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Antirheumatic Drug Effects on Neutrophil Response to Chemotactic Factors: A Comparison of Analytical Techniques[1] (41631)

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Abstract. A recently reported technique employing the leukotactic index which represents all migrating cells in *in vitro* neutrophil chemotaxis systems, was compared to the leading front technique for assaying antirheumatic drug effects on this important neutrophil function. Normal human neutrophils were treated with therapeutic concentrations of aspirin, gold sodium thiomalate, D-penicillamine, and azathioprine. The responses of these cells and of control cells to neutrophil-immune complex-serum-derived chemotactic factors were assayed in Boyden chambers. Significant ($P < 0.05$) inhibition was observed by the leading front technique only for D-penicillamine at high concentrations. Significant ($P < 0.01$) inhibition was seen with D-penicillamine at therapeutic plasma levels with the leukotactic index technique. Gold sodium thiomalate and aspirin at high concentrations also produced significant ($P < 0.01$ and $P < 0.05$) inhibition of chemotaxis as assayed by the leukotactic index procedure. Azathioprine had no significant effects when studied with either technique. These results indicate that the leukotactic index may be a more sensitive technique for quantitating neutrophil migration in response to chemotactic factors and may therefore provide useful additional information for determining the effects of antirheumatic drugs on this important neutrophil function.

The use of the micropore filter assay of Boyden to quantitate the *in vitro* migration of human neutrophils is a well-accepted technique (1). Several modifications have been incorporated into this assay to facilitate the measurement of migrating cells. These modifications involve measurement of the distance of migration of the fastest cells in the filters (the leading front technique) (2), and a more sensitive system incorporating the number of cells and their distance of migration into an index of leukocyte migration (the leukotactic index) (3).

We have adapted the leukotactic index as a more sensitive method of measurement in our *in vitro* neutrophil migration assay and expanded it for use in drug studies involving certain antirheumatic agents. In the studies reported here, aspirin, gold sodium thiomalate, D-penicillamine, and azathioprine in therapeutically attainable plasma levels were added to our *in vitro* systems and their effects on neutrophil migration were determined using the leukotactic index and leading front techniques.

Materials and Methods. *Cell isolation and insoluble IgG aggregate preparation.* Normal human neutrophils (PMN) were isolated in 70–90% purity as previously described (4) and resuspended in Earle's balanced salt solution. Insoluble immunoglobulin G aggregates (IgG Agg) were prepared by heat aggregation at 63°C from Cohn Fraction II human gamma globulin (U.S. Biochemical Corp., Cleveland, Ohio) as previously described (5). The IgG Agg were assayed for protein content by the Lowry technique (6) and resuspended to 2.4 mg/ml.

Chemotaxis. Neutrophil-IgG Agg-serum derived chemotactic factors (CTF) were generated from a suspension of 2.5×10^6 PMN/ml and 1.2 mg/ml IgG Agg in 10% autologous serum incubated for 30 min at 37°C on a rotating wheel. Supernatants obtained from consecutive 10 min centrifuging at 120*g* and 10,000*g* were frozen and stored at −70°C for use as the positive stimulus in further chemotactic studies.

Chemotaxis was assayed in Boyden chambers separated into upper and lower compartments with 3-μm pore size micropore fil-

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ters (Millipore Corp., Bedford, Mass.). Neutrophils (3×10^6/ml) were supplemented with 6 mg/ml bovine serum albumin (BSA) and added to the upper compartments. Lower compartments contained the chemoattractant consisting of 10% CTF; 10% autologous serum.

In vitro exposures of aspirin, gold sodium thiomalate (Myochrisine, Merck Sharp & Dohme, West Point, Pa.), D-penicillamine (Cuprimine, Merck Sharp & Dohme, West Point, Pa.), and azathioprine (Imuran, Burroughs Wellcome, Research Triangle Park, N.C.), were studied in therapeutically attainable plasma levels for their effects on neutrophil migration. Test cells were suspended, after the isolation procedure, in the following concentrations of drug: 2, 20, 200, or 2000 μg/ml aspirin; 1, 10, 100, or 1000 μg/ml gold sodium thiomalate; or 1, 10, or 100 μg/ml D-penicillamine. Test and untreated cells were incubated for 30 min at 37°C prior to addition to the upper compartments of Boyden chambers. Cells suspended in 0.2, 2, or 20 μg/ml azathioprine and untreated cells were incubated for 30 min at 37°C prior to the isolation procedure.

