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Inhibition of *in Vitro* Neutrophil Chemotaxis and Spontaneous Motility by Anti-Inflammatory Agents[1] (39518)

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Acutely inflamed tissues are rich in neutrophils and current evidence suggests that chemotactic stimuli may be responsible for the entry of this cell into sites of inflammation (1, 2). Many anti-inflammatory agents are available which dampen the inflammatory response. One possible mode of action of these drugs could be to modulate the response of the neutrophil to chemotactic stimuli. In order to test this hypothesis we undertook to study *in vitro* the effect of anti-inflammatory agents on the responsiveness of rabbit and rat peritoneal and human blood neutrophils to chemotactic material obtained from *Escherichia coli* culture filtrates. To study the specificity of these agents on chemotactic stimuli this study also investigated the effect of anti-inflammatory agents on neutrophil spontaneous motility.

Materials and methods. Drugs were obtained from the following sources: triamcinolone acetonide, triamcinolone acetonide dipotassium phosphate, halcinonide, ciclo-profen, procainamide, and niflumic acid from Squibb; diazepam from Roche; hydrocortisone succinate, prednisolone, and methyl prednisolone from Upjohn; hydrocortisone acetate and indomethacin from Merck; cytoxan from Mead Johnson; imuran from Burroughs-Wellcome; chloroquine phosphate from Winthrop; ibuprofen from Boots Pure Drug; phenylbutazone from Ciba-Giegy; naproxen from Syntex; aspirin from Monsanto; sudoxicam from Pfizer; ethynylestradiol and progesterone from Schering.

Peritoneal exudates containing 85-95% neutrophils were induced in female New Zealand white rabbits with 0.02% shellfish glycogen (Sigma) as previously described (3). Rat peritoneal exudates, also 85-95% rich in neutrophils, were induced by injecting 20 ml of 12% sterile sodium caseinate (DIFCO) into the peritoneal cavity and aspirating the exudate 24 hr later through the use of an 18-gauge needle. Human blood neutrophils 94-100% pure were obtained by the Ficoll-Hypaque method (4) from healthy males and females who had not taken any medication for at least 1 month prior to donation of the blood.

The neutrophils obtained from the above three sources were washed and suspended at a concentration of 5×10^6 cells/ml in Hanks' balanced salt solution, pH 7.2, containing 0.02 *M* Tris buffer and 0.2% bovine serum albumin (BSA, Fraction IV, Sigma). The test drugs were dissolved at twice their final concentration in the above buffer without BSA, the Ph readjusted to 7.2 if necessary, and the solution diluted 1:1 with the cell suspension so that the final cell concentration was 2.5×10^6/ml in 0.1% BSA. This cell suspension was added directly to the chemotaxis chambers without a preincubation step unless otherwise indicated. The control consisted of a cell suspension free of drug but containing the vehicle used to dissolve the test drug.

The chemotactic agent for the rabbit and rat neutrophil experiments was a butanol extract of an *E. coli* culture filtrate, diluted 1/8000 with buffer when used with rabbit neutrophils, and 1/500 when used with rat neutrophils. The crude supernatant of the above *E. coli* culture filtrate diluted 1/25 served as the positive chemotactic factor in the human blood neutrophil experiments.

Chemotaxis and spontaneous motility

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[2] Some of the data presented here have been taken from a dissertation submitted by C. H. Rosen in partial fulfillment of the requirements for the degree of Bachelor of Arts, Department of Biology, Princeton University.

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were measured by the Boyden chamber method as previously described (5). For chemotaxis experiments a Millipore filter of 0.65- or 0.8-μm average pore size divided the chemotaxis chamber into the upper and lower compartments. Cell suspension with or without added drug was placed in the upper compartment immediately after injection of bacterial chemotactic factor into the bottom compartment. Each experiment was carried out in triplicate along with duplicate chambers that contained only buffer in the bottom compartment for assessment of background activity. The chambers were incubated for 90 min at 37°, and the filters were removed and histologically stained for microscope scoring at 40× magnification. Three random fields of each filter were counted consisting of all the cells that moved into the filter from the initial top monolayer. The mean activity in the three filters from each experimental condition was then determined, and the background activity was subtracted. The final results, unless otherwise indicated, are reported as the molar concentration of drug which inhibited chemotaxis by 50% (I_{50}).

Spontaneous motility was measured in triplicate exactly as outlined above except that a 5-μm average pore size Millipore filter was used and only buffer was placed in the bottom compartment.

