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In vitro EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON HUMAN POLYMORPHONUCLEAR CELLS AND LYMPHOCYTE MIGRATION

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1 The action of the non-steroidal anti-inflammatory drugs (NSAID), sodium salicylate, aspirin, phenylbutazone and indomethacin was investigated on the migration of human polymorphonuclear cells (PMNs) and lymphocytes, using the system of migration of leucocytes from glass capillary tubes.

2 All NSAID produced a dose-dependent inhibition of cell migration, and were more effective on the migration of the PMN than on lymphocytes.

3 Drugs optimally suppressed PMN migration after 20 to 24 h incubation, and lymphocytes after 3 to 6 h.

4 Prolonged incubation of cells with several concentrations of NSAID demonstrated an 'escape' from inhibition in PMNs prepared from one subject.

Introduction

Several hypotheses have been presented for the mode of action of non-steroidal anti-inflammatory drugs (NSAID) (Adams & Cobb, 1958; Mizushima, Sakai & Yamoura, 1970; Vane, 1971), but the relevance of the experimental system to acute and, in particular, chronic inflammation is not always certain. One of the main morphological events of inflammation is a prominent leucocyte infiltration of the inflammatory site. Since all leucocytes are carriers of potential inflammatory mediators, it seems reasonable to infer that an inhibition of cell movement by the NSAID would restrict the extent of leucocyte entry and suppress, but not stop, the ensuing inflammation.

NSAID inhibit *in vitro* migration of total human leucocytes by a dose-dependent action (Brown & Collins, 1977). Studying two experimental models of inflammation, Di Rosa, Papadimitriou & Willoughby, (1971) suggested that an important anti-rheumatic effect of the NSAID was to prevent the entry of mononuclear cells into an inflammatory focus. Lymphocytes and polymorphonuclear cells are two of the main effector cells of rheumatoid arthritis, and this study investigates whether their migration differs in susceptibility to the inhibitory action of the NSAID.

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Methods

Heparinised peripheral blood samples were obtained from healthy male and female volunteers, whose ages ranged from 20 to 57 years.

Lymphocyte separation

Lymphocytes were isolated by a modification of the method of Bøyum (1968): 5 ml 1% methyl cellulose dissolved in 0.9% w/v NaCl solution (saline) and 60 mg carbonyl iron were added to every 20 ml blood and shaken for 30 min. Following incubation at 37°C for 30 min, the iron-containing polymorphonuclear cells (PMNs) and large mononuclear cells were removed by a magnetic field, while the erythrocytes were sedimented by the methyl cellulose. The lymphocyte-rich supernatant was applied to a previously prepared ficoll-triosil gradient and centrifuged for 30 min at 220 g. The lymphocyte band was removed, washed twice in Hanks' physiological salt solution (HPSS) (Wellcome Reagents Ltd) and concentrated to 4×10^7 cells/ml. PMN concentration rarely exceeded 2% and cell viability was always >98% as determined by trypan blue exclusion.

Polymorphonuclear cell separation

Following the method of Dioguardi, Agostoni, Fiorelli & Lomanto (1963), 30 ml samples of blood were

divided into equal volumes, added to cellulose nitrate tubes containing 70 ml 0.83% NH_4Cl, gently mixed and left at room temperature for 8 to 10 min. After twice centrifuging at 120 *g* for 10 min and discarding the supernatant, contaminating erythrocytes were removed. Lymphocytes remaining in the supernatant were removed by resuspending each PMN pellet in 50 ml HPSS and centrifuging for 10 min at 55 *g*. This washing procedure was repeated twice. The final cell pellet, suspended in a small volume of HPSS was adjusted to a concentration of 4×10^7 cells/ml.

