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PROTECTIVE EFFECT OF INDOMETACIN AGAINST CHEMOTACTIC DEACTIVATION OF HUMAN NEUTROPHILS INDUCED BY FORMYLATED PEPTIDE

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Abstract—The effects of the nonsteroidal antiinflammatory drug indometacin on the parameters relating to the migration and respiratory burst of human polymorphonuclear leukocytes (PMN) were studied in an attempt to clarify the mechanism of this drug's action on PMN.

At various concentrations below 200 μg/ml, indometacin partially inhibited the spontaneous migration of PMN but did not alter the directional migration induced by C5a-activated serum. In the presence of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) as chemoattractant, directed PMN migration was either inhibited or stimulated by indometacin, depending on FMLP concentration. When PMN migration was induced by the optimal and suboptimal FMLP concentrations of 10^{-7} and 10^{-8} M, indometacin inhibited this migration, but when the high FMLP concentration of 10^{-6} M depressed this migration by chemotactic deactivation, indometacin restored it to its maximum. Both the inhibitory and stimulatory effects of indometacin on FMLP-induced PMN migration were due to changes in the migration speed. Indometacin also inhibited FMLP-induced changes in the shape of floating PMN, and in respiratory burst, as well as specific FMLP binding to PMN. In contrast, indometacin did not alter the PMN respiratory burst induced by phorbol myristate acetate or C5a-activated serum. These data show that indometacin is able to prevent the loss of PMN chemokinetic activity induced by formylated peptides and suggest that it might be useful for investigating the mechanism of peptide-induced chemotactic deactivation.

Polymorphonuclear leukocytes (PMN) and mononuclear phagocytes are effector cells of inflammation. These cells are able to migrate directionally in response to a gradient of chemotactic factors. This chemotactic mechanism plays an important role in the accumulation of leukocytes at inflammatory sites. High doses of chemotactic factors deactivate PMN migration and trigger the optimal release of oxygen-derived radicals and lysosomal enzymes by PMN [1]. These products, which normally serve to kill and degrade foreign particles such as bacteria, may be responsible for tissue destruction [2]. Since leukocytes play an important part both in host defence and in the progression of inflammatory diseases, the development of drugs able to induce selective alteration of leukocyte functions, could help to control inflammatory disorders. Antiinflammatory agents possess this property, since they efficiently reduce leukocyte emigration to inflammatory sites [3, 4]. Some of them also induce a concomitant *in vivo* alteration in the functions of exudate PMN [3, 4]. To gain insight into the mechanism by which antiinflammatory agents alter PMN functions, we explored the *in vitro* effects of indometacin, a nonsteroidal antiinflammatory drug, on various parameters relating to the migration and superoxide anion production by human PMN. For this purpose, the cells were stimulated by various doses of the chemotactic peptide-*N*-formyl-methionyl-leucyl-phenylalanine (FMLP). The results indicate that indometacin both inhibited the optimal PMN migration induced by FMLP and had a protective influence against the loss of migration induced by high doses of this peptide, a process known as chemotactic deactivation.

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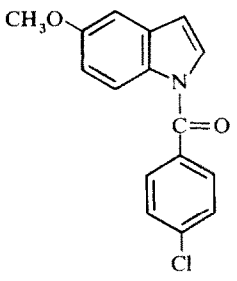
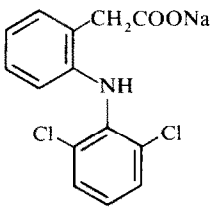
MATERIALS AND METHODS

Drugs and chemoattractants. Indometacin (a gift from Merck, Sharp & Dohm, Paris, France) was diluted extemporaneously in dimethylsulfoxide (DMSO) and then in 0.15 M Krebs Ringer phosphate buffer (KRP), pH 7.4, to the desired final concentration. The final concentration of DMSO in the assays was less than 0.5% and had no effect on PMN functions. The structural formula of indometacin is described below, with that of diclofenac sodium (Voltaren), another antiinflammatory drug whose previously reported effects on human PMN (see Refs. 7, 10, 15 and 30) will be discussed here.



