NEUTROPHIL GRANULOCYTES: ADHESION AND LOCOMOTION ON COLLAGEN SUBSTRATA AND IN COLLAGEN MATRICES

A. F. BROWN
Department of Cell Biology, The University, Glasgow G12 8QQ, Scotland

SUMMARY

Neutrophil granulocytes (PMNs) adhere poorly to and are unable to locomote on collagen-coated glass; they are able to attach to and invade three-dimensional matrices of collagen fibres. Invasion is largely independent of adhesion to the fibres, does not occur by proteolysis of the gel, and is not affected by the presence of fibronectin or chondroitin sulphate. Invasion is reduced by increasing the concentration of collagen in the gel or by the presence of hyaluronic acid. It is proposed that in both these situations there is physical obstruction of PMN movement. The ability to locomote through tissues of very variable properties in vivo is important in the role of the PMN in inflammation.

INTRODUCTION

Visual studies of cell locomotion have traditionally been performed using protein-coated glass and plastic substrata. In addition, fibrin clots and, more recently, three-dimensional gels of native collagen fibres have been used, as these provide an environment more like the physiological environment of the cells, and can be used to test the ability of cells to invade the fibrin and collagen matrices (Elsdale & Bard, 1972; Schor, 1980; Kleinman, Klebe & Martin, 1981).

The mechanism of fibroblast locomotion seems to be similar on two-dimensional substrata and in three-dimensional collagen gels; i.e. while the cell morphology is different in the two situations, fibroblasts form strong adhesions with protein-coated glass (Abercrombie & Dunn, 1975) or collagen fibres within a gel (Grinnell & Bennett, 1981), allowing the locomotory force of the cell to be translated into forward movement. Some deformation of the collagen fibres in gels, as a result of fibroblast traction, has also been noted (Bard & Hay, 1975).

Neutrophil granulocytes (PMNs) locomote over surfaces by a mechanism similar to that of fibroblasts, although these cells form broad areas of weak adhesion (as shown by interference reflection microscopy) rather than strong focal adhesions (Armstrong & Lackie, 1975). The weak forces generated by PMNs on the substratum have been demonstrated by the ability of these cells to locomote over protein films adsorbed to silicone oil substrata that are five orders of magnitude less viscous than that required to support fibroblast movement (Harris, 1973). Harris (1982) has also shown that while normal fibroblasts can deform silicone rubber substrata, forming wrinkles, PMNs do not.
A. F. Brown

In response to an inflammatory stimulus, PMNs leave the circulation and migrate through the collagenous extracellular matrix towards the focus of inflammation. It is demonstrated here that PMNs are unable to locomote over collagen-coated glass due to lack of adhesion, and thus traction, between the cells and collagen, but can locomote within three-dimensional gels of collagen: a possible mechanism for locomotion in the latter situation is discussed.

The effects of altering PMN adhesion to collagen, varying the composition of the collagen matrix, and the effects of collagenase inhibitors are also discussed with respect to the role of PMNs in inflammation.

**MATERIALS AND METHODS**

**Isolation of PMNs**

Peritoneal exudates were elicited in female New Zealand White rabbits by injection of 500 ml of sterile 0.9% (w/v) NaCl containing 0.1% (w/v) oyster glycogen (Sigma Chemical Co.). Peritoneal fluid, containing > 95% pure PMNs, was collected after 4 h (Lackie, 1974). Rabbit peritoneal PMNs were used in all experiments described here, but human and rabbit peripheral blood PMNs were also used and found to behave in a similar manner to rabbit peritoneal PMNs. These cells were isolated by standard methods as described previously (Brown & Lackie, 1981).

**Preparation of PMNs for adhesion and invasion assays**

Cells isolated by the above methods were washed successively in calcium- and magnesium-free salts solution (CMF, pH 7.4), CMF/EDTA (as CMF, with 1 mM-EDTA, pH 7.4), and balanced salts solution (BSS, pH 7.4) (Brown & Lackie, 1981). The cells were then resuspended at the desired concentration, usually 1 x 10^6 cells/ml.