The Boyden chambers were incubated for 90 min in a humid environment at 37°C. Filters were removed, fixed in methanol, stained in hematoxylin, cleared in xylene, and permanently mounted on glass slides.

Migration distances (MD) were determined at 400× magnification for the leading front of cells (2) and for the average population of cells traveling through the filter as calculated by the leukotactic index (3). Cell counts were done at every 10 μm depth of the filter for determination of the leukotactic index (LI) which was calculated with the following formula:

$$LI = \frac{\sum_{i=1}^{n} MD_i \times \text{No. PMN}_i}{\text{Total No. PMN}}.$$

The farthest field observed was recorded as the leading front.

A television system, electronic bacterial colony counter, and data interfacer (Model 980, Artek Systems Corp., Farmington, N.Y.) were adapted to a microscope to facilitate cell counting and data entry for calculations done on a TI-59 calculator (Texas Instruments, Lubbock, Tex.). The value of the leading front or leukotactic index per filter was expressed as a mean of three measured fields.

Percentage of control chemotaxis was calculated for both determinations by comparing test cell values to the mean untreated cell values for each experimental day utilizing the formula: Percentage control = test cell migration/$\bar{x}$ untreated cell migration × 100. Data were analyzed by one-way analysis of variance and applied to the Tukey's multiple comparison test to determine significantly different relationships.

Results. Untreated neutrophils migrated a mean distance of 118 μm through the filters as analyzed by the leading front technique ($118 \pm 2 = \bar{x} \pm$ SEM; $n = 40$ for all cell populations included in this study). The average ($\bar{x} \pm$ SEM) migration distance for these cells calculated by the leukotactic index technique was 40 ± 2 μm. Table I shows the effects of aspirin on neutrophil response to chemotactic factors analyzed by the leading front and leukotactic index techniques with control cells for this experiment compared to cells treated with different concentrations of the agent. As shown in this table, a significant ($P < 0.05$) inhibition of response to chemotactic factors was seen only with the leukotactic index at the 2000 μg/ml concentration. In Table II, gold sodium thiomalate is shown to have caused significant ($P < 0.01$) inhibition of re-

TABLE I. EFFECTS OF ASPIRIN ON NEUTROPHIL RESPONSE TO CHEMOTACTIC FACTORS ANALYZED BY LEADING FRONT AND LEUKOTACTIC INDEX TECHNIQUES

Drug concentrations			$\bar{x}$ percentage of control[a] ± SEM	
μg/ml	Molarity	N	Leading front[c]	Leukotactic index[d]
0.0	0	10	100.0 ± 1.3	100.0 ± 3.2
2.0	6.7×10^{-6}	10	92.0 ± 3.0	85.3 ± 7.6
20.0	6.7×10^{-5}	10	98.1 ± 2.0	102.0 ± 10.5
200.0[b]	6.7×10^{-4}	10	86.8 ± 7.9	76.2 ± 13.6
2000.0	6.7×10^{-3}	10	81.2 ± 12.3	60.0 ± 8.5*

[a] Percentage of control = test cell migration/$\bar{x}$ untreated cell migration × 100.
[b] Therapeutically attainable plasma level.
[c] One-hundred percent of control = 119 ± 5 μm ($\bar{x} \pm$ SEM).
[d] One-hundred percent of control = 29 ± 3 μm ($\bar{x} \pm$ SEM).
* Significantly different from 0.0 μg/ml at $P < 0.05$.