Indometacin



Diclofenac sodium

Formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA) from Sigma Chem. Co. (St Louis, MO), were prepared in aliquots in DMSO and stored at −80° until use. Sera obtained from 10 healthy volunteers were pooled and stored in aliquots at −80°. Zymosan-activated serum (ZAS) was prepared by incubating 1 ml serum with 10 mg of heat-boiled zymosan for 30 min at 37° in the presence of 5 mM ε-aminocaproic acid. Zymosan suspension was centrifuged at 2000 *g* for 15 min at 4° and the supernatant stored in ice until use.

Isolation of PMN. Venous blood was obtained from healthy adults, in preservative-free lithium heparin (10 IU/ml of blood). Leukocytes were isolated by a one-step centrifugation of the blood on a mixture of Ficoll-Hypaque (monopoly resolving medium, Flow Laboratories, France) as previously described [5]. The final cell suspension contained 10^8 PMN/ml. All experiments were completed within 4 hours of blood collection.

PMN function tests. PMN migration under agarose was investigated by two different methods [6, 7]. One served to measure directed PMN migration (i.e. the combined effect of both speed and direction of PMN) in the presence of either zymosan-activated human serum (i.e. C5a-activated serum) or of FMLP placed in the chemoattractant wells. The other was used to measure undirected PMN migration (i.e. speed only) in the presence or absence of chemokinetic factors incorporated into the agarose gel.

Directed PMN migration was assessed by the leading front of PMN migration, using a modified version of the agarose technique, as previously described [6], except that the agarose contained various amounts of indometacin.

Undirected PMN migration was evaluated under similar conditions of agarose preparation, except that four sets of one well only instead of three were cut in the agarose with a template. The agarose contained various amounts of FMLP as previously described [7] and indometacin whose concentrations are given in the results. Five microlitres of leukocyte suspension (5×10^5 PMN) was placed in each well and incubated at 37° for 90 min. At the end of incubation, the surface occupied by the migrating PMN was a circle with the well in its centre. The radius of this circle minus that of the well was used to estimate undirected migration. The variation in the results obtained with this technique was less than 5% for duplicate experiments (two petri dishes) of four measurements each.

Changes in the shape of floating PMN stimulated by FMLP were assessed by a modified version of the method of Smith *et al.* [8]. Suspensions of 5×10^5 PMN/500 μl were exposed for 10 min at 37° to various concentrations of indometacin or buffer. FMLP at the final concentration of 10^{-8} M was then added and the PMN were fixed 4 min later with 1% (final concentration) of ice-cold glutaraldehyde (v/v). PMN were examined by phase contrast microscopy (magnification: 400) and about 200 cells per field were classified into two groups: those which remained round and those which had at least one pseudopod (polarized cells).

Superoxide anion production by PMN stimulated by FMLP was measured according to Cohen and Chovaniec [9] by continuously recording the reduction of cytochrome *c* with a Uvikon 810/820 spectrophotometer as reported previously [10]. Control experiments were performed by incubating the PMN for 10 min at 37° with the drug solvent (DMSO) which was not removed during the measurements. Drug solvent (less than 0.5%) did not affect PMN functions. More than 95% of the PMN incubated for 30 min at 37° with the largest amount of indometacin used in the assays (i.e. 100 μg/ml) excluded Trypan blue.

FMLP binding assays. ^{3}H-FMLP binding assays were performed as described [10] except that various amounts of indometacin or solvent solution (control) were present in the incubation medium. Each determination was performed in duplicate.

The significance of the differences between the experiments performed in the absence (control) or presence of indometacin was assessed by paired Student-*t*-test. A $P < 0.05$ was considered significant.

