INVERSE CORRELATION BETWEEN NEUTROPHIL MICROTUBE NUMBERS AND ENHANCED RANDOM MIGRATION

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SUMMARY
The random migration of neutrophils under agarose as measured by the number of cells leaving the well, is enhanced when the pH or the osmolality of the medium is reduced or when microtubule agents are used. Concentrations of colchicine above $5 \times 10^{-7} \text{ M}$ increased the number of cells migrating and decreased the mean number of centriolar microtubules in a dose-dependent fashion from 16 to 4 per $4 \mu\text{m}^3$ at $10^{-6} \text{ M}$. The distance that colchicine-treated neutrophils migrated from the well was not different from the control. Lowering the pH from 7.4 to 6.0 also increased random migration and decreased pericentriolar microtubules from a mean of 16 to a mean of 10. At pH 6.0, both the number of cells that migrated and the distance the cells forming the leading edge travelled from the well were increased. Since peripheral microtubules may play a greater role in cell migration than centriolar ones, we examined the numerical density of microtubules in the peripheral cytoplasm. Lowering the medium pH reduced the mean number of microtubules per $10 \mu\text{m}^3$ from 6 to 2. Colchicine reduced microtubules in the same area to 1. At the low pH, colchicine reduced even further the numbers of both centriole-associated and peripheral microtubules but the migration pattern was the same as that seen at pH 6.0 without colchicine. Lowering medium osmolality from 280 to 230 m-osmol increased random migration but did not affect microtubule numbers. The addition of colchicine to this system decreased microtubule numbers and increased migration even further. Conditions that enhanced neutrophil migration also affected cell shape. Whereas cells at pH 7.4 were generally fan-shaped with a broad, smooth leading edge, cells at pH 6.0 with or without colchicine were long and narrow. Neutrophils at pH 7.4 but 230 m-osmol had a scalloped edge, which often appeared thickened. This too was not altered by colchicine. The morphology of cells treated with colchicine was similar to controls except for the more frequent presence of long retraction fibres. Each of these treatments thus appears to act on a different aspect of the cell's locomotory apparatus. The mechanisms by which colchicine and lowered pH enhance migration may partially overlap since both significantly decrease peripheral microtubules. The data suggest that microtubules play a constraining role within the cell, limiting the ability of the cell to move and change direction.

INTRODUCTION
One of the methods used for examining neutrophil motility is the under-agarose technique. In this technique, originally devised by Nelson, Quie & Simmons (1975), neutrophils are placed in wells cut in an agarose-medium gel and allowed to settle on to a substrate and migrate out of the well under the agarose. This system has been used to study both random and directed migration and, in general, the results are comparable to other systems used to measure cell migration, such as the Boyden chamber. The advantages of the under-agarose system are many. It allows direct
observation of living cells and analysis of morphological changes. It also allows alterations of the medium and the substrate and requires relatively few neutrophils.

Antimicrotubular drugs have been shown to increase the numbers of neutrophils that spontaneously migrate from the wells (Dziezanowski, DeStefano & Rabinovitch, 1980). Lowering the pH or the osmolality of the medium also increases spontaneous migration (Rabinovitch, DeStefano & Dziezanowski, 1980). Whereas the effects of colchicine and lowered osmolality are additive, those of lowered pH and colchicine are not, suggesting that microtubule function might already be affected by extracellular pH. In this paper, we show that colchicine and low pH but not low osmolality reduced microtubule numbers. However, examination of cell shape and migration pattern suggest that these agents affect locomotion by different means. Colchicine appears to alter the proportion of motile cells within the population whereas lowered pH or osmolality seem to alter both the proportion of motile cells and the distance the cells travel.

MATERIAL AND METHODS

Media and chemicals
Powdered bicarbonate-free RPMI 1640 was obtained from Gibco; agarose and PIPES (piperazine-N,N'-bisc(ethane sulphonic acid)) from Calbiochem; colchicine and pig skin gelatin from Sigma; dextran T250 from Pharmacia Fine Chemicals.