Leucocyte migration from capillary tubes

Cells were packed into capillary tubes (Drummonde Scientific Co, USA, vol 8.5 µl) which were sealed at one end and centrifuged at 240 *g* for 5 min. The resulting cell pellets were cut from the tubes and mounted in migration chambers (Sterilin) containing a nutrient medium of 10% horse serum, Eagle's MEM (Wellcome Reagents Ltd) and penicillin-streptomycin (Flow Laboratories) buffered to a pH of 7.35 with 0.2 M Hepes buffer (Wellcome Reagents Ltd). All NSAID investigated were dissolved in this medium to a final pH of 7.35. Each migration chamber was sealed with a glass cover-slip and incubated at 37°C for 20 h. The fan-shaped migration area along the floor of the chamber was drawn with the aid of a photographic enlarger and measured by planimetry. Results of the effect of NSAID on leucocyte migration were expressed as % inhibition of control migration areas, i.e. chambers with no NSAID present. At least four migration chambers were prepared for every investigation.

Statistical significance was assessed by Student's *t* test.

Results

The effects of NSAID on polymorphonuclear cell migration

All NSAID investigated produced a dose-dependent inhibition of PMN migration (Table 1). The lowest concentrations that produced significant inhibition of migration were as follows: 500 µM salicylate ($P < 0.001$), 50 µM phenylbutazone ($P < 0.02$) and 10 µM indomethacin ($P < 0.05$). There was no further modification of cell migration by 1 µM to 1 pM NSAID (not shown in Table 1). The greatest inhibition was produced by 10 mM aspirin, though indomethacin was more effective over a wider concentration range.

The effects of NSAID on lymphocyte migration

NSAID inhibited lymphocytes far less than PMNs (Table 1). The lowest concentrations which produced significant inhibition of migration were as follows: 5 mM aspirin ($P < 0.005$), 5 mM salicylate ($P < 0.02$), 500 µM phenylbutazone ($P < 0.005$) and 1 mM indomethacin ($P < 0.005$). Salicylate and aspirin produced a stimulation of migration at 1 mM and 100 µM.

Time of onset of inhibition of polymorphonuclear cell and lymphocyte migration by NSAID

At least ten capillaries were prepared for every drug investigated in the PMN study and at least five capillaries for the lymphocyte study. On completion of each experiment, no change was found in the pH of any of the nutrient media.

Polymorphonuclear cell study

Four subjects were investigated. The results obtained from Subject 1 (Figure 1a) were typical of the migration responsiveness of PMNs prepared from three subjects. NSAID inhibited optimally after 20 h of incubation. An almost identical response was elicited by 1 mM indomethacin and 500 µM phenylbutazone. Comparable to this action in time, but not intensity, was the analagous action of 100 µM indomethacin and 1 mM salicylate. For the first 20 h of incubation 10 mM salicylate and 1 mM phenylbutazone evoked the same quantitative response from the migrating PMNs, but towards the end of the incubation period the inhibition by both drugs was slightly less pronounced.

An atypical migration response was obtained with the PMNs of Subject 4 (Figure 1b) where a persistent inhibition was only demonstrable with 1 mM phenylbutazone. A 27% inhibition induced by 500 µM phenylbutazone after 3 h incubation gradually declined to a 7% inhibition at 70 h. The cells also 'escaped' from inhibition by 1 mM indomethacin and 10 mM salicylate (a maximum migration inhibition of 28% and 39% respectively was progressively reduced to an 11.5% and 10% inhibition at 70 h incubation).

Lymphocyte study

In typical experiments (Figure 2a), the NSAID significantly inhibited lymphocyte migration after 3 h incubation. The extent of inhibition produced by all drugs was well sustained throughout the incubation period.