RESULTS

Effect of indometacin on spontaneous and serum-induced PMN migration

Incorporation of indometacin into the agarose gel at concentrations ranging from 25 to 100 μg/ml inhibited spontaneous PMN migration by 50% at most (Fig. 1). This inhibition was not due to any incidental cytotoxic property of indometacin, since when PMN were preincubated for 90 min at 37° with 100 μg/ml indometacin, the highest concentration tested, they excluded Trypan blue in the same proportion (95%) as control PMN not treated with indometacin. In other respects, the observation illustrated in Fig. 1, that indometacin did not significantly alter the PMN migration induced by C5a-activated serum confirms that indometacin was not toxic for PMN under our experimental conditions.

Inhibition and stimulation by indometacin of FMLP-induced directed PMN migration

When the synthetic peptide FMLP was used as the chemoattractant, indometacin displayed both inhibitory and stimulatory effects, depending on the peptide concentrations applied, as indicated in Fig. 2. Addition of FMLP to attractant wells at concentrations ranging from 10^{-8} M to 10^{-5} M induced optimal directed PMN migration at 10^{-7} M. Above

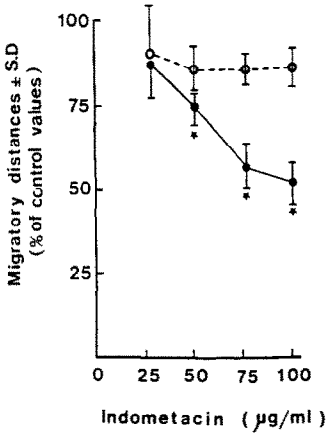


Fig. 1. Dose effect of indometacin on spontaneous and serum-induced PMN migration. Control values (100%) for spontaneous (●) and serum-induced (○) PMN migration were 0.47 ± 0.07 and 1.52 ± 0.14 mm (mean ± 1 SD, 5 experiments) for periods of incubation at 37° for 90 min and 120 min respectively. A significant difference between the values obtained in the absence and presence of indometacin is designated by * ($P < 0.05$).

and below this concentration, directed PMN migration towards the attractant well diminished, and the resulting migration patterns differed as previously described [11]. Under optimal and suboptimal conditions of induction of directed PMN migration by FMLP (i.e. FMLP at 10^{-7} M and 10^{-8} M respectively), indometacin inhibited PMN migration in a dose-dependent manner (Fig. 2).

By contrast, the PMN migration induced by a deactivating FMLP concentration of 10^{-6} M was enhanced by 25 μg/ml indometacin while this migration was restored to its maximum in the presence of 50, 75 and 100 μg/ml indometacin (Fig. 2). By protecting PMN against chemotactic deactivation, indometacin also partially protected the PMN migration induced by the even higher FMLP concentration of 10^{-5} M. This protective influence of indometacin against FMLP-induced loss of PMN migration provides additional evidence that indometacin was not cytotoxic under our experimental conditions. In other respects, these data seem to indicate that the different effects of indometacin on PMN migration might be due to a cellular modification rather than to changes in the agarose gel. This possibility was supported by the observation that the modulating effects of indometacin on FMLP-induced PMN migration were also observed when the PMN were preincubated for 10 min at 37° with various doses of indometacin and placed in the PMN wells (data not shown). Furthermore, if the preincubated PMN were washed twice and allowed to migrate, normal PMN migration was observed, suggesting that the interaction of indometacin with the PMN was reversible (results not shown).

Effect of indometacin on FMLP-induced chemokinesis and on changes in the shape of PMN

The above results indicate that indomethacin did not interfere with C5a-induced PMN migration but modulated FMLP-induced directional migration, as a function of the indometacin concentration in the gel and of the FMLP concentration added to the PMN wells. To gain insight into the mechanisms by which indometacin modifies PMN migration, we explored its effect on a migration component, i.e. PMN chemokinesis, by a method we described previously [7]. Incorporation of FMLP into the gel at constant concentrations ranging from 10^{-9} M to 10^{-6} M induced maximal PMN chemokinetic activity at 10^{-8} M FMLP (Fig. 3). Under optimal and subop-

