1·92 ±0·14 |
| | Dexamethasone | 45 μg kg$^{-1}$ | **174·4 ±24·6 | ****299·5 ±22·1 | 1·90 ±0·24 |
| | Hydrocortisone | 10 mg kg$^{-1}$ | 230·6 ±19·0 | ****356·2 ±12·7 | 1·66 ±0·15 |
| | Indomethacin | 3 mg kg$^{-1}$ | 217·1 ±20·6 | *404·8 ±20·5 | 2·01 ±0·18 |
| | (+)-Naproxen | 20 mg kg$^{-1}$ | **174·4 ±26·0 | ****292·3 ±24·6 | 1·84 ±0·22 |
| | Benoxaprofen | 40 mg kg$^{-1}$ | *166·3 ±34·7 | ****312·8 ±24·5 | 2·28 ±0·37 |
| | Nabumetone | 50 mg kg$^{-1}$ | **192·3 ±17·5 | ****321·2 ±18·9 | 1·72 ±0·09 |

Figures represent means ± s.e.m.
Significantly different from controls as assessed by Student's *t*-test.
*$P$ <0·05, **$P$ <0·02, ****$P$ <0·001.

Table 2. The effect of nabumetone on cell infiltration at 24 h in saline-soaked sponges compared with dexamethasone, hydrocortisone and some non-steroidal anti-inflammatory drugs.

| Group | Dose (per kg) | Total cell counts ($\times 10^6$) | PMNL % | PMNL $\times 10^6$ | Mononuclear cells % | Mononuclear cells $\times 10^6$ |
|---|---|---|---|---|---|---|
| Methylcellulose | | 20·5 ±1·8 | 74 ±1 | 15·1 ±1·3 | 26 ±1 | 5·3 ±0·6 |
| Hydrocortisone | 10 mg | ****8·4 ±1·3 | 74 ±4 | ****6·4 ±1·3 | 26 ±4 | ****1·8 ±0·2 |
| Nabumetone | 50 mg | ***11·0 ±2·2 | 73 ±2 | ***8·3 ±1·8 | 26 ±3 | ***2·7 ±0·5 |
| Methylcellulose | | 15·3 ±1·8 | 77 ±2 | 11·6 ±1·3 | 23 ±2 | 3·7 ±0·7 |
| Indomethacin | 3 mg | ***6·6 ±1·6 | 78 ±1·6 | ***5·1 ±1·2 | 21 ±1 | *1·5 ±0·4 |
| Benoxaprofen | 50 mg | ***6·7 ±2·0 | 67 ±8 | **5·2 ±2·1 | 33 ±8 | 1·9 ±0·4 |
| Methylcellulose | | 22·0 ±2·0 | 87 ±1 | 18·1 ±1·8 | 13 ±1 | 3·0 ±0·4 |
| Dexamethasone | 45 μg | ****4·8 ±0·6 | ****53 ±3 | ***2·6 ±0·4 | ****42 ±3 | 2·3 ±0·2 |
| (+)-Naproxen | 20 mg | 16·4 ±2·0 | 82 ±3 | **11·8 ±1·6 | 18 ±3 | 2·9 ±0·7 |

Significantly different from control levels as assessed by Student's *t*-test.
*$P$ <0·05, **$P$ <0·02. ***$P$ <0·01, ****$P$ <0·001.

number of PMNLs in response to carrageenan, with a highly significant increase in the percentage of PMNLs. Mononuclear cell numbers were also increased, but the effect was less marked.

*The effect of nabumetone at 24 h in the carrageenan-impregnated sponge compared with some NSAID's*

Groups of 10 Olac Wistar rats were dosed orally with indomethacin (2 mg kg$^{-1}$), benoxaprofen (50 mg kg$^{-1}$), BW 755C (50 mg kg$^{-1}$) or nabumetone (50 mg kg$^{-1}$) or vehicle alone, 24 h before and again 1 h before implantation on day 0, and killed after exactly 24 h. Total and differential cell counts were obtained on the trypsinized sponges; the results

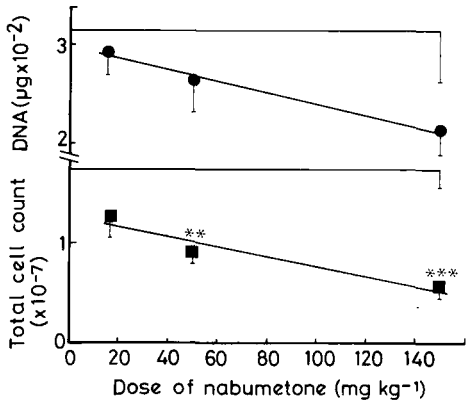


FIG. 3. Dose-response to nabumetone at 24 h. (means ± s.e.m.). ● Total DNA (μg/2 sponges). ■ Total cell counts (× 10$^{-7}$). ** $P$ <0·01, *** $P$ <0·001.

are in Table 4 (control values are in Table 3). All four compounds markedly reduced total and PMNL cell numbers, and in three instances the mononuclear cells. The decrease in mononuclear cells in response to BW 755C was not significant.