Preparation of agarose gels
Gels on glass slides were prepared as previously described (Rabinovitch et al. 1980) with the addition of 2 mg/ml gelatin to the RPMI agarose.

Preparation of neutrophils
Venous blood was drawn from healthy adult volunteers and anticoagulated with citrate or heparin. The erythrocytes were gravity sedimented for 30 min after mixing with half vol. of 6% dextran dissolved in phosphate-buffered saline. The leukocyte-rich supernatant was collected, centrifuged and resuspended in RPMI medium buffered with 5 mM PIPES adjusted to 280 m-osmol and pH 7.4.

Pretreatment
Cells were treated in suspension with medium with or without colchicine (Sigma) at concentrations from $10^{-7}$ to $10^{-5}$ M for 45 min at 37 °C. Suspensions were then centrifuged and resuspended in medium without colchicine. Each suspension was divided in 2:1 suspension was used in the under-agarose migration assay and the other for electron-microscopic analysis of the microtubules. The latter was centrifuged and resuspended in medium adjusted to 280 or 230 m-osmol and pH 7.4 or 6.0 according to the requirements of the experiment and incubated for 1 h at 37 °C. These cells were then fixed.

Under-agarose migration assay
Aliquots containing $10^4$ neutrophils were placed in each well and the number that spontaneously migrated from the wells after 1 h was determined as described by Rabinovitch et al. (1980). In addition, the distance that cells forming the leading front travelled was measured using a 10 x 10 mm grid with 0.5-mm divisions in the microscope ocular. We defined the leading front as a line between 2 adjacent cells that had migrated farthest from the edge of the well. The line between adjacent cells was essentially parallel to the edge of the circular well and therefore the distance to that line was easily measurable using a Polaron graticule with 0.1-mm divisions (Ted Pella Co.) in the eye piece. The measurements were always made on the left side
Neutrophils: microtubules and migration

of the well to eliminate any possible differences caused by unequal migration around the well. Six to 10 wells were measured for each treatment. Representative micrographs were taken using a Zeiss microscope equipped with Normarski interference optics, courtesy of Dr Joseph F. Gennaro, Jr.

Electron microscopy

Fixative for electron microscopy was made by combining equal amounts of stock solutions of 2 % acrolein and 3 % glutaraldehyde in 0.2 M cacodylate buffer with 2 % osmic acid in distilled water. One millilitre of cell suspension was mixed with 8 ml freshly prepared fixative for 5 min and then centrifuged (1000 g for 3 min). Cells were washed 3 times with distilled water, stained with 2 % uranyl acetate for 30 min and dehydrated with ethanol. Spurr's low molecular weight epoxy was used as the embedding medium. Sections were stained with lead citrate and viewed in a Zeiss EM 9S.

All centrioles visible in 6-8 sections from each of 4 experiments were photographed at x 17000 and printed at x 50000 on high-contrast paper. Pericentriolar microtubules were counted as previously described (Goldstein, Hoffstein, Gallin & Weissmann, 1973) from all the centrioles photographed. The numerical density of peripheral microtubules was determined following the procedure of Hoffstein, Goldstein & Wissmann (1977).

RESULTS

Effect of colchicine on microtubule numbers and random migration

Colchicine inhibits microtubule assembly (Borisy & Taylor, 1967; Hoffstein et al. 1977) and increases the number of neutrophils that spontaneously migrate from the wells in the under-agarose assay (Dziezanowski et al. 1980). We compared the effect of a range of colchicine concentration from $10^{-7}$ to $10^{-5}$ M on both migration and

![Graph showing the effect of colchicine on neutrophil migration and pericentriolar microtubule numbers.](image)