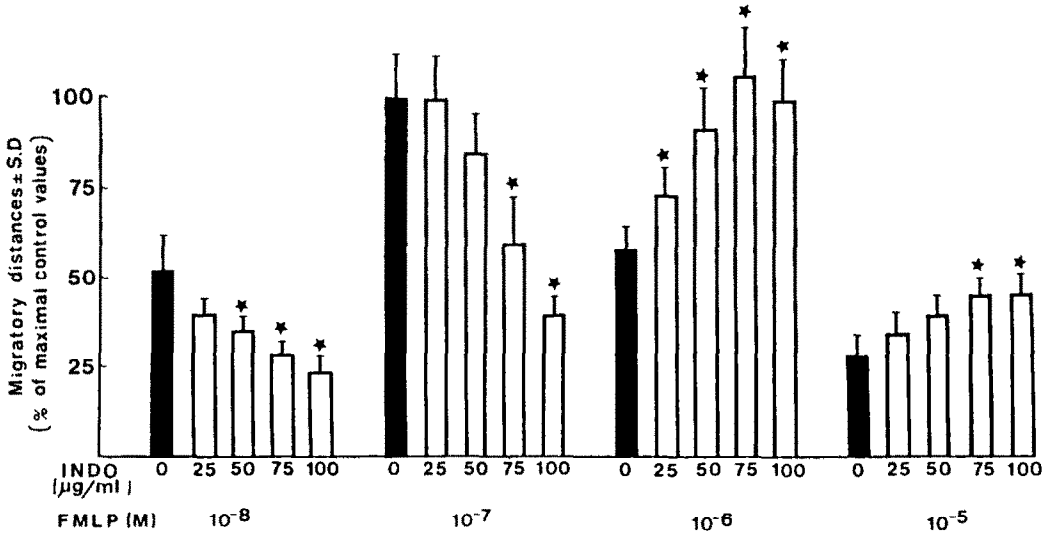


Fig. 2. Dose effect of indometacin on the directed PMN migration induced by various FMLP concentrations. The PMN migration induced by FMLP in the absence of indometacin was optimal for an FMLP concentration of 10^{-7} M, and covered a distance of 1.82 ± 0.17 mm (mean ± 1 SD, 8 experiments) for an incubation period of 90 min at 37°. Results are expressed in percent of the maximal control value. Statistically significant differences were calculated between the values obtained in the absence and presence of indometacin for each FMLP concentration, and are designated by * ($P < 0.05$).