None of the drugs used in this study affected neutrophil viability as determined by the ability of the cells to exclude eosin-Y.

Results. Table I demonstrates that both steroidal and nonsteroidal anti-inflammatory agents inhibit neutrophil chemotactic responsiveness to bacterial chemotactic factor. Except for indomethacin in the human neutrophil system, the inhibition of chemotaxis observed with a particular drug was consistent. Each drug reported in Table I was assayed at least twice. Phenylbutazone was assayed twice with human blood neutrophils with identical I_{50} values being observed each time. This drug was assayed another four times with rabbit neutrophils and each time there was significant inhibition of chemotaxis at the concentrations tested. Prednisolone at a concentration of 1×10^{-5} *M* was employed in each experiment as a positive drug control. This drug also has consistently inhibited chemotaxis.

TABLE I. EFFECT OF ANTI-INFLAMMATORY AGENTS ON NEUTROPHILS FROM RABBIT AND RAT PERITONEAL EXUDATES AND HUMAN BLOOD.

Drug[a]	Neutrophil source[b]		
	Rabbit	Rat	Human
Hydrocortisone succinate	4×10^{-6}	2×10^{-6}	5×10^{-6}
Hydrocortisone acetate	No effect	NT[c]	NT
Methyl prednisolone	4×10^{-6}	5×10^{-7}	NT
Prednisolone	4×10^{-6}	NT	1×10^{-7}
Triamcinolone acetonide	1×10^{-5}	4×10^{-6}	4×10^{-6}
Triamcinolone acetonide dipotassium phosphate	$>10^{-3}$	1×10^{-4}	6×10^{-3}
Halcinonide	7×10^{-5}	NT	3×10^{-5}
Cytoxan	2×10^{-5}	1×10^{-4}	1×10^{-5}
Imuran	7×10^{-5}	7×10^{-5}	7×10^{-5}
Sudoxicam	3×10^{-4}	1×10^{-6}	NT
Naproxen	1×10^{-6}	3×10^{-4}	6×10^{-5}
Phenylbutazone	9×10^{-7}	3×10^{-7}	1×10^{-5}
Cicloprofen	1×10^{-5}	NT	5×10^{-6}
Ibuprofen	5×10^{-6}	NT	2×10^{-5}
Niflumic acid	4×10^{-7}	NT	2×10^{-7}
Aspirin	1×10^{-2}	1×10^{-2}	2×10^{-4}
Chloroquine PO_4[d]	No effect	NT	No effect
Indomethacin[d]	No effect	No effect	Variable

[a] Test agents were mixed with the cells and immediately added to the chemotaxis chambers and assayed as indicated in Materials and Methods.

[b] Values are reported as I_{50} (*M*), the molar concentration of drug which inhibited chemotaxis by 50%.

[c] Not tested.

[d] Highest concentration tested = 1×10^{-4} *M*.

The data in Table I also demonstrate that the two immunosuppressive agents, imuran and cytoxan, inhibit chemotaxis.

The steroidal anti-inflammatory agent hydrocortisone succinate significantly inhibited neutrophil chemotaxis as shown in Table I, whereas the acetate of hydrocortisone had no effect at concentrations up to $1 \times 10^{-3} M$. Note also that the anti-inflammatory steroid triamcinolone acetonide inhibited chemotaxis whereas its phosphate ester was effective only at very high concentrations. It should be pointed out that diazepam and procainamide, drugs totally unrelated to anti-inflammatory agents, were tested and found to have no effect on neutrophil chemotaxis. The steroid hormones progesterone and ethinylestradiol were tested on human blood neutrophils and found to have no effect.

Indomethacin, at concentrations up to $1 \times 10^{-4} M$, had no effect on rabbit and rat neutrophil chemotaxis even when preincubated with the cells for 1/2 hr at 37° prior to their addition to the chemotaxis chambers. This is shown in Table II with rabbit peritoneal neutrophils where it can be seen that indomethacin at the concentrations tested did not inhibit chemotaxis even upon preincubation with the cells. Similar negative results were obtained with rat peritoneal neutrophils. With respect to the human neutrophil system, indomethacin at concentrations below $1 \times 10^{-4} M$ had no appreciable effect on chemotaxis; at $1 \times 10^{-4} M$ indomethacin gave variable results.