Fig. 1. Effect of colchicine on neutrophil migration and pericentriolar microtubule numbers. Abscissa: concentration of colchicine in the medium during the 45-min pretreatment. ●, migration of neutrophils expressed as mean no. of cells that left the well ± s.e. ○, mean no. of pericentriolar microtubules in 4 μm² ± s.e.
Fig. 2. Micrographs of pericentriolar regions of neutrophils at pH 7.4 (A); after pretreatment in medium containing $10^{-3}$ M colchicine (B); at pH 6.0 (C); and after pretreatment in medium containing $10^{-4}$ M colchicine and subsequent incubation at pH 6.0 (D). Note microtubules in A and C but not after colchicine treatment, B, D. x 64,000.
microtubule numbers. In the absence of colchicine, microtubule numbers in the pericentriolar region were high and few neutrophils migrated out of the wells (Fig. 1).

At 10^{-7} M colchicine, there was no significant change in either migration or microtubule numbers (P > 0.05, Student's t test). However, microtubule numbers decreased and the number of cells migrating increased at 5 \times 10^{-7} M colchicine (P < 0.01). Microtubule numbers were even lower at 10^{-8} and 10^{-9} M colchicine and the number of cells migrating increased. Thus, the number of microtubules in the pericentriolar region of the neutrophils was inversely correlated with the number of cells that migrated (Fig. 2A, B).

![Graph showing effect of pH and colchicine on neutrophil migration and pericentriolar microtubule numbers. Abscissa: incubation/migration conditions. Y, migration of neutrophils expressed as mean no. of cells that left the well ± S.E. ■, mean no. of pericentriolar microtubules in 4 μm² ± S.E.](image)

**Effect of pH**

At pH 7.4, migration was low and the mean number of pericentriolar microtubules was high. After 10^{-5} M colchicine pretreatment, the number of neutrophils that migrated from the wells was increased approximately 7-fold and the mean number of microtubules greatly reduced. When no colchicine was present in the preincubation medium, reduction of the pH of the migration medium from 7.4 to 6.0 enhanced migration approximately fourfold and decreased the mean microtubule number from 16 to 9.5 (Fig. 3) (P < 0.01). In this system, colchicine reduced even further the number of centriole-associated microtubules (Fig. 2C, D). However, the migration of colchicine-treated cells at pH 6.0 was approximately 4 times the pH 7.4 control. This migration is equivalent to that of untreated cells at pH 6.0. Since microtubule numbers were virtually zero, colchicine's inability to affect migration at pH 6.0 was not due to lack of penetration.
The reduction in pericentriolar microtubule numbers at the low pH was reproducible and consistent. In 4 separate experiments reducing the pH decreased the mean number of pericentriolar microtubules (Table 1). Whereas the mean number of microtubules in the control cells varied from experiment to experiment, in any given experiment the mean number of microtubules at pH 6.0 was approximately two thirds the number seen at pH 7.4. Regardless of the pH of the medium, colchicine reduced the number of microtubules to almost zero.

Table 1. Effect of colchicine and pH on pericentriolar microtubule number* in neutrophils

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH 7.4</th>
<th>pH 6.0</th>
<th>pH 7.4 + 10^-6 M colchicine</th>
<th>pH 6.0 + 10^-6 M colchicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.0 ± 2</td>
<td>13.0 ± 3</td>
<td>—</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>14.6 ± 1</td>
<td>9.0 ± 1</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>22.2 ± 2</td>
<td>16.6 ± 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>15.9 ± 1</td>
<td>9.6 ± 1</td>
<td>2.1 ± 0.4</td>
<td>1.0 ± 0.5</td>
</tr>
</tbody>
</table>

Cells were incubated at pH 7.4 with and without 10^-5 M colchicine and at pH 6.0 with and without 10^-5 M colchicine for 1 h at 37 °C. In experiment 4, cells were pretreated with 10^-5 M colchicine for 1 h and then exposed to medium of pH 7.4 or 6.0.
* Microtubules within a 2 μm x 2 μm square area centred on a centriole. Mean ± S.E.M. (n = 6–10).