Cells labelled with 61 Cr were prepared by washing in CMF and CMF/EDTA as described above. The cell pellet was resuspended in 0.5% (w/v) bovine serum albumin (BSA, Behringwerke) in BSS and 100 µCi of Na_2^{61}CrO_4 activity (Radiochemical Centre, Amersham) were added. The cells were incubated for 30–45 min at 37 °C, washed three times in BSS to remove BSA and free 61 CrO_4, and resuspended in the appropriate medium at the required concentration.

**Isolation of collagen and fibronectin**

Type I collagen was prepared from rat tail tendons as described by Schor (1980). Tendons were stripped and solubilized in 3% (v/v) acetic acid for 2 days at 4 °C. Insoluble material was removed by centrifugation at 3000 g for 30 min and the clear tropocollagen solution was mixed with an equal volume of 20% (w/v) NaCl to precipitate the collagen. The collagen was pelleted by centrifugation at 3000 g for 45 min, washed twice in distilled water, and resuspended in 3% (v/v) acetic acid at a final concentration of 3 mg/ml as determined by measuring absorbance at 230 nm compared with a calibration curve prepared from standard solutions of a freeze-dried sample of the collagen preparation. The collagen solution was then dialysed exhaustively against distilled water adjusted to pH 4.0 with HCl.

In some experiments commercially prepared type I bovine dermal collagen (Vitrogen 100, Flow Laboratories, Irvine, Scotland) was used. This gave similar results to rat-tail tendon collagen.

Fibronectin was isolated from rabbit, human or bovine serum by affinity chromatography on a gelatin-Sepharose column (Engvall & Ruoslahti, 1977). Fibronectin was eluted with 8 M-urea, dialysed against BSS and stored for up to 3 weeks at 4 °C at a final concentration of 1 mg/ml. Fibronectin purity was checked by SDS–polyacrylamide gel electrophoresis.

Other reagents used were neutral dextran (M_r 5 x 10^6, Pharmacia), hyaluronic acid (M_r 10^6, Miles), chondroitin sulphate from whale cartilage (Sigma), alpha-1-antitrypsin (Sigma), and human alpha-2-macroglobulin (a generous gift from Dr J. V. Forrester).
Preparation of collagen-coated coverslips

Glass coverslips of 13 mm diameter were immersed in 1 ml of a solution of collagen at a concentration of 100 \( \mu \text{g/ml} \) in 1 % (v/v) acetic acid. Collagen was precipitated onto the glass by raising the pH of the solution with 0.1 ml 20 % (w/v) Trizma base (Tris(hydroxymethyl) aminomethane) or by salting out the collagen with 0.1 ml 20 % (w/v) NaCl. The coverslips were incubated at 37 °C for 1 h, then washed in BSS to remove acetic acid, Trizma base or NaCl. It should be noted that this procedure produces only a thin coat of collagen on the glass, and not a gel of the type described in the following section.

Preparation of collagen gels

The collagen stock solution was mixed at 4 °C with appropriate amounts of 10× BSS and 0.142 M-NaOH to restore pH and ionic strength to physiological values. This was then diluted with BSS or test reagent in BSS to the desired concentration, and 0.5-ml samples were placed in wells of 16 mm diameter. The collagen polymerized in approximately 15 min, but the gels were incubated for 2 h at 37 °C before use, as this seemed to prevent detachment of the gels from the wells on the addition of cell suspension.

Adhesion assay

A total of 10⁶ cells in 1 ml of BSS, or BSS containing test reagent, was added to wells containing clean glass or collagen-coated coverslips. The coverslips were incubated at 37 °C for 30 min, washed ten times through an air/liquid interface to detach non-adherent cells, and the residual gamma-emission of \(^{61}\text{Cr}\)-labelled cells was counted on a Wilj 2001 Gamma counter. Alternatively, cells were stained and counted visually. Adhesion to clean glass was taken as 100%; in all experimental controls (clean glass) 35-45 % of the total cells added adhered to clean glass.

Invasion assay: visual methods

Collagen gels were prepared as described above and 1 ml of cell suspension at 10⁶ cells/ml was added to each well. The gels were incubated, normally for 2 h, fixed in formol saline, and the leading front of the cell population (Zigmond & Hirsch, 1973) was measured using the calibrated fine focus on a Leitz Diavert microscope at a magnification of ×320. Five readings were taken from each of three replicate gels and the mean ± S.D. was calculated for each treatment.

