

EFFECT OF ANTITUBULINS ON SPONTANEOUS AND CHEMOTACTIC MIGRATION OF NEUTROPHILS UNDER AGAROSE

M. A. DZIEZANOWSKI, M. J. DESTEFANO AND M. RABINOVITCH

Department of Cell Biology, New York University School of Medicine, New York, N.Y. 10016 U.S.A.

SUMMARY

The effect of colchicine, Nocodazole or vinblastine on the movement of mouse bone marrow neutrophils was examined by the method of migration under agarose. Migration was estimated by counts of the numbers of cells that left the wells and moved under the gel. Micromolar concentrations of the agents increased spontaneous migration 2- to 4-fold, while lumicolchicine was ineffective. The increase may be due to the disassembly of the microtubular skeleton, making the cells more fluid, or to some other effect of the drugs. Since other agents, such as serum, low osmolality and low pH also enhance migration, these were tried in combination with a pretreatment of the cells with colchicine. Serum and colchicine acted synergistically, low osmolality and colchicine were additive. Possibly, the enhancement of neutrophil migration by serum or lowered osmolality does not require intact microtubules. At low pH, colchicine had very little stimulatory effect, perhaps indicating that the microtubules are more colchicine-resistant at low pH.

Although antitubular drugs showed such marked effects on spontaneous migration, the chemotactic attraction of the neutrophils towards activated serum was unaffected. This indicates that the sensory and control mechanisms of the cells are resistant to antitubulins.

INTRODUCTION

There are conflicting reports on the effect of antitubular agents such as colchicine on spontaneous migration of polymorphonuclear neutrophils or heterophils. Spontaneous migration has been variously reported to be stimulated (Edelson & Fudenberg, 1973), unaffected (Bandmann, Norberg & Rydgren, 1974) or inhibited (Ramsey & Harris, 1973) by the drugs. In contrast, most investigators agree that antitubulins inhibit the chemotactic response of leukocytes in micropore filter assays (for reviews see: Ward & Becker, 1977; Zigmond, 1978; Klebanoff & Clark, 1978). Most of this work preceded the methodological advances and insights that allowed a clear distinction to be made between chemokinesis (stimulated random migration) and chemotaxis (directional migration) (Keller *et al.* 1977).

To test further the effect of antitubulins on spontaneous migration we used mouse bone marrow neutrophils and an under-agarose assay which is particularly useful in studies of spontaneous or chemokinetically stimulated movement (Nelson, McCormack & Fiegel, 1978; Tono-Oka, Nakayama & Matsumoto, 1978). In this assay, cells are placed in wells cut in agarose gels and move over the substrate and under the gels.

In the absence of gradients of chemotactic factors the areas of migration are circular (spontaneous migration). When gradients are established by delivering chemo-attractants into wells (attractant wells) placed near the cell wells, more cells move towards the attractant wells than away from them. An elliptical area of migration is then generated (Nelson *et al.* 1978). We have determined spontaneous migration by counts of the total numbers of cells that leave the wells and chemotactic migration by counts of cells in defined samples of the areas of migration, towards and away from the attractant wells (Rabinovitch & DeStefano, 1978). We found that colchicine and other antitubulins enhance spontaneous migration at concentrations that have no detectable influence on neutrophil chemotaxis. In a series of related experiments, we also examined the role of microtubules in spontaneous migration stimulated by several environmental conditions (i.e. serum, lowered pH or lowered osmolality).

MATERIALS AND METHODS

Animals

Swiss-Webster female mice, 20 g body weight and Fisher outbred rats were used as sources of bone marrow cells or serum respectively.

Media and chemicals

Agarose and PIPES (piperazine-*N-N'*-bis(ethanesulphonic acid)) were obtained from Calbiochem; powdered, bicarbonate-free RPMI 1640 from Gibco; zymosan, ovalbumin, colchicine and vinblastine, from Sigma Chemical, Nocodazole (R 17934 Janssen Pharmaceutica), from Aldrich Chemical. Lumicolchicine was prepared and tested spectrophotometrically according to Obika *et al.* (1978). Chemotactic serum was obtained by activation of fresh rat serum with zymosan (Rabinovitch & DeStefano, 1978).