Table 2. Effect of colchicine and pH on numerical density* of microtubules in peripheral cytoplasm of neutrophils

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH 7.4</th>
<th>pH 6.0</th>
<th>10^-6 M colchicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.2 ± 0.3</td>
<td>2.1 ± 0.8</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>9.8 ± 2.2</td>
<td>2.2 ± 0.9</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>8.4 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>7.0 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>1.4 ± 0.6</td>
</tr>
</tbody>
</table>

* Mean no. of microtubules per 10 μm² of peripheral cytoplasm ± S.E. (n = 6–10). See legend to Table 1 for description of experiments.

Since the peripheral microtubules may play a greater role in cell migration than pericentriolar ones, we examined the microtubule distribution within the neutrophil by analysing the numerical density of microtubules in electron micrographs of the peripheral cytoplasm (Table 2). In control cells, the mean number of microtubules per 10 μm² of peripheral cytoplasm varied from 6 to approximately 10, whereas in medium at pH 6.0 the mean numbers varied from 2 to 3. At 10^-6 M colchicine, the mean microtubule number was approximately 1. Thus, the effect of lowered pH on peripheral microtubules was more drastic than the effect on pericentriolar microtubules.
Effect of osmolality

Reducing the osmolality of the agarose-medium gel also increased random migration (Rabinovitch et al. 1980) but did not alter pericentriolar microtubule numbers (Fig. 4). At 280 m-osmol, approximately 50 neutrophils migrated out of the wells and these cells had a mean of 23 microtubules in their pericentriolar region. When the osmolarity was reduced to 230, random migration was enhanced more than 2-fold without any effect on microtubule numbers. After preincubation with 10⁻⁵ M colchicine, migration at the lower osmolality was greatly enhanced above that seen in the absence of colchicine and microtubule numbers were down to virtually zero. Colchicine alone as always enhanced migration but not as much as the combination of lowered osmolality and colchicine did.

Distance travelled by neutrophils forming the leading front

We also measured the distance travelled by neutrophils forming the leading front (Table 3). The leading front was defined as a line parallel to the edge of the well and through 2 adjacent cells that had travelled farthest from the edge of the well. The mean distance that the leading front neutrophils travelled after colchicine pretreatment was not significantly different from that observed under control conditions. In contrast, when the pH or the osmolality of the medium was reduced, the leading front
was significantly farther from the well than the controls (Student's t test, \( P < 0.001 \)). Colchicine pretreatment did not alter the mean distance travelled by the leading cells in medium of lowered pH or lowered osmolality. Thus, colchicine only increased the number of cells that migrated whereas low pH or osmolality increased both the number that migrated and the mean distance that cells forming the leading front travelled.

Table 3. Effect of pH, osmolality and colchicine on neutrophil migration* under agarose

<table>
<thead>
<tr>
<th></th>
<th>— Colchicine</th>
<th>+ Colchicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4, 280 m-osmol</td>
<td>82 ± 4 ( \mu )m (8)</td>
<td>107 ± 9 ( \mu )m (8)</td>
</tr>
<tr>
<td>230 m-osmol</td>
<td>137 ± 12 ( \mu )m (10)</td>
<td>129 ± 13 ( \mu )m (9)</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>172 ± 17 ( \mu )m (7)</td>
<td>163 ± 22 ( \mu )m (6)</td>
</tr>
</tbody>
</table>

* Data are expressed as the mean distance from the edge of the well of those cells forming the leading front ± S.E.M. \( (n = \text{no. of wells}). \) One hour of incubation at 37 °C.

Light-microscope morphology

We then examined the morphology of the cells by light microscopy to see whether this offered any clues as to what system within the cell these conditions may be affecting. Fig. 5 shows representative neutrophils under the influence of colchicine, pH and osmolality as the cells migrate under the agarose. The shape of both the leading and trailing edges varied with the conditions.