In addition, the distribution of cells through the gels was determined by counting the number of cells in half a field at successive intervals of 30 or 40 \( \mu \text{m} \).

Invasion assay: \(^{61}\text{Cr}\) method

As a supplement to the above methods, \(^{61}\text{Cr}\)-labelled cells were added to collagen gels and incubated for 2 h at 37 °C. The medium was then removed and 1 ml of 0.02 % collagenase added to each well for 10 min at 37 °C. This was removed and the surface of the gel was washed three times with BSS to remove cells on the gel surface. The bulk of the gel was then counted for residual \(^{61}\text{Cr}\) emission on a Wilj 2001 gamma counter. Knowing the total counts added, and the residual counts in the gel, the percentage of cells invading the gel can be calculated. The mean ± S.D. was calculated from four replicates.

Scanning electron microscopy

Collagen gels were fixed in 2.5 % glutaraldehyde, dehydrated through a series of ethanol, taken to absolute acetone, critical-point dried, and sputter-coated with gold. After being taken to acetone some gels were embedded in paraffin wax, sections were cut, and the wax was removed with xylene. These were then processed for scanning electron microscopy as before.
It should be noted that the effects of dehydration may not reflect the true spatial relationships of the fibres to each other or to the cells; comparisons with time-lapse film do, however, suggest a close similarity.

RESULTS

Time course of invasion

The time course of invasion of the collagen matrix by PMNs, using the leading front method, is shown in Fig. 1. The rate of migration is linear for approximately 2 h, after which movement of the leading front slows down. For this reason all assays were carried out over a 2-h period, thus maximizing the displacement.

![Graph showing the time course of invasion of collagen gels (1 mg/ml) by PMNs.](image)

Fig. 1. Time course of invasion of collagen gels (1 mg/ml) by PMNs; $10^8$ cells were added to each well, incubated at 37 °C for 2 h, and the leading front measured. Gels were reconstituted with BSS.

A plot of $\log_{10}N$ against $d^3$, where $N$ is the number of PMNs in half a $\times 320$ field at a distance of $d \mu m$ into the gel, is shown in Fig. 2. The plot is linear, indicating that the distribution of cells within the collagen matrix is close to that expected for a random walk (Zigmond & Hirsch, 1973). In addition, the leading front measurements after 1 h and 2 h lie close to the linear plots of cell distribution; thus the leading front is probably a good approximation of the population movement within the matrix.

PMNs were shown not to be falling through the collagen gels under the influence of gravity, in several ways: erythrocytes and fixed PMNs did not penetrate the gels; PMNs fixed within the matrix or cooled to room temperature did not continue to move within the gel; and time-lapse film occasionally showed cells entering the gel and subsequently moving against gravity and returning to the gel surface.
Neutrophil granulocyte invasion of collagen matrices

![Graph](image)

Fig. 2. Distribution of cells through collagen gels (1 mg/ml) after 1 h and 2 h. \( N \) is the number of cells in half a \( \times 320 \) field at a distance of \( d \ \mu \text{m} \) from the gel surface. Circled data points represent the leading front of the population after 1 h (\( \times \) ) and 2 h (\( \bullet \)).

**Effect of collagen concentration**

It was found that gels could be formed satisfactorily only at concentrations between 1-0 and 2-5 mg/ml; below 1 mg/ml they were unstable and tended to rupture easily; above 2-5 mg/ml the high viscosity of the collagen solution caused trapping of air bubbles during reconstitution, rendering the optical properties of the gels very poor.

Fig. 3 shows that increasing the collagen concentration reduces PMN invasion; this would be consistent with a reduction in the number of gaps in the matrix large enough for PMNs to move through. Time-lapse analysis has shown that constriction rings formed by cells within the collagen gels remain stationary relative to the matrix while the cells move forward (Fig. 4): these may be the result of cells squeezing through small gaps between collagen fibres.

**Effects of adhesion on invasion**

It has been shown previously (Brown & Lackie, 1981) that the adhesion of PMNs to collagen-coated glass is very low; time-lapse analyses have shown that on such a substratum cells gain insufficient traction to translocate over the collagen-coated glass (Table 1). It was found that the adhesion of PMNs to collagen-coated glass could be altered by the removal of \( \text{Mg}^{2+} \) or addition of \( \text{Mn}^{2+} \) to BSS. Thus a comparison could be made between PMN–substratum adhesion and the ability of PMNs to locomote over the surface of collagen, and the ability to locomote within a three-dimensional collagen matrix (Table 1). The absence of \( \text{Mg}^{2+} \) or the presence of \( \text{Mn}^{2+} \) did not
Fig. 3. Effect of collagen concentration on PMN invasion. The leading front was measured after 2 h.

