Action of nonsteroidal, anti-inflammatory drugs on human and rat peripheral leucocyte migration *in vitro*

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SUMMARY Using an *in vitro* system of cell migration from glass capillary tubes, the nonsteroidal, anti-inflammatory agents sodium salicylate, aspirin, ibuprofen, phenylbutazone, and indomethacin were shown to inhibit the migration of human peripheral leucocytes in a dose-related manner. This drug action was not confined to one species, as shown by the modification of rat peripheral leucocyte motility by sodium salicylate and aspirin. The relevance of the human findings to the clinical effectiveness of these agents is discussed.

An increase in capillary permeability followed by a leucocytic exudation to remove the inciting agent and/or damaged tissue, constitute two of the main morphological events of an inflammatory response. Leucocyte participation, in particular the polymorphonuclear cells, is not only confined to an acute inflammatory episode, such as gout, but also extends to the chronic inflammatory reaction as in rheumatoid arthritis. Should leucocyte infiltration into an inflammatory focus be restricted, then the powerful mediators that these cells are capable of releasing would be impeded and the inflammation suppressed.

The inhibition by indomethacin of an inflamed dog knee joint, initiated by the deposition of sodium urate crystals, has been correlated with a decreased fluid exudate and polymorphonuclear cell accumulation (Phelps and McCarty, 1967). The authors concluded that the mode of drug action was a direct inhibitory effect on the movement of the invading polymorphonuclear cell.

In the rat, carrageenan-induced oedema Van Arman *et al.*, (1971) showed that an anti-oedematous dose of indomethacin reduced the number of mobilized neutrophils. Similarly, an analogous finding was obtained with aspirin (Vinegar *et al.*, 1971) and other nonsteroidal anti-inflammatory drugs (Di Rosa *et al.* 1971; Nakanishi and Kazuhiro, 1974). However, objection to the hypothesis that nonsteroidal, anti-inflammatory drugs (NSAID) inhibited cell movement, stemmed from the failure

of indomethacin to reduce polymorphonuclear cell infiltration in the local Schwartzman reaction (Van Arman *et al.*, 1970), together with the failure of phenylbutazone and indomethacin to modify the *in vitro* chemotactic activity of rabbit and human polymorphonuclear cells (Keller and Sorkin, 1965; Borel, 1973).

Using a technique originally described in the Leucocyte Migration Test (George and Vaughan, 1962), the action of several NSAID agents were investigated on the *in vitro* migration of human and rat peripheral leucocytes. Previously a similar system had shown NSAID to suppress the movement of rat peritoneal leucocytes (Di Rosa, 1974).

Methods

PREPARATION OF HUMAN PERIPHERAL LEUCOCYTES
From each subject, 20 ml of blood were collected from a cubital vein into a syringe containing 400 units heparin. After centrifugation at 800 *g* for 10 minutes, the plasma and leucocyte layer were transferred to a clean syringe which was stood upright in a 37°C incubator. After 1 hour of incubation, during which most of the remaining erythrocytes had spontaneously sedimented, the leucocyte-rich plasma was aspirated and centrifuged at 240 *g* for 10 minutes. The leucocytes were washed twice with Hanks's Balanced Salt Solution (HBSS) (pH 7.35) and adjusted to a final concentration of 4×10^7 cells/ml.

PREPARATION OF RAT PERIPHERAL LEUCOCYTES
Blood was obtained from ether-anaesthetized male

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Wistar rats, either by withdrawal from the ventral aorta or by cardiac puncture. Unlike human erythrocytes, rat erythrocytes do not sediment spontaneously and, consequently, 3% solutions of the sediment-inducing agent dextran (MW 200 000–250 000), containing 0.9% NaCl and 20 units/ml heparin, were added in equal volumes to each rat's blood sample. After incubation at 37°C for 1 hour, leucocytes from 15 rats were pooled. After washing twice with HBSS, the final cell suspension was adjusted to a concentration of 1×10^8 cells/ml.

MIGRATION OF LEUCOCYTES FROM CAPILLARY TUBES

This method was modified from that described by Maini *et al.* (1973). Leucocytes, prepared as above, were packed into glass capillary tubes (Drummond, diameter 0.4 mm, length 75 mm), sealed at one end, and centrifuged at 2400 *g* for 5 minutes. Each capillary tube was cut at the resultant cell-supernatant interface and its leucocyte pellet mounted into migration chambers (Sterilin) containing tissue culture media composed of 10% horse serum, Eagles's medium (MEM) and 100 IU/ml penicillin-streptomycin, buffered with 20 mmol/l Hepes buffer to a final pH of 7.35. Chambers were rendered air-tight by applying cover-slips on to previously greased edges. At least four replicate chambers were prepared for every drug concentration investigated. All migration plates, which contained 12 chambers each, were carefully layered on to perfectly horizontal incubator shelves and left for 20 hours at 37°C.