Aspirin, when added to neutrophil suspensions and placed immediately in the chemotaxis chamber, inhibited chemotaxis of rat and rabbit peritoneal neutrophils only at extremely high concentrations (Table I). However, Fig. 1 illustrates that preincubation of aspirin with rabbit peritoneal neutrophils for 1/2 hr at 37° prior to their addition to the chemotaxis chamber increases inhibition of chemotaxis 100-fold. The I_{50} for aspirin was $2 \times 10^{-2} M$, however, it increased to $2 \times 10^{-4} M$ when the drug was in contact with the cells prior to their addition to the chemotaxis chambers. Similar results, not shown here, were obtained using rat neutrophils. It should be pointed out that preincubation of neutrophils with triamcinolone acetonide dipotassium phosphate did not improve its ability to inhibit chemotaxis.

TABLE II. LACK OF EFFECT OF INDOMETHACIN ON RABBIT PERITONEAL NEUTROPHIL CHEMOTAXIS.

Drug	Experimental condition[a]	
	No preincubation	1/2-Hr preincubation
Control		
No drug added	140 ± 2	120 ± 12
Indomethacin		
$1 \times 10^{-6} M$	149 ± 8	107 ± 3
$1 \times 10^{-5} M$	138 ± 10	110 ± 11
$1 \times 10^{-4} M$	126 ± 8	109 ± 10
Prednisolone		
$1 \times 10^{-5} M$	63 ± 3	47 ± 3

[a] Test agents were mixed with the cell suspensions and either added immediately to the upper compartment of the chemotaxis chamber or incubated for 1/2 hr at 37° and then added to the chambers. The chambers were then incubated for 90 min at 37° and the activity determined as indicated in Materials and Methods. The chemotactic activity is reported as cells per three high-powered fields minus background activity ± SEM.

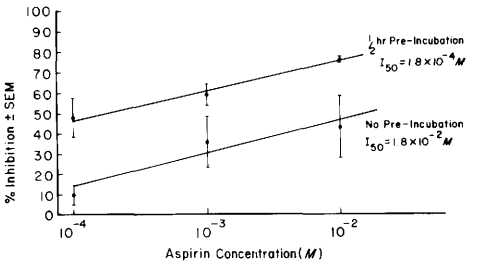

FIG. 1. Inhibition of chemotaxis by aspirin. The drug was mixed with cell suspension (rabbit peritoneal neutrophils) and either added immediately to the upper compartment of the chemotaxis chambers or incubated for 1/2 hr at 37° and then added to the chambers. The chambers were then incubated for 90 min at 37° and the activity determined as indicated in Materials and methods.

Table III presents our findings with respect to the effect of anti-inflammatory agents on neutrophil spontaneous motility. Similar results were obtained with rabbit peritoneal neutrophils; rat neutrophils were not used in the motility studies. The data in Table III demonstrate that the anti-inflammatory agents fall into two categories with respect to their effect on neutrophil movement. All the steroidal anti-inflammatory agents tested affected neutrophil spontaneous motility at concentrations which inhibited chemotaxis (Table I). The immunosuppressive agents cytoxan and imuran also

TABLE III. EFFECT OF ANTI-INFLAMMATORY AGENTS ON HUMAN BLOOD NEUTROPHIL SPONTANEOUS MOTILITY.

Drug[a]	Spontaneous motility I_{50} (M)[b]
Prednisolone	4×10^{-5}
Triamcinolone acetonide	5×10^{-5}
Hydrocortisone succinate	4×10^{-5}
Halcinonide	2×10^{-4}
Cytoxan	1×10^{-3}
Imuran	2×10^{-4}
Phenylbutazone	3×10^{-4}
Niflumic acid	No effect
Naproxen	No effect
Cicloprofen	No effect
Ibuprofen	No effect
Aspirin	No effect

[a] Test agents were mixed with the cells and immediately added to the chemotaxis chamber for the measurement of spontaneous motility. A 5-μm Millipore filter was used and only buffer placed in the bottom compartment of the chamber as indicated in Materials and methods.

[b] I_{50} (M) = the molar concentration of drug which inhibits spontaneous motility by 50%.

inhibited spontaneous motility (Table III). However, the nonsteroidal anti-inflammatory agents tested, with the exception of phenylbutazone, specifically inhibited the chemotactic responsiveness of the neutrophil at concentrations that had no effect on spontaneous motility.