Collagen concentration (mg/ml)

Leading front (mm) (mean ± 1 s.d.)

Fig. 4. PMN moving within a collagen gel in the direction of the large arrow. The cell can be seen extending a pseudopod (p), which dilates beyond the stationary constriction ring (c). The dilated pseudopod may behave like an anchor, allowing the cell to gain purchase against the collagen fibres and thus pull the cell body forward. The interval between frames was 30 s. Nomarski optics. × 1250.
Neutrophil granulocyte invasion of collagen matrices

affect cell viability as determined by trypan blue exclusion, or by the ability of cells to ruffle and attempt to translocate.

It can be seen from Table 1 that conditions of very low and very high adhesion prevent cell locomotion on two-dimensional collagen, owing to lack of traction and anchorage of cells, respectively. However, movement in the three-dimensional collagen matrix seems to be largely independent of adhesion to the collagen; only at very high values of adhesion, in the presence of $10^{-2}$ M-Mn$^{2+}$, is movement reduced, presumably by adhesive drag between cell and substratum, although sub-lethal toxicity of $10^{-2}$ M-Mn$^{2+}$ cannot be ruled out.

**Table 1. Effects of adhesion on locomotion**

<table>
<thead>
<tr>
<th>Medium</th>
<th>% Adhesion (mean ± S.E. (n))</th>
<th>% Motile cells</th>
<th>% Anchored cells</th>
<th>Three-dimensional collagen gels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Leading front (μm) (mean ± S.D. (n))</td>
</tr>
<tr>
<td>Mg$^{2+}$-free</td>
<td>4 ± 1 (5)</td>
<td>0</td>
<td>0</td>
<td>164 ± 13 (15)*</td>
</tr>
<tr>
<td>BSS</td>
<td>14 ± 3 (5)</td>
<td>0</td>
<td>0</td>
<td>202 ± 16 (15)</td>
</tr>
<tr>
<td>$10^{-3}$M-Mn$^{2+}$</td>
<td>36 ± 9 (5)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>189 ± 14 (15)</td>
</tr>
<tr>
<td>$10^{-2}$M-Mn$^{2+}$</td>
<td>66 ± 7 (5)</td>
<td>74</td>
<td>&gt; 20†</td>
<td>160 ± 15 (15)†</td>
</tr>
<tr>
<td>$10^{-1}$M-Mn$^{2+}$</td>
<td>110 ± 5 (5)</td>
<td>5</td>
<td>&gt; 68†</td>
<td>57 ± 8 (15)†</td>
</tr>
</tbody>
</table>

The adhesion and locomotion of PMNs on collagen-coated glass was determined in Mg$^{2+}$-free BSS, BSS and BSS + Mn$^{2+}$; the invasion of collagen gels reconstituted with these media was also measured as described in the text. N.D., not done.

* The reduction in invasion in the absence of Mg$^{2+}$ is probably not due to lower PMN-substratum adhesion, but a result of PMN aggregation on the gel surface reducing the number of cells available for invasion.

† PMNs were scored as being anchored if translocation was attempted but hindered by a highly adhesive tail. These values are therefore minimum percentages as some cells may have been too adhesive to attempt translocation.

‡ $P$ (no different from BSS control) < 0.001.

**Scanning electron microscopy**

Scanning electron micrographs of PMNs within collagen matrices clearly show cells with pseudopodia extended between collagen fibres (Figs. 5, 6). The direction of cell locomotion in three dimensions cannot be determined by cell morphology as can generally be done for cells moving on a two-dimensional substratum; however, comparison with time-lapse film suggests that the cell in Fig. 5 is probably moving towards the top of the photograph.