Preparation of agarose gels

Gels contained 1% agarose in RPMI 1640, 2 mg/ml ovalbumin and 5 mM PIPES (Nelson *et al.* 1978; Rabinovitch & DeStefano, 1978). Control gels were adjusted to pH 7.0 and 280 mosmol/kg water. Osmolalities were measured with a Fiske model H osmometer. Other pH levels and osmolalities were as noted in the text. In some experiments antitubulin drugs were added to the gels in the concentrations indicated. Two or 5 ml of molten agarose were poured into 35- or 60-mm tissue-culture dishes, respectively. Alternatively, 3 ml were delivered on microscope slides and the gels covered with thin plastic sheets to minimize evaporation. Wells were cut with a 2.4-mm LKB punch.

Bone marrow cells

Marrow cell suspensions were prepared as described elsewhere (Rabinovitch & DeStefano, 1978). The final cell suspension contained 2×10^7 cells per ml with between 5 and 15% neutrophils as determined by differential counts. In some experiments marrow cells were treated with colchicine or Nocodazole in RPMI medium for 1 h at 37 °C, sedimented by centrifugation and resuspended in fresh medium. Control cells were treated with RPMI medium alone.

Migration assays

Chemotactic wells were loaded with zymosan-activated serum 30–60 min prior to the addition of the bone marrow cells to the appropriate wells. Five microlitres of cell suspensions

containing 10000 to 20000 mature neutrophils were pipetted into each cell well and the plates or slides incubated for 1-3 h at 37 °C in a humidified atmosphere (Rabinovitch & DeStefano, 1978). Cells were fixed with glutaraldehyde and stained with 0.01 % crystal violet. For assays of spontaneous migration, the total numbers of cells that left the wells and moved over the glass or plastic substrates were counted with a light microscope at 100 or 200 × magnification. To measure chemotactic migration, neutrophils were counted in 2 areas of equal size. One (area *A*) was on the side closer to the chemotactic factor well; the other (area *B*) was on the opposite side of the cell well. Chemotactic ratios were calculated as $(A-B)/(A+B)$ (Rabinovitch, DeStefano & Dziezanowski, 1980).

RESULTS

Enhancement of spontaneous migration by microtubule inhibitors

In micromolar concentrations colchicine, Nocodazole and vinblastine increased both the numbers of neutrophils that migrated under agarose and the net distances travelled by the cells. In the experiments summarized in Table 1, either colchicine

Table 1. *Enhancement of spontaneous migration of neutrophils by anti-tubulin drugs.*

Drug concentration	Ratio exp./control
Colchicine ^a , μM	
1	3.06 ± 0.36 (10) ^c
3	4.33 ± 1.02 (5)
10	4.18 ± 0.52 (4)
Colchicine ^b , μM	
3	4.04 ± 0.58 (8)
Nocodazole ^a , μM	
1	3.38 ± 0.61 (3)
Vinblastine ^a , μM	
3	2.46 ± 0.08 (3)
Lumicolchicine ^a , μM	
3	1.11 ± 0.16 (4)
10	1.3 ± 0.07 (4)

^a drug added to the agarose.
^b neutrophils pretreated with the drug.
^c Mean \pm s.e. (no. expts.). In each experiment 5 to 10 wells were counted per treatment.

was added to the agarose gels or the marrow cells were pretreated with colchicine and allowed to migrate beneath drug-free agarose. Under both conditions the total number of neutrophils that left the wells was 3 to 4 times that of cells not exposed to colchicine, with near maximal enhancement by 1.0 μM colchicine. 0.1 μM was not stimulatory (not shown). The areas of migration were also noticeably increased. In contrast, lumicolchicine preparations (devoid of antitubulin activity) were not stimulatory, even at 10 μM . Nocodazole and vinblastine also enhanced migration when added to the agarose. However, pretreatment with Nocodazole did not increase migration (not shown), suggesting that the effect of the drug was rapidly reversible.