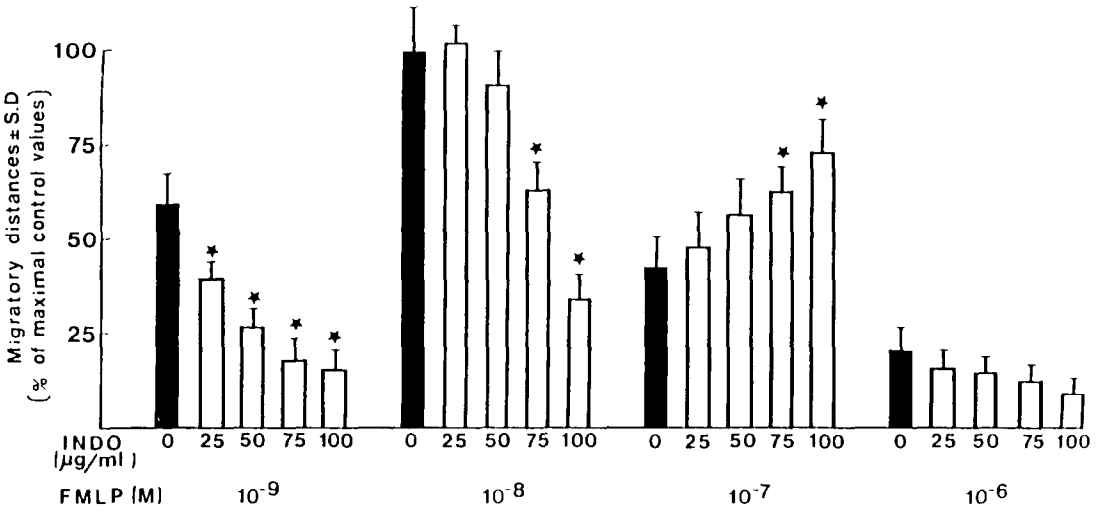


Fig. 3. Dose effect of indometacin on the chemokinetic activity of PMN induced by various FMLP concentrations. FMLP was incorporated into the agarose gel at various concentrations and induced optimal PMN migration at 10^{-8} M. The corresponding distance migrated by PMN was 1.64 ± 0.21 mm (mean ± 1SD of 6 experiments) for an incubation period of 90 min at 37°. Results are expressed in percentages of the maximal control value. For each series of PMN migrations induced by a given FMLP concentration, statistically significant differences were calculated between control and assay values and are designated by * ($P < 0.05$).

timal conditions of PMN stimulation by FMLP concentrations of 10^{-8} M and 10^{-9} M respectively, indometacin caused a dose-dependent reduction of the PMN chemokinetic responses which, however, was more marked in the presence of 10^{-9} M FMLP. On the other hand, in the presence of the chemokinetically deactivating FMLP concentration of 10^{-7} M, indometacin stimulated PMN migration in a dose-dependent manner, whereas at the higher FMLP concentration of 10^{-6} M, the effect of indometacin was not significant, although a slight tendency towards inhibition was observed.

These data strongly support the possibility that the modulation by indometacin of FMLP-induced directed PMN migration (Fig. 2) mainly affected the speed of PMN migration. Because the chemokinetic activity of PMN depends on the rate of lamellipodia formation and deformation by the cells, we attempted to measure these parameters by assessing cell polarity, as previously described [7]. The results shown in Fig. 4 indicate that in the presence of optimal chemokinetic stimulation by FMLP, which was observed at 10^{-8} M (Fig. 3), approximately 20% of the control PMN incubated in the absence of indometacin did not exhibit any changes in shape (Fig. 4). Of the remaining 80% of responsive PMN about 20% exhibited a bipolar shape. The presence of indometacin in the incubation medium inhibited FMLP-induced changes in PMN shape in a dose-dependent manner. When a higher FMLP concentration of 10^{-7} M was applied, inhibition by indometacin diminished (maximal inhibition: 30%). The inhibition by indometacin of changes in PMN shape induced by 10^{-8} M FMLP is consistent with the decrease in PMN chemokinesis induced by the same concentration. On the other hand, in the presence of 10^{-7} M FMLP, indometacin still decreased cell polarity, but enhanced PMN chemokinesis. These data suggest that the mechanisms of PMN deactivation by chemotactic factors in adherent and non adherent PMN might be different.

Interference by indometacin with the FMLP-induced PMN respiratory burst and FMLP binding to PMN

The loss of PMN migration induced by high concentrations of chemoattractants is believed to be related to the concomitant stimulation of the PMN respiratory burst [12, 13]. We measured this burst to determine whether or not it was responsible for the stimulation of FMLP-induced PMN chemokinesis by indometacin. As shown in Fig. 5, indometacin

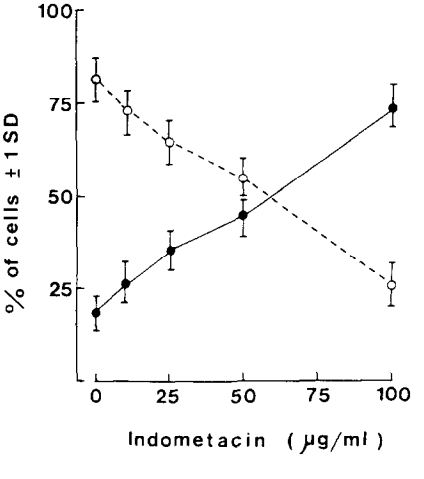


Fig. 4. Dose effect of indometacin on FMLP-induced changes in PMN shape. PMN were preincubated at 37° for 10 min in the absence or presence of indometacin, stimulated for 4 min with 10^{-8} M FMLP and fixed with glutaraldehyde. Two groups of PMN were distinguished according to shape: those which were round (●) and those with at least one pseudopod (○). Results are means ± 1 SD of the percentages of cells examined.