The response of lymphocytes from Subject 3 was atypical (Figure 2b). An apparent enhancement of inhibition at 43 h and 50 h with phenylbutazone, was

Table 1 The *in vitro* effects of non-steroidal anti-inflammatory drugs on polymorphonuclear cells (PMNs) and lymphocyte migration

		Concentration of NSAID in chamber							
		10 mM	5 mM	1 mM	500 μM	100 μM	50 μM	10 μM	1 μM
Salicylate	PMNs (8)	61.0 ± 6.5	41.8 ± 6.1	22.0 ± 4.8	16.0 ± 6.3	†2.0 ± 6.1	†0	†0	†0
	Lymphocytes (9)	37.0 ± 3.2	20.6 ± 5.0	†+4.4 ± 3.2		† +7.0 ± 3.7		†7.0 ± 10.1	
Aspirin	PMNs(5)	79.2 ± 4.5	55.2 ± 4.3	25.3 ± 3.6	10.7 ± 0.7	†3.2 ± 12.0	†0	†0	†0
	Lymphocytes (5)	38.0 ± 3.5	16.2 ± 3.5	† +12.0 ± 10.8		† +11.0 ± 14.2			
Phenylbutazone	PMNs (9)			61.4 ± 4.5	44.0 ± 3.6	14.0 ± 4.4	6.0 ± 4.7	†4.0 ± 3.2	†0
	Lymphocytes (6)			38.7 ± 4.6	34.0 ± 4.2	†0		†0	†0
Indomethacin	PMNs (9)			47.2 ± 2.7	35.1 ± 2.7	19.2 ± 2.9	10.0 ± 4.4	9.0 ± 4.6	†3.0 ± 3.2
	Lymphocytes (6)			20.5 ± 5.5	†5.0 ± 3.9	†0		†0	†0

† No significant inhibition of migration ($P > 0.05$).
Results after 20 h incubation are expressed as % inhibition of control values (mean ± s.e.mean). At least four replicate chambers were prepared for each subject's drug-containing and control chambers (s.e. mean of the replicates <1). Numbers in parentheses denote number of subjects investigated.

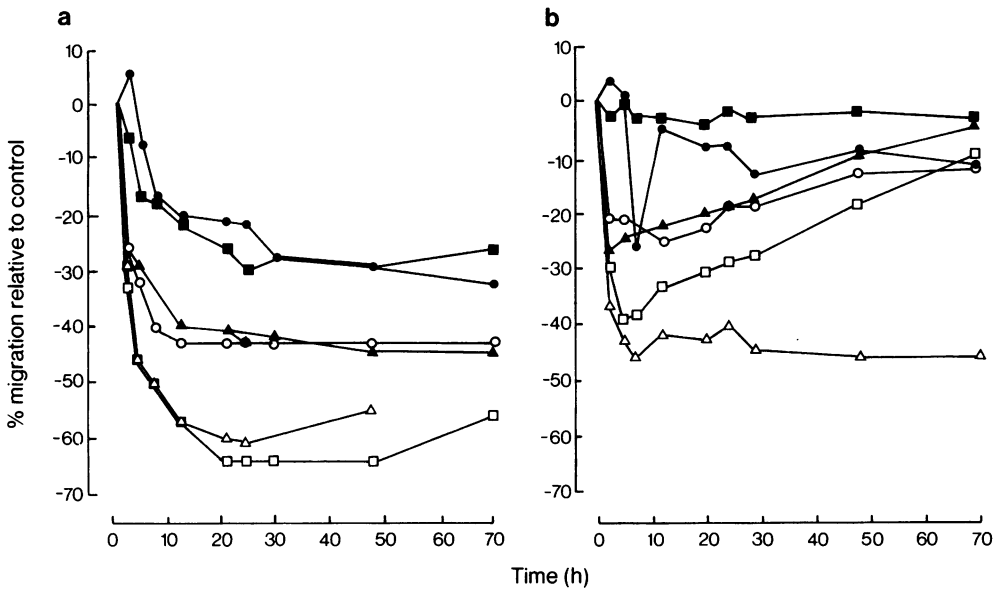

Figure 1 Extent of inhibition of polymorphonuclear cell (PMN) migration by 10 mM (□) and 1 mM (■) salicylate, 1 mM (○) and 100 μM (●) indomethacin, 1 mM (△) and 500 μM (▲) phenylbutazone during 70 h incubation. A typical PMN migration response is illustrated by the cells of Subject 1 (a), and an atypical response by PMNs of Subject 4 (b). Each symbol represents the mean response from at least ten migration chambers.