Effect of microtubular agents on the chemotactic response

Fig. 1 summarizes the results of a representative experiment in which the effect of colchicine on directional migration was examined with C5a-rich zymosan-activated serum as an attractant. Concentrations of activated serum between 2.5 and 20% were placed in chemotactic factor wells prior to the addition of marrow cell suspensions to the cell wells. Neutrophils were counted in selected fields of the migration areas and

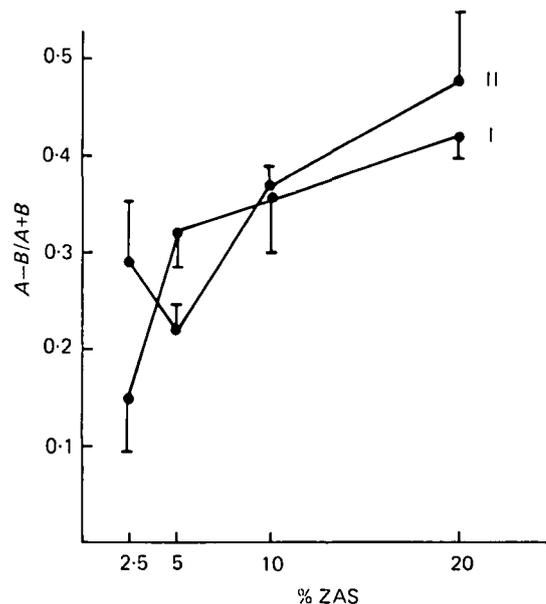


Fig. 1. Chemotactic ratios of colchicine-treated ($1 \mu\text{M}$ curve II) or control (curve I) neutrophils as a function of the concentration of zymosan-activated serum (ZAS) placed in the attractant wells. Plate method. 1.8×10^3 neutrophils per well, 6 wells per point. Migration time 105 min. Vertical bars indicate standard errors.

chemotactic ratios calculated as given in Materials and methods. It can be seen that chemotactic ratios increased with the concentration of activated serum (Rabinovitch & DeStefano, 1978; Orr & Ward, 1978) and that colchicine had no demonstrable effect on the chemotactic responses of the neutrophils.

In other experiments gels were prepared without (controls) or with 1 or 10 μM colchicine and chemotaxis was evaluated with 25% activated serum as the attractant. Spontaneous migration was determined in separate wells cut in the same dishes. With the chemotactic ratios in control gels normalized to 100, the chemotaxis of neutrophils in the presence of 1.0 or 10 μM colchicine was, respectively, 100.9 ± 6.4 (mean \pm standard error, 4 experiments) and 91.3 ± 9.6 (3 experiments). In the same experiments spontaneous migration was enhanced over 3-fold by both colchicine concentrations. Similar results were obtained with 1 μM Nocodazole (2 experiments). Thus, the chemotactic response to zymosan-activated serum was unaffected by concentrations of antitubulins which markedly enhanced the spontaneous migration of neutrophils.

Effect of colchicine on stimulated migration

Migration of neutrophils can be stimulated by the addition of heat-inactivated serum to the agarose or by adjustment of the gel to lower pHs or osmolalities (Rabinovitch & DeStefano, 1978; DeStefano, Dziezanowski & Rabinovitch, 1978; Rabinovitch *et al.* 1980). We used colchicine to determine whether such stimulated migration requires intact microtubules. Since the effects of colchicine persist for several hours