Table 3. Comparison of the cell content of saline-soaked and carrageenan-impregnated sponges 24 h after implantation. The results of three separate experiments.

| Group | Total cells $\times 10^6$ | PMNLs % | PMNLs $\times 10^6$ | Mononuclear % | Mononuclear $\times 10^6$ |
|---|---|---|---|---|---|
| 1. Saline | 9·8 ±1·0 | 74 ±3 | 7·3 ±0·8 | 26 ±3 | 2·6 ±0·4 |
| *Carrageenan* | ****26·4 ±2·6 | ***84 ±2 | ****22·1 ±2·2 | ***16 ±2 | *4·3 ±0·6 |
| 2. Saline | 11·1 ±0·7 | 74 ±2 | 8·0 ±0·5 | 26 ±2 | 2·9 ±0·3 |
| Carrageenan | ****28·2 ±2·4 | ****84 ±1 | ****23·9 ±2·1 | ****16 ±1 | ***4·3 ±0·4 |
| 3. Saline | 19·3 ±1·2 | 75 ±2 | 14·4 ±1·3 | 24 ±2 | 4·6 ±0·3 |
| Carrageenan | ****39·3 ±3·1 | ****86 ±1 | ****33·8 ±2·8 | ****14 ±1 | 5·5 ±0·6 |

Significantly different from saline soaked sponge values as assessed by Student's *t*-test.
*$P$ <0·05, ***$P$ <0·01, ****$P$ <0·001.

Table 4. The effect of nabumetone on the cell content of carrageenan-impregnated sponges at 24 h compared with some NSAIDs.

| Group | Dose (mg kg$^{-1}$) | Total Cell Count ($\times 10^6$) | PMNLs % | PMNLs $\times 10^6$ | Mononuclear Cells % | Mononuclear Cells $\times 10^6$ |
|---|---|---|---|---|---|---|
| Methyl-cellulose | | 28·2 ±2·4 | 84 ±1 | 23·9 ±2·1 | 16 ±1 | 4·3 ±0·4 |
| Nabumetone | 50 | ***18·0 ±1·1 | 85 ±1 | ***15·2 ±1·1 | 16 ±1 | ****2·0 ±0·2 |
| Benoxaprofen | 50 | ****11· ±1·2 | 88 ±2 | ****10·0 ±1·1 | 12 ±2 | ****1·4 ±0·2 |
| Methyl-cellulose | | 26·4 ±2·6 | 84 ±2 | 22·1 ±2·2 | 16 ±2 | 4·3 ±0·6 |
| Indomethacin | 2 | ****13·5 ±1·2 | 83 ±2 | ****11·1 ±1·1 | 18 ±2 | ***2·3 ±0·3 |
| Methyl-cellulose | | 39·3 ±3·1 | 86 ±1 | 33·8 ±2·8 | 14 ±1 | 5·5 ±0·6 |
| BW 755† | 50 | ***24·0 ±3·8 | 86 ±1 | ***20·4 ±3·2 | 14 ±1 | 3·5 ±0·7 |

Significantly different from controls as assessed by Student's *t*-test.
*** $P$ <0·01, **** $P$ <0·001.
† Freshly made up just before dosing. (3-Amino-1-[(m-trifluoromethyl)phenyl]-2-pyrazoline.

## DISCUSSION

The time-course of cellular infiltration into the saline-soaked sponge differs from that described by Higgs (1979) in his carrageenan-impregnated sponges. He describes a low plateau of leucocytes from 4 to 48 h, followed by a steep and steady increase in cell numbers. We found a very steep increase in PMNL infiltration, accompanied by mononuclear cells, which reached a peak at about 24 h after implantation, and then fell sharply, to be followed by a much shallower plateau around 3–4

days, predominantly of macrophages. Our preliminary comparisons with carrageenan-impregnated sponges at 24 h showed an increase in the total number of cells, with a greater proportion of PMNLs. The pattern of cell infiltration into our saline-soaked sponge implants is similar to that described by Freeman et al (1979) in cotton pellet implants, where PMNLs predominated at 48 h (they did not look at 24 h pellets), and macrophages predominated at 4 days.

