Biochemical Pharmacology, Vol. 33, No. 14, pp. 2239–2243, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

IN VIVO INTERACTION OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON THE LOCOMOTION OF NEUTROPHILS ELICITED BY ACUTE NON-SPECIFIC INFLAMMATIONS IN THE RAT—EFFECT OF INDOMETHACIN, IBUPROFEN AND FLURBIPROFEN

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(*Received* 12 *December* 1983; *accepted* 31 *January* 1984)

Abstract—The *in vivo* effects of Flurbiprofen, Ibuprofen and Indomethacin (1.5, 6 and 3 mg/kg respectively) were studied on two acute non-specific pleurisies induced by calcium pyrophosphate crystals (CaPP) or decomplemented isologous rat serum (DIRS) in the rat. Drug effects on the exudation phase (pleural exudate volume), leukocyte emigration (number of leukocytes in the fluid) and on random and directed locomotion of elicited neutrophils (PMN) under agarose were investigated.

In the CaPP model, Indomethacin, Flurbiprofen and Ibuprofen reduced the pleural exudate volume by approximately 48, 57 and 22% respectively while leukocyte emigration was inhibited 50, 45 and 50% respectively. In the DIRS model Indomethacin and Flurbiprofen reduced the exudate volume by 54 and 52% and leukocyte emigration by 51 and 31% respectively. Ibuprofen administration produced a decrease in exudate volume of only 27%. The three drugs did not alter *in vitro* locomotion of DIRS-elicited PMN. On the other hand, Flurbiprofen reduced both random and directed locomotion of CaPP-elicited PMN stimulated with peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), isologous rat serum (IRS) or cell-free exudates. Ibuprofen induced a slight increase in random migration of CaPP-elicited PMN while Indomethacin was without effect. None of the three drugs altered the chemotactic activity of inflammatory exudate. These data suggest that therapeutic doses of anti-inflammatory drugs interfere with PMN at inflammatory sites and induce modifications in their movement *per se* which persist after cell washing.

Polymorphonuclear leukocytes (PMN) play an important role in inflammation [1, 2]. Neutrophils are mainly involved in the early stages of inflammatory reactions owing to their ability to leave blood vessels rapidly and to migrate towards injured and infected tissues [3]. Neutrophil influx at inflammatory sites can be reduced by pharmacological agents such as nonsteroidal anti-inflammatory drugs (NSAID). However, some of these compounds have been shown to interfere *in vitro* with neutrophil locomotion [4–8]. On the other hand, there are limited data on the degree of *in vivo* NSAID interaction with neutrophils, particularly with PMN directly involved in inflammatory reactions (i.e. present at inflammatory sites). The purpose of this study was to compare the reactivity of neutrophils elicited by two different inflammatory reactions and to investigate whether therapeutic treatment with NSAID interferes with these cells or not. We therefore used two experimental models of pleural inflammation induced either by calcium pyrophosphate crystals (CaPP) or decomplemented isologous rat serum (DIRS) in the rat treated or not, with three different nonsteroidal anti-inflammatory drugs. Drug effects on inflammation reaction development and on the reactivity of elicited neutrophils, as measured by their *in vitro* random and directed locomotion under agarose, using different chemotactic stimuli, were analysed. In order to assess the influence of pleural fluid on PMN locomotion, migrations were performed with PMN suspended in their original pleural exudate and compared to those of washed PMN suspended in buffer.

MATERIALS AND METHODS

Drugs and chemicals. Flurbiprofen and Ibuprofen were obtained from Laboratoire Boots-Dacour (Courbevoie, France) and Indomethacin was provided by Merck, Sharp & Dohme (Paris, France). FMLP was obtained from Sigma Chemical Co. (St. Louis, MO). Indubiose A37 was supplied by Industrie Biologique Française (France). Calcium pyrophosphate (CaPP) was synthetized by the laboratoire de Chimie des Solides des Hautes Pressions (Toulouse, France).