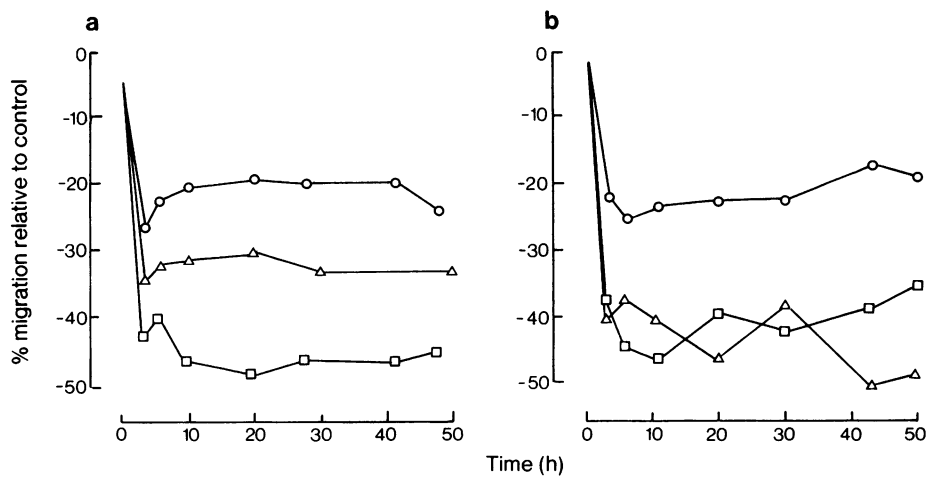

Figure 2 Extent of inhibition of lymphocyte migration induced by 10 mM salicylate (□), 1 mM indomethacin (○) and 1 mM phenylbutazone (△) during 50 h incubation. A typical lymphocyte migration response to the non-steroidal anti-inflammatory drugs is illustrated by the cells of Subject 1 (a), and an atypical response by the lymphocytes of Subject 3 (b). Each symbol represents the mean response from at least five migration chambers.

possibly not attributable to an enhanced pharmacological action of the drug, since there was also a reduction in the control migration areas (Table 2). This phenomenon was also seen at 50 h in chambers containing other drugs.

Discussion

Our data suggest that the *in vitro* migration of the human PMN is more susceptible to the inhibitory action of the NSAID than that of the lymphocyte. In contrast, Di Rosa *et al.* (1971) demonstrated that in the rat carrageenan-induced paw oedema and turpentine-induced pleurisy, the migration of the mononuclear cell was more sensitive to NSAID than that of PMN. Meacock & Kitchen (1976), using similar models of acute inflammation, concluded that NSAID did not directly impede the migration of either the PMN or mononuclear cell. Phelps & McCarty (1967) suggested that an important action of indomethacin was its inhibition of canine and human PMN chemotaxis. The differences in the effect of NSAID on PMN migration are probably due to species differences.

Lymphocyte migration was stimulated by 0.1 and 1 mM aspirin and salicylate. Previously, both drugs had been observed to stimulate the *in vitro* migration of rat peripheral blood leucocytes (Brown & Collins, 1977), where the lymphocyte is the main cell type. It appears that at concentrations lower than those which induce migration inhibition, aspirin and salicylate enhance mononuclear cell migration. An interesting feature of the NSAID effect on the migration of lymphocytes, but not of PMNs, was the rapid onset and prolonged maintenance of maximum inhibition. Optimal suppression of PMN migration was frequently evident after 20 to 24 h incubation, in contrast to 3 to 6 h for the lymphocytes. Of particular interest was the 'escape' from an early inhibition by the PMNs of Subject 4 (Figure 2b). Such an 'escape', though not from the inhibitory action of anti-inflammatory drugs, has been reported in the leucocyte migration test (Hughes, 1972; Brostoff, 1974; Nathan, Karnovsky & David, 1971). We found this 'escape' with four different drug preparations at several incubation periods, which implies that the observation was not an artifact, but a transient sensitivity to NSAID. Whether or not this 'escape' from inhibition is an *in vitro* phenomenon or a decreased sensitivity to NSAID in certain subjects remains to be investigated.