Discussion. This report is the first demonstration that a wide variety of anti-inflammatory drugs inhibits the *in vitro* chemotactic responsiveness of the neutrophil. This effect was specific for anti-inflammatory and immunosuppressive agents. Drugs not employed as anti-inflammatory or immunosuppressive agents had no effect on chemotaxis.

Our results are in agreement with those reported by Ward (6, 7) for hydrocortisone, methyl prednisolone, phenylbutazone, and aspirin. He also reported that chloroquine chloride inhibited rabbit peritoneal neutrophil chemotaxis. We tested chloroquine phosphate (the chloride salt was not available) and found it to be inactive.

The phosphate ester of triamcinolone acetonide was found to be active only at very high concentrations (Table I), whereas the parent molecule was considerably more active. This is in keeping with our findings with chloroquine phosphate and suggests that the phosphate group renders the molecule impermeable to the cell membrane. The acetate derivative of hydrocortisone was found to be inactive, whereas hydrocortisone itself effectively inhibited neutrophil chemotaxis. This is in keeping with the report that hydrocortisone acetate is impermeable to leukocyte membranes (8).

Our results (and those of Ward (6, 7)) are in contrast to those reported by Borel (9) who tested seven anti-inflammatory agents for their effect on rabbit peritoneal neutrophil chemotaxis and found no effect with phenylbutazone and naproxen and variable effects with hydrocortisone succinate and aspirin. The reason or reasons for these discordant results are essentially unknown. Conditions under which neutrophil migration were assayed might well explain the divergence in results between Borel and ourselves. We are currently investigating this problem in an attempt to resolve the reasons for our different findings with respect to these agents.

Indomethacin was found to have no significant effect on *in vitro* rabbit and rat neutrophil chemotaxis and had a variable effect on human neutrophil chemotaxis, the reasons for which are currently under investigation. Similar results were reported by Borel (9) using rabbit neutrophils. Indomethacin is a potent inhibitor or carrageenin-induced rat paw edema (10) and adjuvant-induced arthritis in rats (11) and is used clinically as an effective anti-inflammatory agent. Our findings with respect to indomethacin and neutrophil chemotaxis suggest that the anti-inflammatory activity of this agent may possibly not entail a direct effect on neutrophil migration.

On the other hand, many of the anti-inflammatory agents listed in Table I were found to inhibit *in vitro* neutrophil chemotaxis at concentrations that may have *in vivo* clinical significance (12–15). This suggests that one of the possible mechanisms of their anti-inflammatory activity is to inhibit neutrophil migration. We are currently studying an *in vivo* model of chemotaxis to investigate the significance of these *in vitro* findings.

Our data also demonstrate that, for most of the agents we tested, the source of the neutrophil is not critical for the observation

of a drug effect on chemotaxis; an effective agent inhibited neutrophil chemotaxis from rabbit and rat peritoneal exudates and human blood and an ineffective agent had no effect on neutrophils from any of these three sources.

This study has shown also that with respect to neutrophil chemotaxis the anti-inflammatory agents tested, with the exception of phenylbutazone, fell into two categories. The effective steroidal anti-inflammatory agents which were tested inhibited both chemotaxis and spontaneous motility, whereas the effective nonsteroidal agents tested inhibited only chemotaxis. It is interesting to note that aspirin which inhibited neutrophil chemotaxis, albeit at very high concentrations, had no effect at all on neutrophil motility, even at concentrations as high as 1×10^{-2} *M*. The two immunosuppressive agents tested, cytoxan and imuran, inhibited both chemotaxis and motility. These results suggest that the effect of steroidal anti-inflammatory and immunosuppressive agents on neutrophil chemotaxis is probably due to the effect of these agents on the cell's spontaneous motility. On the other hand, the nonsteroidal anti-inflammatory agents may act on parameters concerned with the ability of the neutrophil to respond to chemotactic stimuli.

Summary. A number of anti-inflammatory agents were tested for their effect on the chemotaxis responsiveness and on the spontaneous motility of neutrophils obtained from rabbit and rat peritoneal exudates and human blood. The majority of these agents, both steroidal and nonsteroidal, inhibited the chemotactic responsiveness of neutrophils obtained from all three sources to a bacterial chemotactic factor. The effective steroidal anti-inflammatory agents tested inhibited both chemotaxis and spontaneous motility, whereas the nonsteroidal agents, with the exception of phenylbutazone, inhibited only chemotaxis and were without effect on motility.

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