TABLE II. EFFECTS OF GOLD SODIUM THIOMALATE ON NEUTROPHIL RESPONSE TO CHEMOTACTIC FACTORS ANALYZED BY LEADING FRONT AND LEUKOTACTIC INDEX TECHNIQUES

Drug concentrations			$\bar{x}$ percentage of control[a] ± SEM	
μg/ml	Molarity	N	Leading front[c]	Leukotactic index[d]
0.0	0	10	100.0 ± 1.1	100.0 ± 2.9
1.0[b]	2.5×10^{-6}	10	98.0 ± 2.4	78.7 ± 6.8
10.0	2.5×10^{-5}	10	98.1 ± 2.0	79.9 ± 7.2
100.0	2.5×10^{-4}	10	96.7 ± 3.4	66.0 ± 7.2**
1000.0	2.5×10^{-3}	10	97.8 ± 3.9	54.9 ± 3.9**

[a] Percentage of control = test cell migration/$\bar{x}$ untreated cell migration × 100.
[b] Therapeutically attainable plasma level.
[c] One-hundred percent of control = 116 ± 4 μm ($\bar{x}$ ± SEM).
[d] One-hundred percent of control = 54 ± 2 μm ($\bar{x}$ ± SEM).
** Significantly different from 0.0 μg/ml at $P < 0.01$.

sponse to chemotactic factors only as measured by the leukotactic index technique at concentrations of 100 μg/ml and higher. As depicted in Table III, significant ($P < 0.01$) inhibition of this response was measured for D-penicillamine at 100 μg/ml drug concentration by the leading front technique. Greater inhibition was measured at the same drug concentration by the leukotactic index technique which also revealed a significant ($P < 0.05$) inhibition produced by 10 μg/ml of D-penicillamine. As shown in Table IV, no effects of azathioprine on neutrophil response to chemotactic factors could be demonstrated by the techniques employed in this study.

TABLE III. EFFECTS OF D-PENICILLAMINE ON NEUTROPHIL RESPONSE TO CHEMOTACTIC FACTORS ANALYZED BY LEADING FRONT AND LEUKOTACTIC INDEX TECHNIQUES

Drug concentrations[b]			$\bar{x}$ percentage of control[a] ± SEM	
μg/ml	Molarity	N	Leading front[c]	Leukotactic index[d]
0.0	0	10	100.0 ± 0.9	100.0 ± 3.1
1.0	6.7×10^{-7}	10	101.4 ± 1.7	91.7 ± 5.4
10.0	6.7×10^{-6}	10	100.1 ± 0.9	76.5 ± 7.4*
100.0	6.7×10^{-5}	10	91.6 ± 3.0**	72.1 ± 6.7**

[a] Percentage of control = test cell migration/$\bar{x}$ untreated cell migration × 100.
[b] Therapeutically attainable plasma level = approximately 20 μg/ml.
[c] One-hundred percent of control = 121 ± 3 μm ($\bar{x}$ ± SEM).
[d] One-hundred percent of control = 43 ± 3 μm ($\bar{x}$ ± SEM).
* Significantly different from 0.0 μg/ml at $P < 0.05$.
** Significantly different from 0.0 μg/ml at $P < 0.01$.

Discussion. The most utilized *in vitro* method of examining neutrophil chemotaxis continues to be the micropore-filter assay introduced by Boyden (1). The leading front technique, a commonly used method for quantitation of neutrophil migration in Boyden chambers was initially described by Zigmond and Hirsch (2). It is based on the concept that cell populations are homogenous in their migration characteristics through micropore filters so that the fastest cells are representative of the total neutrophil population. However, this may not be true since Harvath and Leonard (7) and Klempner *et al.* (8) have described differing subpopulations among human peripheral blood neutrophils with respect to cellular chemotactic capabilities. The leukotactic index, a recently developed modification in the measurement of neutrophil chemotaxis, incorporates into its calculations the number of cells migrating and the distances of migration into filters. It reflects the average migration distance for all cells traveling into the filters, while the leading front technique only measures the migration distance for the fastest cells (3).