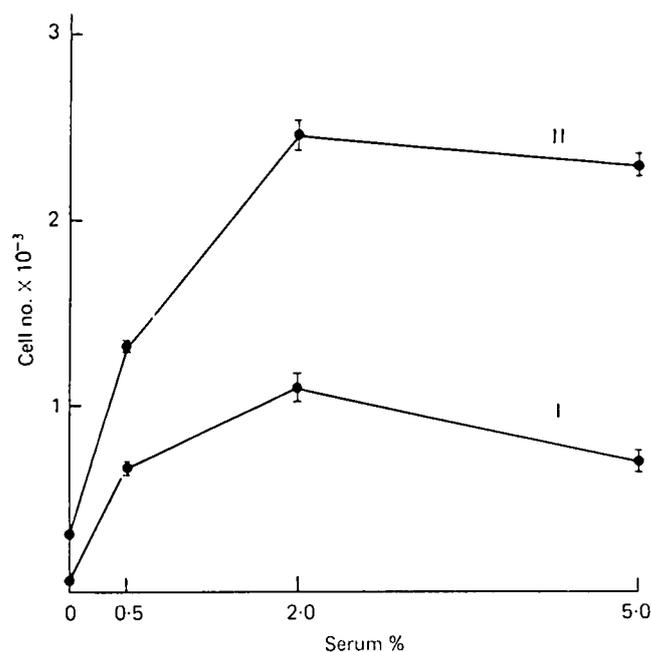


Fig. 2. Spontaneous migration of colchicine-treated ($3 \mu\text{M}$, curve II) or control (curve I) neutrophils under agarose with different concentrations of heat-inactivated rat serum. Slide method. 14×10^3 neutrophils per well. Migration time 65 min.

after its removal from the medium, cells could be loaded with colchicine under uniform conditions, usually RPMI medium adjusted to pH 7.0 and 280 mosmol/kg water, prior to migration in drug-free agarose gels of any desired composition. Fig. 2 shows the results of an experiment involving colchicine and serum. Colchicine-treated or control neutrophils migrated under agarose gels containing a range of concentrations of heat-inactivated serum (56°C , 30 min). Migration of control neutrophils was enhanced by serum, with a peak of 2%. Pretreatment with colchicine increased migration at all serum concentrations, suggesting that stimulation by serum is not dependent on intact microtubules. Moreover, the results indicate a synergistic interaction of serum and colchicine. Thus, in absolute counts, the migration in the presence of serum of cells pretreated with colchicine exceeded the migration estimated by adding the effect of colchicine in serum-free agarose (ordinate values at 0% serum in Fig. 2) to the effect of serum on cells not treated with colchicine (lower curve, Fig. 2). However, the proportionate increase of migration by colchicine was higher in

the absence (5.5-fold) than in the presence of serum (2.0, 2.2, or 3.3 \times respectively, at 0.5, 2.0 or 5.0% serum). To determine if the cells were already maximally stimulated early in the assay by the combination of serum and colchicine, time-course experiments were performed. It can be seen in Fig. 3, from the slopes of the time courses, that the highest rate of migration was displayed by cells pretreated with colchicine and allowed to migrate in 2% serum. Furthermore, the movement of colchicine-treated cells in medium with or without serum was linearly related to time between 1 and 3 h. Therefore there was no evidence to suggest a plateau of the migration of cells treated with both colchicine and serum.