PMNs locomoting on a two-dimensional substratum have a broad area of weak adhesion at the anterior of the cell (Armstrong & Lackie, 1975), which is probably a result of the requirement for traction with the substratum. The ability of PMNs to locomote in three-dimensional matrices without a broad leading edge is further evidence that adhesion to the fibres of the matrix is not necessary for locomotion; it could be argued, however, that very weak adhesive forces exist between the whole cell surface and the collagen fibres.
Fig. 5. PMN within a 30 μm section of collagen gel cut perpendicularly to the surface of the gel. The direction of locomotion cannot be determined, but comparison with time-lapse film suggests movement towards the top of the photograph. Bars, 1 μm.

Fig. 6. PMNs on the surface of a collagen gel. As for Fig. 5 the direction of locomotion cannot be determined. Bars, 1 μm.
Anti-protease effects

To determine whether PMNs moved through the three-dimensional collagen matrix by proteolysis of the collagen fibres, two broad-spectrum plasma anti-proteases, alpha-1-anti-trypsin and alpha-2-macroglobulin, were incorporated into gels at concentrations near physiological values. Both these protease inhibitors are known to inhibit PMN collagenase (Ohlsson & Olsson, 1977). No effect on invasion was seen in the presence of alpha-1-antitrypsin or alpha-2-macroglobulin (Table 2). It has also been shown that fibronectin can inhibit a variety of mammalian collagenases, including PMN collagenase (Biswas, Hynes & Gross, 1979; Uitto et al. 1979). Fibronectin at concentrations from 1-500 \( \mu \text{g/ml} \) had no effect on invasion (Table 3). In addition, scanning electron micrographs showed no apparent changes in the structure of the collagen gels after invasion by PMNs. It was concluded, therefore, that invasion of collagen gels by PMNs is not dependent on proteolytic digestion of the collagen fibres.

Effect of extracellular matrix components

Three components of the extracellular matrix, fibronectin, chondroitin sulphate and hyaluronic acid, were tested for their effect on invasion of collagen gels by PMNs.
Fibronectin at concentrations of 1–500 μg/ml and chondroitin sulphate at concentrations of 100 μg–5 mg/ml had no effect on movement, judged by the distance of the leading front, or percentage of ³¹Cr-labelled cells entering the gels (Table 3). The distribution of cells through the matrix after 2 h was not significantly different from the control shown in Fig. 2.

Hyaluronic acid (M, 10⁴), at concentrations of 1 μg/ml–1 mg/ml, had no effect on the leading front (Fig. 7), or distribution of cells through collagen gels with a collagen concentration of 1 mg/ml. However, hyaluronic acid at a concentration of 1 mg/ml did inhibit the invasion of collagen gels at a higher collagen concentration of 2·5 mg/ml (Fig. 7). This effect might be explained in three ways: increased PMN adhesion to the collagen fibres producing adhesive drag; increased bulk viscosity of the medium caused by hyaluronic acid; or some other physical obstruction. These will be dealt with in turn.

Invasion might be reduced if hyaluronic acid increased PMN–collagen adhesion, thus increasing adhesive drag; indeed, hyaluronic acid was found to increase PMN adhesion to collagen from 10% to about 40% (taking adhesion to clean glass as 100%). However, an equivalent increase in adhesion produced by the addition of Mn²⁺ did not reduce invasion of the gels; it would also be expected that reduced invasion, if caused by adhesive drag, would be seen in gels containing 1 mg/ml collagen. Reduced invasion due to increased adhesive drag seems unlikely.
Reduced invasion due to the increased bulk viscosity of the medium caused by hyaluronic acid can be ruled out since it would be expected that this effect would be seen regardless of the concentration of collagen in the gel; in addition, neutral dextran solution of the same viscosity as 1 mg/ml hyaluronic acid (Forrester & Lackie, 1981) did not affect invasion (Fig. 7). Thus increased bulk viscosity of the medium in the interstices of the collagen matrix was probably not the cause of reduced invasion.

The third explanation involves the interaction of hyaluronate molecules with each other. It is known that hyaluronate molecules can interact to form a matrix with gel-like properties (Comper & Laurent, 1978). It is possible that hyaluronate within the collagen matrix might form local areas of hyaluronate gel, which could block gaps in the collagen fibre mesh and obstruct PMN movement. Smaller gaps, such as those likely in a gel of 2.5 mg/ml collagen, would be blocked more easily than those in a 1 mg/ml collagen gel.