Administration of NSAID to rats. Flurbiprofen, Indomethacin and Ibuprofen were suspended in 1% methylcellulose and administered *per os* (1.0 ml per rat) to male Sprague–Dawley rats (weighing 180–200 g from Depré, St Doulchard, France) at doses of 1.5, 3 and 6 mg/kg respectively. These doses were chosen in comparison with clinical doses used in human therapeutics. Control rats received 1.0 ml of methylcellulose only. The three drugs were tested simultaneously using rats from the same batch. Twelve rats were randomly allocated to experimental or control group.

Experimental inflammation models. One hour after administration of the appropriate drug, 1.0 ml of a suspension of 1% calcium pyrophosphate crystals (CaPP) in saline or decomplemented isologous rat serum (DIRS) was injected into the pleural cavity of each rat, as previously described [9, 10]. After a period of 4 hr, the rats were sacrified by ether inhalation and the pleural exudate harvested with a Pasteur pipette. Each pleural fluid was placed in a plastic tube standing in ice and was supplemented with heparin (10 IU/ml). The pleural exudate volume was measured and its leukocyte number assessed with a HYCEL 202 counter.

PMN migration assay. PMN from each sample of pleural fluid were tested for, by *in vitro* migration both in their own exudate and after being washed twice and resuspended in 0.1 M Krebs Ringer phosphate buffer (KRP) pH 7.4. Random and directed migrations were measured by a modification of the agarose method, as described previously [11, 12]. Briefly, 4 ml of 0.75% indubiose (in KRP pH 6.8), containing 10% heat inactivated fetal calf serum, was poured into a small tissue culture Petri dish (Falcon 15 × 15 mm, Lab. Exp. Service, France). The agarose was allowed to gel at +4°, and four sets of three wells, 2.5 mm in diameter and spaced 2.5 mm apart, were cut out using a template. Five microlitres of the leukocyte suspension (i.e. 5×10^5 PMN) was placed in each of the middle wells (PMN wells), 5 μl of chemoattractant was placed in each of the outer wells (chemoattractant wells) and 5 μl of KRP pH 7.4 was placed in each of the inner wells (control wells).

The chemoattractants used were FMLP (at the optimal concentration of 10^{-6} M), isologous rat serum (IRS) and cell-free pleural fluid. FMLP was prepared as a stock solution of 10^{-3} M in 0.15 N NaOH and stored at −20° in aliquots. All dilutions were made in KRP pH 7.4 immediately before use. IRS from the same batch was stored at −20° and used undiluted throughout this study. The cell-free exudates used as chemoattractants were obtained on the day of the experiment. For migration assays with all chemoattractants used, plates were incubated at 37° in a 95% air/5% CO_2 humidified atmosphere for 150 min. Random and directed migrations, that is, the distance traversed by the cells from the border of the middle wells in the direction of the chemoattractant wells (directed migration) or the control wells (random migration), were measured at the leading front of migration (at least 10 PMN) under a microscope (magnification 40) using a calibrated eyepiece. Results are expressed in arbitrary units; one unit representing approximately 360 μm.

Statistics. Means, standard error of the means and standard deviations were calculated for each series of experiments (see tables and figures). Student's *t*-test was used to assess differences between control and experimental values.

RESULTS

Effects of drugs on the development of pleurisy induced by calcium pyrophosphate crystals and decomplemented serum

Table 1 shows the effects of NSAID on exudate volume and number of emigrating leukocytes collected in the pleural cavity of rats 4 hr after injection of 1.0 ml of CaPP of DIRS. The volume of exudate in these two models was not significantly different. In contrast, the number of pleural leukocytes elicited by CaPP was approximately 60% lower ($P < 0.001$) than in the DIRS model. Ibuprofen administration had different effects in these two models. Although, exudate volume was similar, inhibition of leukocyte emigration was only significant in the CaPP model. The other drugs, Indomethacin and Flurbiprofen, lowered the values for these two measured parameters with similar effectiveness in both models. Leukocytes collected from control rats after intrapleural injection of CaPP or DIRS were mainly neutrophils (approximately 90%) and mononuclear cells (10%) including macrophages, monocytes and a few lymphocytes. These proportions were not modified by treatment of animals with NSAID.