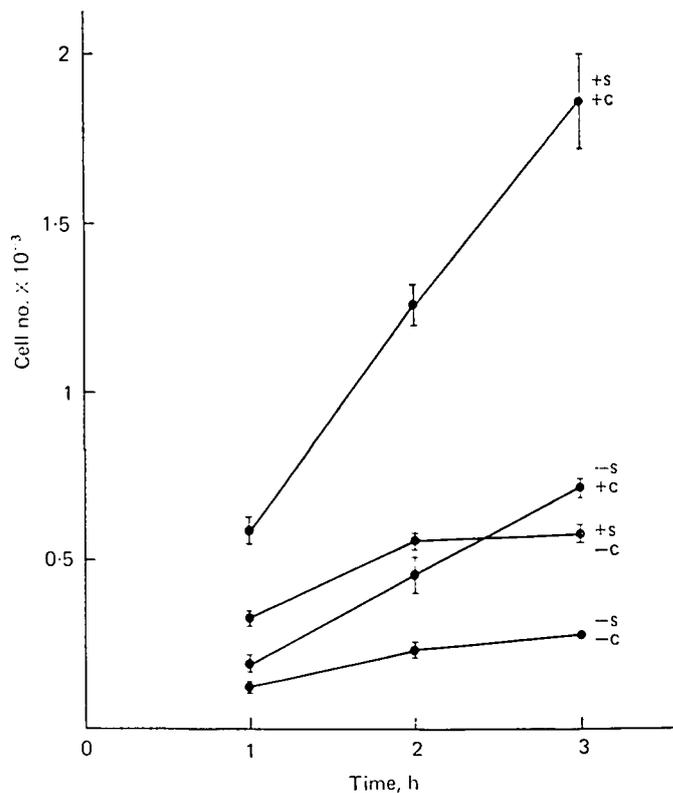


Fig. 3. Time course of the spontaneous migration of 3 μ M colchicine-treated (+c) or control (-c) neutrophils. Two percent heat-inactivated serum was present (+s) or absent (-s). Slide method. 13×10^3 neutrophils per well, 6 wells per point.

Fig. 4 shows the results of an experiment involving colchicine and lowered osmolality of the medium. It can be seen that control cells moved maximally under agarose adjusted to 230 mosmol/kg, and that pretreatment with colchicine increased migration at all osmolalities tested. The absolute increment of migration due to colchicine was approximately constant, as shown by the parallelism of control and colchicine lines between 200 and 280 mosmol/kg and the effects of colchicine and lowered osmolality were roughly additive. Again, the results suggest that the osmolality effect does not require polymerized tubulin.

A third set of experiments involved colchicine and pH. Fig. 5 shows, as previously reported (DeStefano *et al.* 1978; Rabinovitch *et al.* 1980) that migration of control neutrophils was maximal at pH 6.0 and decreased as the pH was increased to 7.5 but that migration of neutrophils pretreated with colchicine exhibited a different pH dependence. The effect of colchicine was maximal at pH 7.5, while the drug was least stimulatory at pH 5.5 or 6.0. It was therefore of interest to determine, first, whether the influx of colchicine would be reduced at pH 6.0, and second, whether the efflux of

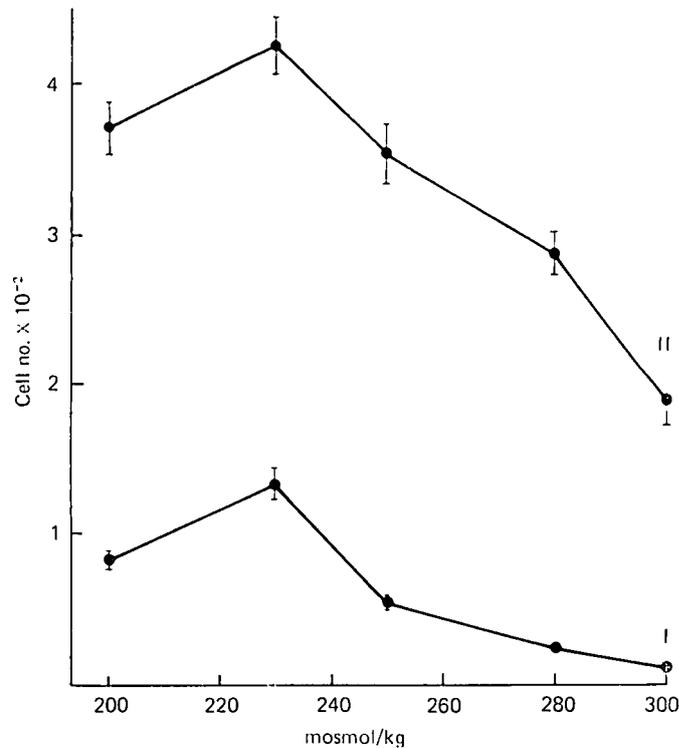


Fig. 4. Spontaneous migration of colchicine-treated ($3 \mu\text{M}$, curve II) or control (curve I) neutrophils as a function of the osmolality of the agarose medium. Plate method. 17.5×10^3 neutrophils per well, 10 wells per point. Migration time 90 min.