After 50 h incubation, the absolute lymphocyte migration areas in the control and NSAID containing chambers of Subject 3 were smaller. A similar 'recall' of cells after prolonged incubation was reported by Hughes (1972) studying cellular sensitivity in guinea-pigs. Lymphocytes release mediators of cellular immunity (lymphokines) which possess diverse biological actions. The reversed migration may be due to the lymphokine migration inhibitory factor (MIF) which is spontaneously synthesized by lymphocytes following three days culture (Arvilommi & Rasanen, 1975).

Two possible mechanisms by which NSAID inhibit the migration of leucocytes are a suppression of cell glycolysis and an alteration of cell surface charge. Glycolysis is the major energy source for cell movement as demonstrated by the inhibition of cell migration by iodoacetate, an inhibitor of glycolysis (Carruthers, 1966). Phenylbutazone suppresses leucocyte homogenate glycolysis (Strauss, Paul & Sbarra, 1968), and could impede by this action cell migration. Lymphocytes and PMNs possess a net negative surface charge (Brown, Collins & Holborow, 1977). The movement of leucocytes on the floor of a migration chamber may be governed to a certain extent by a repulsion between the like-charges of adjacent cells.

Table 2 The *in vitro* migration of lymphocytes from Subject 3 in the presence of non-steroidal anti-inflammatory drugs for 50 h at 37°C

Incubation (h)	*Salicylate* 10 mM	*Phenylbutazone* 1 mM	*Indomethacin* 1 mM	*Control*
3.5	4.1 ± 0.2	3.9 ± 0.1	5.1 ± 0.2	6.5 ± 0.25
6	5.0 ± 0.2	5.6 ± 0.15	6.7 ± 0.5	8.9 ± 0.4
11	7.4 ± 0.2	8.2 ± 0.2	10.5 ± 0.8	13.6 ± 0.7
19.5	11.2 ± 0.2	10.0 ± 0.3	14.4 ± 0.9	18.4 ± 1.0
29.5	12.2 ± 0.2	13.0 ± 0.3	16.5 ± 0.8	21.0 ± 1.1
43	14.4 ± 0.7	11.4 ± 0.4	19.3 ± 0.4	23.2 ± 0.8
50	14.2 ± 1.0	11.1 ± 0.2	17.7 ± 0.8	21.7 ± 0.5

All migration values are expressed as absolute migration areas (cm^2) ± s.e. mean. Areas smaller than in earlier reading are underlined.

An interference with cell surface charge could therefore alter leucocyte migration. Using the technique of cell electrophoresis preliminary studies have demonstrated that NSAID modify the surface charge of human PMNs and lymphocytes (Brown, unpublished observations).

In rheumatoid arthritis, the PMN is the predominant cell in articular effusions. Phagocytosis of immune complexes by PMNs with concurrent release of lysosomal enzymes probably mediates the proliferative and destructive changes in rheumatoid arthritis. Inhibition of PMN migration from the vascular endothelium to the inflammatory focus may reduce disease activity. In the present study the migration of PMNs, but not of lymphocytes was inhibited by concentrations of salicylate (Ansell, 1963), phenylbutazone (Burns, Rose, Chenkin, Goldman, Schubert & Brodie, 1953), and indomethacin (Hvidberg, Lausen & Jansen, 1972) required for anti-inflammatory effects. We conclude that an inhibition of PMN migration may be an important anti-inflammatory property of certain NSAID.

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