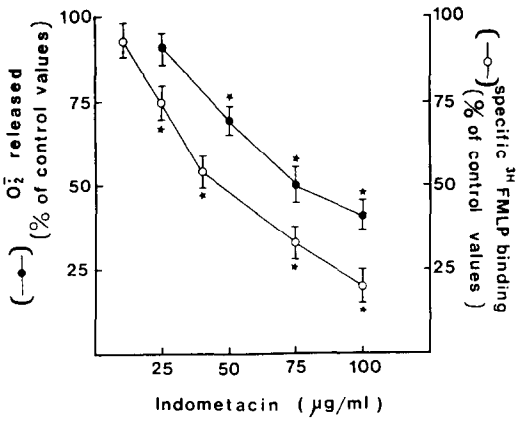


Fig. 5. Dose effect of indometacin on FMLP-induced superoxide anion release by PMN and FMLP binding. PMN were preincubated for 10 min at 37° with various concentrations of indometacin. The production of superoxide anion (●) by PMN (5×10^5 cells in one ml KRP containing 80 μM cytochrome *c*) was induced by FLMP at the final concentration of 10^{-7} M. Results are the means ± SD of 5 experiments. Control value was 2.3 ± 0.2 nmole O_2^- 5×10^5 PMN per min (mean ± 1 SD of 5 experiments). FLMP binding to PMN (○) was measured as previously described [10] at 37° for 20 min with tritiated FLMP at the final concentration of 10^{-7} M. Non specific FLMP binding was assessed in the presence of cold FMLP at 10^{-4} M and did not exceed 20% of the total binding. Control value for the specific FMLP binding was 1.09 ± 0.08 pmoles FMLP per 2×10^6 PMN. Statistically significant differences between control and assay values are designated by * ($P < 0.05$)

inhibited the production of superoxide anion by PMN stimulated with 10^{-7} M FMLP, in a dose-dependent manner. This inhibition correlated to the impairment of the binding of 10^{-7} M tritiated FMLP to PMN (Fig. 5). FMLP binding to human PMN has already been reported to be inhibited in a competitive manner by indometacin [14]. In other respects, the PMN respiratory burst induced by other soluble stimulants, such as phorbol myristate acetate or zymosan-activated serum, was not affected by indometacin (results not shown). These data indicate that indometacin did not interfere with the NADPH oxidase system and may inhibit FMLP-induced PMN responses by altering FMLP binding to its specific receptors and/or transductional events mediated by FMLP.

DISCUSSION

The present results demonstrate that indometacin partially inhibits the spontaneous migration of human PMN without affecting the directed migration induced by C5a-activated serum. Indometacin also inhibited directed PMN migration when it was induced by optimal and suboptimal concentrations of FMLP, and had a protective effect against the loss of PMN migration induced by high deactivating doses of this peptide. Both the inhibitory and stimulatory effects of indometacin on FMLP-induced directed PMN migration were mainly chemokinetic. The mechanism by which indometacin diminished or enhanced FMLP-induced PMN migration may well be related to the initial interference of indometacin with FMLP binding to PMN, and to its subsequent impairment of the PMN respiratory burst.