Table 1. Effects of non-steroidal drugs on exudate volume and number of emigrating leukocytes following pleurisy induced by CaPP and decomplemented serum (DIRS)

	Parameters measured (% of control values) DIRS model		CaPP model	
	Exudate volume	No. of pleural leukocytes	Exudate volume	No. of pleural leukocytes
Control	100 ± 25 (1.24 ± 0.32)	100 ± 16 (127 ± 21)	100 ± 12 (1.02 ± 0.12)	100 ± 18 (51 ± 9)
Indomethacin (3 mg/kg)	46 ± 8***	49 ± 10***	52 ± 7***	50 ± 11***
Flurbiprofen (1.5 mg/kg)	48 ± 12***	63 ± 13**	43 ± 8***	55 ± 15***
Ibuprofen (6 mg/kg)	73 ± 19*	100 ± 30	78 ± 10**	50 ± 17**

Each result is expressed as a percentage of the control. The corresponding control value is given in brackets. The exudate volume is expressed as ml per rat and the number of pleural leukocytes as 10^6 cells per rat. Each result is the mean ± 1 SD of 8 experiments. Significant differences between control and assay values are indicated by *($P < 0.05$). **($P < 0.01$) and ***($P < 0.001$).

Table 2. Random and directed locomotion of CaPP and DIRS elicited PMN

	Migratory distances ± SEM (in arbitrary units)	
	DIRS-elicited PMN	CaPP-elicited PMN
Random	1.80 ± 0.13	1.93 ± 0.05
FMLP	2.35 ± 0.08	2.48 ± 0.06
IRS	5.74 ± 0.17	5.80 ± 0.20

Each result is expressed in arbitrary units and is the mean ± 1 SEM of 6–8 different experiments. One unit represents 360 μm.

Random and directed migrations of PMN elicited by calcium pyrophosphate crystals and decomplemented serum

Table 2 shows the locomotion of pleural PMN after they had been washed and suspended in Krebs Ringer phosphate buffer, pH 7.4. These data indicated that PMN elicited by CaPP and DIRS exhibited similar random migration and directed locomotion stimulated by the peptide FMLP or isologous rat serum (IRS). The migration induced by optimal concentrations of FMLP was weak compared to that induced by IRS and suggests that this peptide is a less powerful chemoattractant than serum for rat PMN. This is in contrast to its effect on human neutrophils [7].

Effects of drugs on random and directed migrations of PMN elicited by calcium pyrophosphate crystals and decomplemented serum

PMN elicited by DIRS in the pleural cavity of animals treated with Indomethacin, Ibuprofen and Flurbiprofen exhibited similar random migration and FMLP and IRS induced chemotactic response compared to PMN taken from control animals (results not shown). On the other hand, PMN taken from drug-treated rats with CaPP-induced pleurisy displayed variable migrations described in Fig. 1. PMN taken from Flurbiprofen treated animals, compared to those of control animals, showed decreased random locomotion as well as a depressed FMLP and IRS-induced directed migration. Contrastingly, PMN taken from Ibuprofen-treated animals exhibited an increased random locomotion while no enhancement of FMLP and IRS-induced chemotactic responses of PMN was observed. Indomethacin did not interfere with elicited neutrophil locomotion in this model. These data indicate that the three drugs interfere in a variable manner with CaPP-elicited PMN reactivity although reducing pleurisy development with similar efficiency. In order to characterize PMN reactivity further, we investigated the effects of pleural exudates on PMN locomotion.