We have adapted the leukotactic index as a more sensitive method to calculate *in vitro* neutrophil chemotaxis and expanded it for use in drug studies involving certain antirheu-

TABLE IV. EFFECTS OF AZATHIOPRINE ON NEUTROPHIL RESPONSE TO CHEMOTACTIC FACTORS ANALYZED BY LEADING FRONT AND LEUKOTACTIC INDEX TECHNIQUES

Drug concentrations			$\bar{x}$ percentage of control[a] ± SEM	
μg/ml	Molarity	N	Leading front[c]	Leukotactic index[d]
0.0	0	10	100.0 ± 0.5	100.0 ± 5.5
0.2	7.0×10^{-7}	10	100.2 ± 2.4	109.1 ± 7.2
2.0[b]	7.0×10^{-6}	10	97.6 ± 1.9	100.5 ± 6.9
20.0	7.0×10^{-5}	10	97.7 ± 2.5	85.6 ± 10.5

[a] Percentage of control = test cell migration/$\bar{x}$ untreated cell migration × 100.
[b] Therapeutically attainable plasma level.
[c] One-hundred percent of control = 114 ± 4 μm ($\bar{x}$ ± SEM).
[d] One-hundred percent of control = 33 ± 3 μm ($\bar{x}$ ± SEM).

matic drugs. These drugs have been studied extensively in *in vitro* systems of neutrophil migration since one possible mode of action of these agents could be to modulate the response of neutrophils to chemotactic stimuli (9). Wide differences in reported results concerning the effects of these drugs on neutrophil chemotaxis (10) suggest the existence of important problems with the techniques and methodologies previously utilized to study this important neutrophil function. We therefore used the more sensitive method of measurement of neutrophil migration, the leukotactic index, and compared it to the standard leading front technique for quantitating the response of neutrophils to chemotactic factors in the presence of varying concentrations of aspirin, gold sodium thiomalate, D-penicillamine, and azathioprine. Because the present studies were designed to reflect as closely as possible events occurring in rheumatoid joints where neutrophils are exposed to both cell- and serum-derived chemotactic factors, neutrophil-IgG Agg-serum-derived factors, previously described and partially characterized in a preliminary study (11), were employed in the present work.

Aspirin at high concentrations was shown to produce significant inhibition of neutrophil response to chemotactic factors only with the leukotactic index. Similar effects have been reported by Rivikin *et al.* (9), whereas Pécoud *et al.* have reported (10) no effect of aspirin on neutrophil chemotaxis.

Gold sodium thiomalate at high concentrations was shown to produce significant inhibition of neutrophil response to chemotactic factors only with the leukotactic index procedure. Although the concentrations producing these results are rarely attainable in plasma during gold therapy, similar concentrations have been shown to exist in the lysosomes of phagocytic cells in the synovial membranes of patients with rheumatoid arthritis treated with gold (12). Our results are similar to those of Pécoud *et al.* (10) and Mowat (13) who have shown *in vitro* inhibition of neutrophil chemotaxis in normal subjects' neutrophils exposed to this antirheumatic agent.

D-Penicillamine at high concentrations was shown to produce significant inhibition of neutrophil response to chemotactic factors with both the leading front and the leukotactic index techniques, but at therapeutically attainable concentrations significant inhibition was seen only with the leukotactic index. Similar effects on the inhibition of neutrophil chemotaxis by D-penicillamine have previously been reported in *in vitro* studies by Chwalinska-Sadowska (14), whereas Mowat has reported (13) varying effects of D-penicillamine on neutrophil chemotaxis depending on the time of drug exposure employed in the studies.

Azathioprine produced no effect on the neutrophil response to chemotactic factors with either technique. Previous studies by Losito *et al.* (15) have also shown no effect of azathioprine on neutrophil chemotaxis, whereas Rivikin *et al.* (9) have demonstrated chemotaxis inhibition with this drug.

Our studies have thus shown that the leukotactic index allows improved *in vitro* measurement of human neutrophil response to chemotactic factors when compared to the leading front technique. Studies utilizing a combination of these techniques with several concentrations of well-defined chemotactic agents should provide more quantitative information concerning the effects of specific agents on specific cell populations. The application of sensitive and biologically representative methods for the assay of neutrophil chemotaxis should help define more clearly the effects of antirheumatic drugs on this important component of rheumatoid inflammatory processes.

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