Indometacin is one of the main nonsteroidal anti-inflammatory drugs and has been widely studied for its interaction with granulocyte functions. It has been previously reported that this drug failed to alter C5a induced migration of human PMN [15] but inhibited the FMLP-induced migration [16] and respiratory burst of these cells [17–21]. One explanation for this is the fact that indometacin interfered with FMLP binding to its specific receptors on the PMN membrane. Several authors have shown that indometacin acts as a competitive inhibitor of FMLP binding to PMN [14]. However, FMLP binding to PMN has been reported to be impaired by numerous anti-inflammatory agents which behave like competitive antagonists, such as phenylbutazone [22, 23] or diclofenac [10], and by other types of drugs [24]. One of these agents, i.e. phenylbutazone, resembles indometacin in that it inhibited the optimal PMN migration induced by FMLP and protected PMN against the loss of migration induced by high FMLP doses [2, 3, 6,]. By contrast, diclofenac did not protect PMN against such loss [7] but only displayed inhibitory effects. These results suggest that the interference of indometacin with FMLP binding might not be the sole mechanism accounting for its modulating effects on FMLP-induced PMN migration. The mechanisms by which *N*-formylated peptides induce PMN deactivation are not well known. It has been reported that deactivation by these peptides accompanied internalization of receptors but was greater than could be accounted for by receptor loss [25]. Deactivation is also apparently dependent on the respiratory burst in neutrophils and does not occur in cells from patients with chronic granulomatous disease [12, 13]. This disease is characterised by the inability of PMN to exhibit a respiratory burst. This seems compatible with the observation that protection by indometacin of FMLP-induced PMN chemotactic deactivation was concomitant with a decrease in the FMLP-induced PMN respiratory burst [17–21] as confirmed in Fig. 5. However, our previous observation that diclofenac, which like indometacin, inhibits FMLP binding to PMN and the subsequent respiratory burst [10], did not protect PMN against FMLP-induced deactivation [7] suggests that indometacin and diclofenac might act through different mechanisms, probably on some steps other than FMLP recognition by PMN. One of these steps could be the metabolism of arachidonic acid metabolism since it has been recently shown that indometacin increased the formation of 5-hydroxy-eicosatetranoic acid (5-HETE) and leukotriene B_4 in PMN [26, 27]. By contrast, diclofenac did not induce such an increase of lipoxygenase products and acted by concomitant inhibition of 5- or 15-lipoxygenase [28]. It has also been reported that diclofenac stimulated the uptake of arachidonic acid into lipid pools [27], thus reducing the intracellular level of free arachidonic acid. This differential effect of indometacin and diclofenac on lipid metabolism is likely to be related to other observations that indometacin enhanced the PMN respiratory burst induced by zymosan particles [29] or by the soluble stimuli oleyl-acetyl-glycerol [21]

whereas diclofenac did not alter zymosan-induced PMN oxygen uptake [10]. However, both drugs were unable to modify PMA-induced PMN respiratory burst [10, 19]. This latter result indicates that both drugs did not directly modify the NADPH oxidase system and may have induced alterations of events prior to the activation of protein kinase C. It has been recently shown that both diclofenac and indometacin inhibited increases in cytosolic calcium in PMN stimulated by FMLP [30]. Indometacin also reduced the mobilisation of calcium from membrane stores and enhanced the level of cyclic AMP in FMLP-stimulated PMN at a concentration which did not alter FMLP binding to PMN [30]. These data are compatible with the inhibition by indometacin of PMN responses induced by FMLP or other chemoattractants such as LTB_4 or platelet activating factor [30, 31]. However, other investigations are required to define the biochemical modifications by which indometacin stimulated the PMN respiratory burst induced by ionophore A23187 or zymosan [21, 29], and protected PMN against FMLP-induced chemotactic deactivation (Figs. 2 and 3). While the precise mechanism by which indometacin and diclofenac induced differential alterations of PMN functions remains to be elucidated, the data suggest that these drugs modify enzymes involved in the early signaling events of PMN activation.

The present data show that indometacin is an antiinflammatory drug capable of modulating the PMN chemokinetic activity induced by various concentrations of *N*-formylated peptides. Indometacin effects were related to alterations in FMLP-binding to PMN. However, some modifications of transductional events might not be ignored. These data suggest that indometacin might be a useful tool for exploring the mechanism of PMN deactivation by these peptides.

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