the drug would be increased at the lower pH. Marrow cells were preloaded with $3 \mu\text{M}$ colchicine for 40 min at 37°C in media adjusted to pH 6 or 7, resuspended in RPMI and placed in agarose at pH 7.0. Migration was enhanced 3.3- and 3.1-fold, respectively, indicating similar stimulation by colchicine whether the cells were loaded at pH 6 or 7. In the same experiment, other aliquots of marrow cells were incubated for 40 min in RPMI medium, pH 6, with or without added colchicine, washed, post-incubated for 30 min in pH 6 RPMI medium in the absence of the drug and placed in agarose pH 7. Here migration of colchicine-treated cells was enhanced 2.7-fold in comparison with the control neutrophils. We concluded that the reduced effect of colchicine at low pH is not due mainly to decreased uptake or to increased efflux of the drug in medium of lower pH.

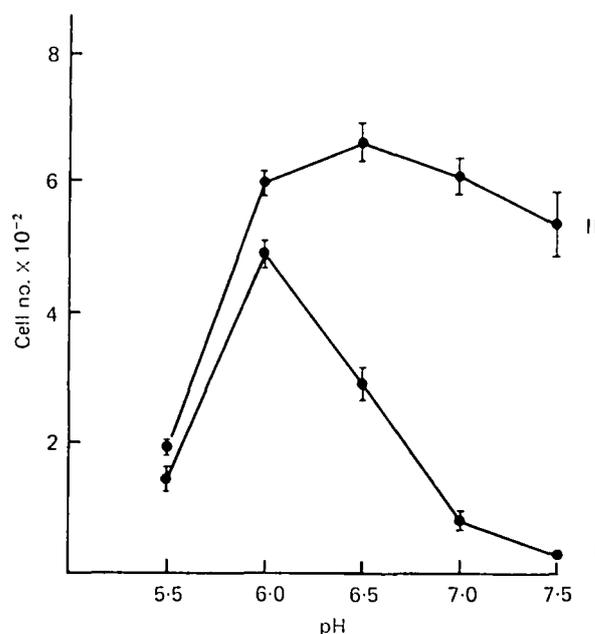


Fig. 5. Spontaneous migration of colchicine-treated ($3 \mu\text{M}$, curve II), or control (curve I) neutrophils as a function of the pH of the agarose medium. Plate method. PIPES buffers. 14×10^8 neutrophils per well, 10 wells per point. Migration time 130 min.

DISCUSSION

Colchicine, Nocodazole and vinblastine are structurally dissimilar agents that bind to monomeric tubulin and inhibit its polymerization (Wilson *et al.* 1974; Hoebeke, Van Nijen & DeBrabander, 1976). In the present experiments the drugs enhanced migration of neutrophils at micromolar concentrations previously shown to decrease the numbers of detectable microtubules in leukocytes and other cells (DeBrabander *et al.* 1976; Hoffstein, Goldstein & Weissmann, 1977). In contrast, lumicolchicine, a product of the photoisomerization of colchicine, devoid of microtubular actions, had no effect on migration. It is thus likely that the enhanced migration of neutrophils was mediated by depolymerization of microtubules. Antitubulins have also been shown to enhance the migration of granulocytes (Edelson & Fudenberg, 1973) or of mononuclear phagocytes (Cheung, Cantarow & Sundharadas, 1978) out of glass capillaries.

The mechanism by which the antitubulins stimulate spontaneous migration need further evaluation. The simplest possibility is that depolymerization of microtubules increases deformability, favouring the movement of the neutrophils in the shallow 'crawl space' under the agarose gels. However, colchicine also affects the anchorage of macromolecules to the plasma membrane (Oliver, Ukena & Berlin, 1974), the net surface charge of cells (Bhisey, Rao & Ranadive, 1977), the levels of neutrophil cyclic AMP (Malawista, Oliver & Rudolph, 1978) as well as the release of endogenous chemotactic factors (Spilberg, Gallacher, Mehta & Mandell, 1976). These or other

cellular effects of colchicine could be involved in the enhanced movement described here.