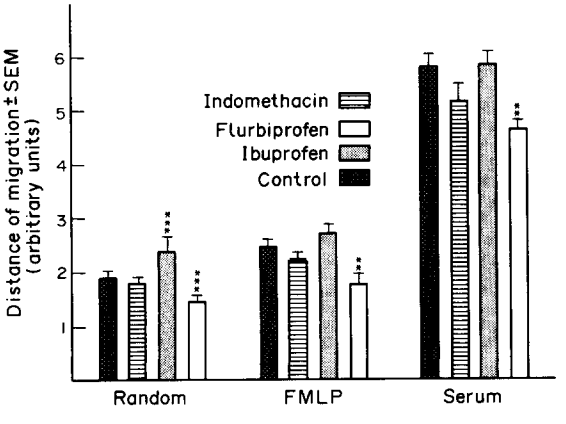

Fig. 1. Effects of non-steroidal drugs on random directed locomotion of CaPP-elicited PMN. Each bar indicates the mean ± SEM of six different experiments. Each result is expressed in arbitrary units. One unit represents 360 μm. Significant differences between control and assay values are indicated by ** ($P < 0.01$) and *** ($P < 0.001$).

Effect of pleural exudate on random and directed migration of elicited PMN

Table 3 shows the locomotion of DIRS and CaPP-elicited PMN, suspended in their original pleural fluid (unwashed cells). CaPP-elicited PMN, compared to DIRS-elicited cells, exhibited least random locomotion (24%, $P < 0.01$), as well as least FMLP-induced directed locomotion (26%, $P < 0.05$). However, there was no significant difference between DIRS and CaPP-elicited PMN chemotactic responses to IRS. Comparison of the migration of unwashed PMN (Table 3) to that of washed PMN (Table 2) showed increased random locomotion of 95% ($P < 0.001$) and 39% ($P < 0.001$) for DIRS and CaPP-elicited PMN respectively. FMLP-induced directed migration of PMN elicited by DIRS and CaPP was also increased by 76% ($P < 0.001$) and 23% ($P < 0.01$) respectively. On the other hand, the chemotactic responses of washed and unwashed PMN stimulated with IRS did not differ. Increased random and FMLP-induced migration of unwashed PMN, as compared to that of washed cells, seemed to be due to positive chemokinetic activity of the pleural exudate, since washed cells resuspended in their original fluid, exhibited a similar pattern of migration to unwashed PMN (results not shown).

Effects of drugs on random and directed migrations of unwashed elicited PMN

In the DIRS pleurisy model, unwashed PMN taken from control and drug-treated animals exhibited similar random locomotion as well as FMLP or IRS-induced chemotactic responses (results not shown). In the CaPP model, PMN taken from Flurbiprofen-treated rats showed a decrease in random migration

Table 3. Effects of pleural exudate on random and directed locomotion of CaPP and DIRS-elicited PMN

	Migrating distances ± SEM (in arbitrary units)	
	DIRS-elicited PMN	CaPP-elicited PMN
Random	3.51 ± 0.07	2.69 ± 0.14**
FMLP	4.14 ± 0.10	3.05 ± 0.13*
IRS	5.64 ± 0.28	5.85 ± 0.16

Migrations were assessed with PMN suspended in their own pleural exudate (unwashed PMN). Each result is expressed in arbitrary units and is the mean of 6–8 different experiments. One unit represents 360 μm. Significant differences between migrations of CaPP and DIRS-elicited PMN, are indicated by *($P < 0.05$) and **($P < 0.01$).

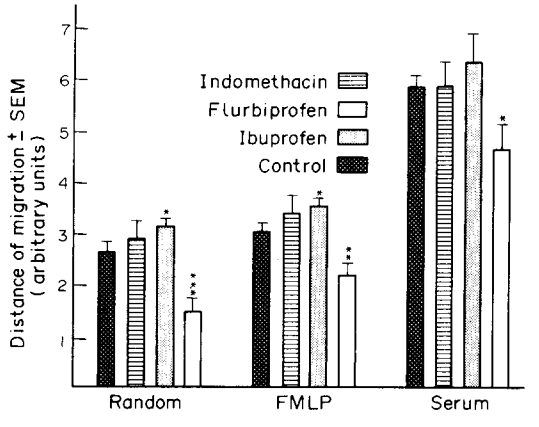

Fig. 2. Effects of non-steroidal drugs on random and directed locomotion of unwashed CaPP-elicited PMN. CaPP-elicited PMN were tested still suspended in their own original pleural exudate. Each result is the mean ± SEM of six different experiments and is expressed in arbitrary units. One unit represents 360 μm. Significant differences between control and assay values are indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