Under control conditions, the cells were generally fan-shaped with a broad, smooth leading edge and a rounded rear that often had small, short retraction fibres trailing behind. The direction of cell migration was easy to establish since the cells were polarized. Colchicine seems to affect the polarity of the cells, allowing the leading edge to be formed anywhere on the periphery. After colchicine treatment, many cells appeared bent. The retraction fibres were often very long, making the direction from which the cell had come particularly obvious. Reducing the osmolality led to a change in the leading edge. Instead of being smooth, it was often scalloped and in many cases appeared thickened. Retraction fibres were often seen in the rear of the cell. Cells exposed to lowered osmolality and colchicine looked similar to those exposed to only low osmolality. Neutrophils exposed to lowered pH were more elongated than control cells. The rear of the cell was often very narrow but usually lacked retraction fibres. Staining differences in the cytoplasm were often visible. Combining colchicine pretreatment with lowered pH did not significantly alter cell morphology from that observed with lowered pH alone.
Fig. 5. Light micrographs of neutrophils under agarose: at 280 m-osmol, pH 7.4 (A); after $10^{-8}$ M colchicine pretreatment (B); at 230 m-osmol, pH 7.4 (C); colchicine pre-treatment at 230 m-osmol pH 7.4, (D); at pH 6.0 (E) and colchicine treatment at pH 6.0 (F). Photographed $\times 400$ and printed $\times 4$. 
DISCUSSION

Microtubules, colchicine and migration

Microtubules seem to play a constraining role within the cell, limiting the ability of the cell to move and to change direction. We have shown that colchicine leads to an increase in the number of neutrophils that migrate under the agarose but does not increase the distance of the leading front from the edge of the well. One possible hypothesis is that colchicine causes an increase in the proportion of the motile cells without altering individual cell velocity. Ramsey & Harris (1972) analysed neutrophil locomotion in time-lapse films and found no change in neutrophil velocity with concentrations of colchicine similar to those used in this study. However, they did find that treatment with colchicine increases surface activity. Allan & Wilkinson (1978) described colchicine-treated cells as more plastic and extending more processes than control cells. There is also an increase in membrane fluidity with colchicine (Oliver, Ukena & Berlin, 1974). An increase in cell plasticity in itself could account for the larger number of cells under the agarose with colchicine than in the control. Without changing cell velocity or the proportion of motile cells within the population, colchicine could allow a greater percentage of cells at the edge of the well to squeeze under the agarose.

Effects of pH

Both colchicine and medium of low pH reduced microtubule numbers. If the increased migration at pH 6.0 were due solely to reduced microtubule numbers, one would expect the responses of colchicine-treated cells at 6.0 to be the same as that of colchicine-treated cells at pH 7.4. This is not the case. The degree of increased migration, the cell shape and the distance of the leading front from the well all duplicated the neutrophil responses at pH 6.0, not those of colchicine-treated cells. In fact, the effects of pH 6.0 override those of colchicine treatment. Lowered pH and colchicine also affect other neutrophil functions differently. Penny, Glaton, Scott & Eisen (1966) found a reduction in cell to substrate adhesion after colchicine treatment whereas Kvarstein (1969) found no change in neutrophil adherence in plasma adjusted to pH 6.3. Colchicine treatment reduces release of endogenous chemotactic factors (Spilberg, Gallacher, Mehta & Mandell, 1976) but neutrophils at low pH release increased amounts of a chemotactic factor (S. Zigmond, personal communication). Therefore, lowered pH does not have the same effects on the neutrophil that colchicine treatment does but increases migration by means of a different mechanism.

Effect of osmolality

Migration enhancement by low osmolality appears to occur via a different mechanism than either low pH or colchicine since both cell shape and microtubule numbers are more similar to the untreated control than to either of the other treatments. The only morphological change seen was at the edge of the lamellipodia, which were scalloped and thickened compared to controls. This localized thickening or swelling suggests
that ion and water fluxes may be important in motility as suggested by Mukherjee & Lynn (1978). The data therefore suggest that each of these treatments enhances the activity of different aspects of the neutrophil motility system.

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REFERENCES


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