At the end of this incubation period, the cells had migrated out from the tube along the floor of the chamber in a fan-like fashion. Each cell migration was projected on to paper by a photographic enlarger, its image drawn, and area measured by planimetry.

There was no change in the media pH of either control (i.e. chambers without a drug present) or drug-containing chambers. Cell viability, as determined by the trypan-blue exclusion test, was always >95%.

PREPARATION OF DRUG SOLUTIONS

All NSAID investigated were dissolved and buffered in tissue culture media to a final pH of 7.35 and passed through a 0.45 μ m filter into sterile glassware. Each drug solution was prepared freshly before starting a migration study and serial dilutions made under aseptic conditions.

Subjects studied

Initial studies had found the human leucocyte migration response to an anti-inflammatory agent to be independent of an age or sex difference. Each

drug was investigated on leucocytes prepared from at least 6 healthy subjects of both sexes, whose ages varied from 20–50 years and who were not receiving any current drug therapy.

Results

All *in vitro* effects of the NSAID on cellular migration were expressed as a percentage inhibition or stimulation of the control migration areas. Student's 't' test was used to assess any significant difference from control and drug-containing chambers.

In the human studies, sodium salicylate, aspirin, ibuprofen, indomethacin, and phenylbutazone inhibited cell movement in a dose-related manner. Phenylbutazone at 1×10^{-4} mol/l induced a mean 14% inhibition of migration ($P < 0.002$), while at lower concentrations no further suppression of cell movement was demonstrable (Fig. 1a). In contrast to the fairly consistent migration response of the individual's leucocytes to phenylbutazone was the response elicited towards indomethacin; inhibition of migration at 1×10^{-3} mol/l varying from 34% to 100% of the control values (Fig. 1b). Compared with the other NSAID, indomethacin produced the most persistent inhibitory activity over a large dilution spectrum. Although indomethacin at 1×10^{-5} mol/l produced a mean 5% inhibition of migration (nonsignificant), leucocytes from 5 individuals expressed a mean 10% inhibition ($P < 0.02$) at this dilution.

Ibuprofen (Fig. 1c) elicited a mean 94% inhibition of leucocyte migration at 1×10^{-2} mol/l, 36% inhibition at 1×10^{-3} mol/l ($P < 0.001$), and 14% inhibition at 5×10^{-4} mol/l ($P < 0.01$), further dilutions failing to modify leucocyte motility. At the highest concentration investigated 1×10^{-2} mol/l aspirin (Fig. 1d) produced a wide variation in the inhibitory migration response (40–100%), a finding that was not comparable with the salicylate studies (Fig. 1e). Also, at 1×10^{-3} mol/l aspirin induced a mean 27% inhibition of leucocyte movement ($P < 0.002$) compared to a mean 15.5% produced by salicylate at the equivalent concentration ($P < 0.01$). Apart from one experiment, in which aspirin stimulated leucocyte motility at 1×10^{-4} mol/l and 1×10^{-5} mol/l, no further significant modification of cell movement was recorded from either the salicylate or aspirin studies at lower drug dilutions.

Paracetamol (Fig. 1f) showed a mean 49% inhibition of migration at 1×10^{-2} mol/l and a 22% inhibition at 5×10^{-3} mol/l ($P < 0.01$). Although not shown graphically, over the spectrum 1×10^{-2} mol/l to 1×10^{-8} mol/l para-hydroxybenzoic acid produced no statistical modification of leucocyte motility.

Fig. 1 Effect in vitro of (a) phenylbutazone, (b) indomethacin, (c) ibuprofen, (d) aspirin, (e) salicylate, and (f) paracetamol on human peripheral leucocyte migration. Each curve illustrates the leucocyte migration response of one individual and every point on that curve the mean response of at least four migration areas. The standard error of the mean (not shown) for each determination was always < 1.0.

Taking the molar concentration of salicylate which produced 50% inhibition as unity, the relative potencies of the other NSAID were calculated graphically from their ID₅₀ values. From the Table the list in decreasing order of potency was indomethacin > phenylbutazone > ibuprofen > aspirin > salicylate.

Table Relative potencies of NSAID on total human leucocyte migration *in vitro*

NSAID	Potency figure	Molar concentration at ID ₅₀ (mol/l)
Salicylate	1	8.0×10^{-3}
Aspirin	2.5	3.2×10^{-3}
Ibuprofen	5	1.7×10^{-3}
Phenylbutazone	11	7.0×10^{-4}
Indomethacin	13	6.3×10^{-4}

A potency figure of 1 was arbitrarily assigned to the concentration of salicylate which produced 50% inhibition (ID₅₀) of leucocyte migration. The relative potencies of the other NSAID were calculated graphically from their ID₅₀ values

NSAID = nonsteroidal anti-inflammatory drugs.