and FMLP and IRS induced chemotactic responses as compared to control PMN (Fig. 2). Ibuprofen enhanced random and FMLP-induced directed locomotion of unwashed PMN while no alteration was observed in IRS-induced directed migration. Indomethacin did not interfere with PMN locomotion. Comparison of washed (Fig. 1), and unwashed (Fig. 2) PMN locomotion showed that random locomotion was increased by 63% ($P < 0.001$) and by 31% ($P < 0.001$) in unwashed PMN taken from animals treated with Indomethacin and Ibuprofen respectively. An enhancement of 56% ($P < 0.05$) and 29% ($P < 0.01$) was also observed, in FMLP-induced directed migration of unwashed cells derived from animals treated with Indomethacin and Ibuprofen respectively. In contrast, PMN taken from Flurbiprofen treated rats exhibited similar random migration and FMLP-induced chemotactic responses both before and after cell washing.

Chemotactic activity of cell-free exudates taken from control and drug-treated animals

The observation that pleural fluid enhances random locomotion of unwashed elicited PMN, compared to washed cells, suggested the presence of chemokinetic and/or chemotactic factors in the exudates. In order to clarify this point further, we tested the ability of cell-free pleural fluid taken from control and drug-treated animals to act as chemoattractant to washed PMN elicited by CaPP. Figure 3 shows that CaPP-elicited PMN taken from control rats or animals treated with Indomethacin or Ibuprofen were highly and similarly stimulated with the cell-free exudate from control rats while responses of PMN taken from Flurbiprofen-treated rats were decreased. PMN taken from control animals also exhibited strong and similarly directed locomotion towards each of the three exudates taken from drug-treated animals. PMN taken from rats treated with Indomethacin or Ibuprofen exhibited high chemotactic responses to their own exudate, but PMN derived from Flurbiprofen treated-rats displayed a depressed response.

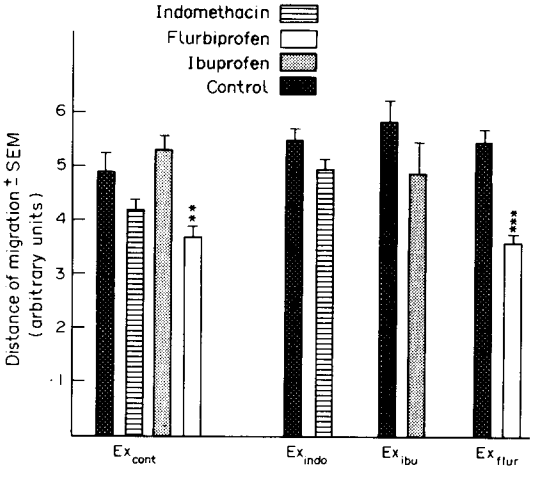

Fig. 3. Chemotactic activity of CaPP cell-free exudate taken from control and drug-treated animals. CaPP cell-free pleural exudate taken from control rats or animals treated with Indomethacin, Ibuprofen or Flurbiprofen are indicated by Ex_{Cont}, Ex_{Indo}, Ex_{Ibu} and Ex_{Flur} respectively. Each exudate was tested as undiluted chemoattractant to washed elicited PMN. Each bar indicates the mean migration ± SEM obtained with six different exudates. Results are expressed in arbitrary units; one unit represents 360 μm. Significant differences between the chemotactic responses of PMN taken from control and drug-treated animals, to a given exudate are indicated by ** ($P < 0.01$) and *** ($P < 0.001$).

DISCUSSION

The pleural inflammation models used here were chosen because they allowed easy quantification of both exudate volume and number of emigrating leukocytes in animals treated or not with NSAID. The results confirm the anti-inflammatory properies of Indomethacin, Ibuprofen and Flurbiprofen previously determined in other models [13–19]. On the other hand, it was shown that *in vivo* administration of NSAID interferes with the locomotion of elicited PMN and that a dissociation in the effects of the three drugs can be made by this approach.