It proved impossible to remove and identify more than a small percentage of the cells after day 5, presumably because of the rapid growth of the granuloma at this stage. We have recently reported the steep increase in DNA and collagen content, with concomitant cell proliferation, between days 5 and 9 (Boyle & Mangan 1980), and trypsinization obviously cannot remove the entrapped cells.

There is some doubt whether or not the glucocorticoids inhibit chemotaxis in vivo, but it is widely acknowledged that they do decrease cell migration in response to inflammation. A review by Fauci et al (1976) summarizes the literature. In our experiments, hydrocortisone and dexamethasone decreased infiltration of both PMNLs and mononuclear cells with the saline-soaked sponge, although hydrocortisone was much less effective than dexamethasone.

Glucocorticoid-induced inhibition of phagocytosis and lysosomal enzyme secretion by PMNLs in vitro has been reported (Mandell et al 1970; Stossel et al 1972), and their experiments were thought to support the concept of steroid-induced membrane stabilization. Recent evidence no longer supports that hypothesis, and Ignarro & Cech (1975) and Weissmann et al (1975) have implicated an effect on the cyclic nucleotides. Ignarro & Cech (1975) showed that hydrocortisone inhibited phagocytosis and the release of β-glucuronidase from human PMNLs in vitro via the reduction of cyclic GMP and inhibition of the influx of calcium.

We found that although NAG release was decreased in the steroid-treated groups, this decrease paralleled the decrease in cell numbers, since the NAG release per cell was the same as that of the controls. Thus, there was no indication of in vivo membrane stabilization, nor reduced phagocytosis in the predominantly PMNL population.

There are reports on the suppression of leucocyte migration by NSAIDs (Blackham & Owen 1975; Walker et al 1976; Ford-Hutchinson et al 1977; Brown & Collins 1978; Higgs 1979; Higgs et al 1979). Most of the in vivo work does not differentiate between the cell types, but Blackham & Owen (1975), using a model of carrageenan-induced pleurisy in the rat, showed that indomethacin and (+)-naproxen inhibited PMNL infiltration, but not that of the mononuclear cells. Warne & West (1978) showed that indomethacin at doses greater than 2·5 mg $kg^{-1}$ inhibited both PMNL and mononuclear cell infiltration into the pleural space in response to carrageenan. We have shown an unequivocal reduction in PMNL infiltration into both saline-soaked and carrageenan-impregnated sponge implants in response to NSAIDs; effects on the mononuclear cells were less marked and more variable, especially in the saline-soaked model. So far we have been unable to confirm the stimulatory effect of low doses of indomethacin on leucocyte migration reported by Eakins et al (1980a,b), as we still obtained maximal inhibition at 0·5 mg $kg^{-1}$ (unpublished observation).

Meacock & Kitchen (1979) and Dawson (1980) have suggested that benoxaprofen has a different mode of action from other NSAIDs in that it selectively inhibits mononuclear cell accumulation. In our experiments benoxaprofen was as effective as other NSAIDs in reducing the initial PMNL infiltration and no more effective than the other drugs in reducing monocyte numbers. A recent report by Smith & Iden (1980) on the effect of NSAIDs on in vitro enzyme release from stimulated human PMNL showed that, in common with indomethacin and other drugs, benoxaprofen inhibited the release of β-glucuronidase and lysozyme in a dose-dependent manner.

We found that the NSAIDs, including benoxaprofen and nabumetone, caused a decrease in the release of lysosomal NAG into the sponge exudate, and, like the steroids, this paralleled the decrease in DNA.

Ford-Hutchinson et al (1977), Walker et al (1978) and Higgs et al (1979) have shown that most NSAIDs only partially reduce cell migration at doses causing complete inhibition of prostaglandin synthesis. Those authors concluded that effects on leucocyte migration were not directly related to effects on prostaglandin synthesis, and indeed, Eakins et al (1979, 1980) reported significantly increased cell numbers in carrageenan-soaked sponges at concentrations of NSAIDs causing partial inhibition of prostaglandin synthesis. Nabumetone is a relatively weak prostaglandin synthetase inhibitor (Boyle et al 1982), and in this respect possibly compares with benoxaprofen which is reported to inhibit white cell accumulation in 9 h sponge exudates only at concen-

trations required to inhibit prostaglandin synthesis (Ford-Hutchinson et al 1977).

In conclusion, we have shown that, in common with other anti-inflammatory drugs, nabumetone effectively reduces cell migration into saline-soaked or carrageenan-impregnated polyvinyl sponges 24 h after implantation in the rat. However, in contrast to most of the compounds tested we have shown elsewhere (Boyle et al 1978; Mangan et al 1981) that it has good activity in the cotton pellet granuloma test without associated toxicity, and lack of gastric irritancy.

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