In the animal studies two different groups of rats were used. The first was comprised of male Wistar rats weighing 220–290 g, and the second of rats weighing 350–430 g of the same sex and strain. At least eight capillary tubes were prepared for each drug dilution.

The extent of suppression induced by sodium salicylate at a range of 1×10^{-2} mol/l to 1×10^{-3} mol/l on the *in vitro* migration of rat peripheral leucocytes was well complemented in both groups (Fig. 2). Leucocytes of group 2 were persistently inhibited in movement at lower concentrations (approximately 20% inhibition from 1×10^{-3} mol/l to 1×10^{-5} mol/l ($P < 0.05$), while cells of group 1 were stimulated to a 23% increase ($P < 0.001$) of their control migration areas in 1×10^{-5} mol/l salicylate.

Though aspirin elicited a mean 52% migration inhibition at 5×10^{-3} mol/l in both groups, thereafter the cellular response of each group to lower concentrations was markedly varied. Group 1 leucocytes showed marked stimulation, culminating in a 31% increase of migration at 1×10^{-5} mol/l ($P < 0.001$), while the migration responsiveness of group 2 leucocytes produced an inconsistent profile.

Discussion

All NSAID investigated inhibited the *in vitro* migration of human and rat peripheral leucocytes in a dose-related manner. The finding that indomethacin was the most potent drug studied agrees with the work of Di Rosa (1974). However, leucocytes used by Di Rosa were collected from the rat peritoneal cavity and were comprised mainly of

Fig. 2 Effect of aspirin and salicylate *in vitro* on rat peripheral leucocyte migration. Group 1 was comprised of pooled peripheral leucocytes isolated from 15 250 g male Wistar rats; and group 2 a concentrate of peripheral leucocytes from 15 400 g rats of the same sex and strain. Each point on a curve represents the mean response from at least eight determinations.

a mononuclear cell population. The migration of these cells was shown to be more susceptible to NSAID inhibitory action than that of the polymorphonuclear cell. This is in contrast to the results obtained from the present investigation in which the outer cell migratory boundary, consisting mainly of polymorphonuclear cells, was the area most vulnerable to drug action. A species difference may account for the disparity of results.

Using the Boyden chamber, Phelps and McCarty (1967) showed that indomethacin suppressed the migration of canine and human polymorphonuclear cells. Even though maximum inhibition occurred at 8×10^{-7} mol/l the suppressive activity persisted in drug concentrations as low as 1×10^{-11} mol/l. These results vary with the ones reported in this study where statistical migration inhibition induced by indomethacin was not seen below 5×10^{-5} mol/l.

A phenomenon that was prevalent in the rat study and occasionally seen in the human experiments was a stimulation of leucocyte migration. Lymphocytes in man constitute approximately 20–40% and polymorphonuclear cells 60–80% of the total leucocyte population, the converse being found in the rat. Hydrocortisone *in vitro* has a dual action on human leucocyte motility, the steroid stimulating the movement of total leucocytes, but inhibiting polymorphonuclear cell migration (Stevenson, 1973). It may therefore be feasible that NSAID exert a selective stimulatory action on a predominantly lymphocyte population and an inhibitory action on a migrating polymorphonuclear cell population. Experiments have begun to examine this possibility.

How pertinent these *in vitro* results are to an *in vivo* action can only be speculated upon. In man, salicylate has been calculated to be clinically effective at blood levels of 25–30 mg/100 ml (Ansell, 1963) and 30–50 mg/100 ml (Smith *et al.*, 1946), which is comparable with an *in vitro* concentration of 1.7×10^{-3} mol/l to 2.5×10^{-3} mol/l, respectively. Similarly, on a molar basis, therapeutic concentrations of 3×10^{-4} mol/l phenylbutazone (Burns *et al.*, 1953) and 1×10^{-4} mol/l ibuprofen (Adams, 1974) are frequently found. At these therapeutic levels cell motility in this study was not totally inhibited, but only partly suppressed, a situation analogous to the use of NSAID in rheumatoid arthritis, where the disease is restrained but not arrested. From several reports stating the clinical effectiveness of indomethacin in blood to be approximately 1×10^{-5} mol/l to 1×10^{-6} mol/l (Caruso, 1971; Champion *et al.*, 1972; Hvidberg *et al.*, 1972) it appears that the inhibitory activity shown by this agent *in vitro* transgressed a therapeutic level.

Paracetamol induced inhibitory activity at concentrations surpassing those obtained in body fluids, while the ineffectiveness of para-hydroxybenzoic acid, in modifying leucocyte motility, added support to the hypothesis that an abrogation of cell migration by NSAID was an anti-inflammatory action.

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