Acute non-specific inflammation induced by CaPP or DIRS differed in a number of ways. CaPP induced lower leukocyte emigration than DIRS while exudation was similar (Table 1). Differences in the locomotion of unwashed elicited PMN (Table 3) and sensitivity to drug interaction were also shown in the two models.

Unwashed CaPP-elicited PMN in control animals displayed a lesser random locomotion than unwashed DIRS cells. These differences may have been related to the positive chemokinetic activity of exudate since both cell types exhibited a lower but similar random locomotion after cell washing (Table 2). The observation that the stimulatory effect of exudate affected PMN chemotactic responses induced by FMLP but not by IRS is difficult to interpret at this stage. Further investigation regarding the nature and concentration of exudate-stimulating factors is re-

quired. Some of these factors might also have been responsible for the strong chemotactic activity of the fluid on CaPP-elicited PMN (Fig. 3) Preincubation of CaPP or DIRS cell-free exudates at 56° for 30 min abolished the ability of the fluids to induce PMN chemotactic responses (results not shown). These data argue in favor of the presence of heat-labile chemotactic agents in the fluid. Some of these may have been derived from complement [20, 21]. The ability of exudate to promote PMN locomotion *in vitro* has been reported by others [22–25]. Chemotaxis is also considered to be a main mechanism giving rise to leukocyte influx at inflammatory sites.

The two pleural models could be differentiated by the lesser sensitivity of DIRS-elicited PMN to *in vivo* drug interaction compared to that of CaPP-elicited PMN. Although the three drugs had similar effects on pleurisy development in the CaPP model, they could be clearly identified by their effects on PMN locomotion. Flurbiprofen induced decreases in both random and directed locomotion of PMN while the structurally related compound, Ibuprofen, tended to act inversely. These data suggest that the drugs induced non-specific alterations in PMN, impairing their movement *per se*. The modification of chemotactic responses might have been a consequence of an alteration in PMN movement *per se* but a drug alteration with PMN sensory mechanisms cannot be ignored. The decrease in chemokinetic (Figs. 1–2) and chemotactic (Fig. 3) responses of PMN taken from Flurbiprofen-treated rats might have been related to PMN impairment rather than an alteration in exudate stimuli. This hypothesis is supported by the observation that pleural fluids taken from control and Flurbiprofen-treated animals induced similar directed locomotion of PMN derived from control animals (Fig. 3). In our models, Indomethacin did not interfere with PMN locomotion or pleural fluid chemotactic activity. These data failed to confirm earlier results [26]. This disagreement in Indomethacin effect could have been due to differences in PMN migration assay, drug dose applied, and inflammation model used.

In conclusion, we have shown that inflammatory PMN elicited by non-specific pleurisies exhibited different locomotory behaviour depending on whether they were tested in their original fluid or buffer and depending on the chemotactic stimuli applied. It was also observed that therapeutic administration of NSAID could induce variable interference with PMN locomotion depending on the type of inflammatory reaction and the drug. The three drugs tested here did not interfere with locomotion of DIRS-elicited PMN. On the other hand, in the CaPP model, Flurbiprofen induced a decrease in PMN movement *per se*, Ibuprofen had a converse effect while Indomethacin was without effect. The three drugs did not modify the chemotactic activity of the fluid. These data indicate that NSAID not only played a beneficial role in limiting the development of inflammation, but also that these compounds alter PMN movement. Therefore it might be of interest to evaluate the extent of NSAID interference with other phagocytic functions and to determine whether this interaction is detrimental to phagocyte activities.

Acknowledgements—The authors are grateful to Mrs M. Lenoir and Mr O. Muntaner for technical assistance and to Miss C. Delpont for excellent secretarial assistance. This work was supported by a grant from Laboratoire Boots-Dacour (Courbevoie, France).

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