We have also found that the chemotactic responses to gradients of activated serum were unaffected by concentrations of colchicine that enhanced the spontaneous migration of neutrophils. This finding is in contrast to previous observations with micropore filter assays (Edelson & Fudenberg, 1973; Bandmann *et al.* 1974; Ward & Becker, 1977; Zigmond, 1978; Klebanoff & Clark, 1978). However, the orientation of neutrophils in chemotactic gradients has been shown not to be influenced by colchicine (Zigmond, 1977).

As in the filter methods (Keller *et al.* 1977), the preferential migration of neutrophils under agarose towards the attractant well results both from chemotaxis (Orr & Ward, 1978) or from stimulated spontaneous migration or chemokinesis (Tono-Oka *et al.* 1978). If chemokinesis represents a major component of the neutrophil responses under agarose, inhibitory effects of antitubulins on chemotaxis may be difficult to demonstrate. Since the agarose method allows studies of the behaviour of individual cells, it should be possible to further delineate the effects of antitubulins on both spontaneous and directional migration of the neutrophils (Allan & Wilkinson, 1978).

Our finding that the effects of colchicine and those of serum or lowered osmolality on spontaneous migration appear to be additive or even synergistic suggests that enhancement of migration by serum or lowered osmolality does not require intact microtubules. In contrast, the effects of colchicine and of lowered medium pH were not additive. Control experiments revealed that this was not due to decreased influx or to increased efflux of the drug. We are testing the possibility that at lower environmental pH colchicine fails to inhibit microtubule depolymerization.

We wish to thank Ms Susan Kirsch for helpful suggestions. The work was supported by grants GM/CA 23093 and AI/CA 10969 from the U.S. National Institutes of Health.

REFERENCES

- ALLAN, R. B. & WILKINSON, P. C. (1978). A visual analysis of chemotactic and chemokinetic locomotion of human neutrophil leucocytes. *Expl Cell Res.* **111**, 191-203.
- BANDMANN, U., NORBERG, B. & RYDGREN, L. (1974). Polymorphonuclear leukocyte chemotaxis in Boyden chambers. Effect of low concentrations of vinblastine. *Scand. J. Haemat.* **13**, 305-312.
- BHISEY, A. N., RAO, S. G. A. & RANADIVE, K. J. (1977). Alterations in electrophoretic mobility of mouse macrophages treated with microtubule inhibitors. *Indian J. exp. Biol.* **15**, 970-972.
- CHEUNG, H. T., CANTAROW, W. D. & SUNDHARADAS, G. (1978). Colchicine and cytochalasin B (CB) effects on random movement, spreading and adhesion of mouse macrophages. *Expl Cell Res.* **111**, 95-103.
- DEBRABANDER, M. J., VAN DE VEIRE, R. M. L., AERTS, F. E. M., BORGERS, M. & JANSSEN, P. A. J. (1976). The effects of methyl[5-(2-thienylcarbonyl) 1 H-benzimidazol-2 Y] carbamate (R 17934, NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cell cultures *in vitro*. *Cancer Res.* **36**, 905-916.
- DESTEFANO, M. J., DZIEZANOWSKI, M. A. & RABINOVITCH, M. (1978). Migration of polymorphonuclear neutrophils under agarose is enhanced by media of low pH or osmolality. *J. Cell Biol.* **79**, 89a.
- EDELSON, P. J. & FUDENBERG, H. F. (1973). Effect of vinblastine on the chemotactic responsiveness of normal human neutrophils. *Inf. Immun.* **8**, 127-129.