**DISCUSSION**

It has been shown that while PMNs adhered poorly to, and were unable to gain traction to locomote over, collagen-coated glass, they were able to locomote within three-dimensional gels of collagen. This suggests there is a fundamental difference in the method of PMN locomotion in three-dimensional matrices compared with that on surfaces; this is in contrast to fibroblasts, which seem to adhere to and locomote on collagen fibres in a similar manner, regardless of whether they are on a surface or in a gel (Bard & Hay, 1975).

It is proposed that PMNs attach to and locomote within collagen gels by extending pseudopodia (into gaps in the matrix) that, when dilated, behave like anchors; the cells could then gain sufficient purchase against the fibres to pull the rest of the cell body through the gaps. Time-lapse films of PMNs moving within collagen gels support this hypothesis: constriction rings in cells have been observed to remain stationary relative to the matrix while the cell itself moves forward; similar observations have been made of lymphocytes moving in fibrin and collagen gels (de Bruyn, 1946; Haston, Shields & Wilkinson, 1982). This evidence is not conclusive, however, as cells have also been observed to form constriction rings on surfaces.

The space between collagen fibres is also important in determining the extent of invasion as shown by the inverse correlation of invasion against collagen concentration. A similar correlation has been shown for the rate of movement of neural crest cells in collagen gels (Davis & Trinkaus, 1981). Haston et al. (1982) found there was a lag period of several hours before lymphocytes invaded gels of high collagen concentration (3.0 mg/ml), and suggested that the surface of the gel might be somewhat different from the bulk of the matrix; this lag period has not been observed for neutrophils, and scanning electron microscopy of gels has shown no obvious difference between the surface and bulk of the gels.

Neutrophil invasion, like that of other tissue cells (Schor, Allen & Harrison, 1980), does not seem to be dependent on digestion of the collagen matrix by protease action. Plasma anti-proteases alpha-1-antitrypsin and alpha-2-macroglobulin had no effect
on the movement of PMNs into collagen gels; fibronectin, which also inhibits PMN collagenase, had no effect either. The capacity to locomote through collagenous matrices without causing degradation may prevent unnecessary damage to healthy tissue in vivo.

Increasing the adhesion of PMNs to the collagen fibres by the addition of Mn²⁺ had no effect on invasion until high values of adhesion, comparable to those between clean glass and neutrophils, were reached; this may be important in vivo where PMNs would have to migrate through different tissues of various adhesive properties towards inflammatory foci. High adhesiveness would tend to immobilize the cells and this effect may operate at the inflammatory focus (Lackie, 1982). It should be noted, however, that while these results may reflect the ability of individual PMNs to cope with a variety of adhesive conditions, it seems likely that the PMN population is broadly heterogeneous in its adhesive properties, so that a proportion of the cells will be capable of moving optimally over substrata of different adhesive properties. For a full discussion of this see Lackie & Brown (1982).

The extracellular matrix components, fibronectin and chondroitin sulphate, did not affect invasion of the gels. Hyaluronic acid did inhibit invasion in gels of high collagen concentration (2.5 mg/ml). It is proposed that this may be due to physical obstruction of gaps in the collagen matrix by areas of hyaluronate gel. The inhibitory effect of hyaluronic acid on the adhesion of PMNs to clean glass may also depend upon this obstructive principle (Forrester & Lackie, 1981).

In conclusion, the invasion of collagen matrices by PMNs is apparently independent of PMN-collagen adhesion, except under conditions of very high adhesion, which are likely to be non-physiological. The composition of the matrix appears to have little effect on invasion, unless the cells are perhaps physically obstructed by a high concentration of collagen (reducing the size and/or number of gaps through which cells can pass) or by the presence of hyaluronate (which may form a second matrix within the collagen gel and inhibit PMN movement by blocking pores in the matrix). It seems that PMNs are capable of locomoting in three-dimensional matrices over a wide range of conditions, an attribute fitting to the role that they play in the inflammatory response in vivo.

The author would like to thank Drs L. Tetley and J. Kerr for assistance with SEM, and Professor A. S. G. Curtis and Dr J. M. Lackie for their critical reading of the manuscript. This work was supported by an MRC research studentship.

REFERENCES


Neutrophil granulocyte invasion of collagen matrices


(Received 29 May 1982)