- HOEBEKE, J., VAN NIJEN, G. & DEBRABANDER, M. (1976). Interaction of Oncodazole (R 17934), a new antitumoral drug, with rat brain tubulin. *Biochem. biophys. Res. Commun.* **69**, 319-324.
- HOFFSTEIN, S., GOLDSTEIN, I. M. & WEISSMANN, G. (1977). Role of microtubule assembly in lysosomal enzyme secretion from human polymorphonuclear leukocytes. A reevaluation. *J. Cell Biol.* **73**, 242-256.
- KELLER, H. U., WILKINSON, P. C., ABERCROMBIE, M., BECKER, E. L., HIRSCH, J. G., MILLER, M. E., RAMSEY, W. S. & ZIGMOND, S. H. (1977). A proposal for the definition of terms related to the locomotion of leukocytes and other cells. *Clin. exp. Immun.* **27**, 377-380.
- KLEBANOFF, S. J. & CLARK, R. A. (1978). *The Neutrophil: Function and Clinical Disorders*, pp. 127-129. Amsterdam: North-Holland.
- MALAWISTA, S. E., OLIVER, J. M. & RUDOLPH, S. A. (1978). Microtubules and cyclic AMP in human leukocytes: on the order of things. *J. Cell Biol.* **77**, 881-886.
- NELSON, R. D., MCCORMACK, R. T. & FIEGEL, V. D. (1978). Chemotaxis of human leukocytes under agarose. In *Leukocyte Chemotaxis* (ed. J. I. Gallin & P. Q. Quie), pp. 25-40. New York; Raven Press.
- OBIKA, M., TURNER, JR., W. A., NEGISHI, S., MENTER, D. G., TCHEN, T. T. & TAYLOR, J. D. (1978). The effects of lumicolchicine, colchicine and vinblastine on pigment migration in fish chromatophores. *J. exp. Zool.* **205**, 95-110.
- OLIVER, J. M., UKENA, T. E. & BERLIN, R. D. (1974). Effects of phagocytosis and colchicine on the distribution of lectin binding sites on cell surfaces. *Proc. natn. Acad. Sci. U.S.A.* **71**, 394-398.
- ORR, W. & WARD, P. A. (1978). Quantitation of leukotaxis in agarose by three different methods. *J. immunol. Meth.* **20**, 95-107.
- RABINOVITCH, M. & DESTEFANO, M. J. (1978). In vitro chemotaxis of mouse bone marrow neutrophils. *Proc. Soc. exp. Biol. Med.* **158**, 170-173.
- RABINOVITCH, M., DESTEFANO, M. J. & DZIEZANOWSKI, M. A. (1980). Neutrophil migration under agarose: stimulation by lowered medium pH and osmolality. *J. reticuloendothel. Soc.* (In Press).
- RAMSEY, W. S. & HARRIS, A. (1973). Leucocyte locomotion and its inhibition by antimetabolic drugs. *Expl Cell Res.* **82**, 262-270.
- SPIELBERG, I., GALLACHER, A., MEHTA, J. M. & MANDELL, B. (1976). Urate crystal induced chemotactic factor. Isolation and partial characterization. *J. clin. Invest.* **58**, 815-819.
- TONO-OKA, T., NAKAYAMA, M. & MATSUMOTO, S. (1978). Enhanced granulocyte mobility induced by chemotactic factor in the agarose plate. *Proc. Soc. exp. Biol. Med.* **159**, 75-79.
- WARD, P. A. & BECKER, E. L. (1977). Biology of leukotaxis. *Rev. Physiol., Biochem. Pharmac.* **77**, 125-148.
- WILSON, L., BAMBURG, J. R., MIZEL, S. B., GRISHAM, L. M. & CRESWELL, K. M. (1974). Interaction of drugs with microtubule proteins. *Fedn Proc. Fedn Am. Socs. exp. Biol.* **33**, 158-166.
- ZIGMOND, S. H. (1977). Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.* **75**, 606-616.
- ZIGMOND, S. H. (1978). Chemotaxis of polymorphonuclear leukocytes. *J. Cell Biol.* **77** 269-287.

(Received 24 July 1979)