Neutrophil Motility in Extracellular Matrix Gels: Mesh Size and Adhesion Affect Speed of Migration

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ABSTRACT Polymorphonuclear leukocyte (PMN) migration through tissue extracellular space is an essential step in the inflammatory response, but little is known about the factors influencing PMN migration through gels of extracellular matrix (ECM). In this study, PMN migration within reconstituted gels containing collagen type I or collagen type I supplemented with laminin, fibronectin, or heparin was measured by quantitative direct visualization, resulting in a random motility coefficient (μ, a quantititive index for rate of cell dispersion) for the migrating cell population. The random motility coefficient in unsupplemented collagen (0.4 mg/ml) gels was $\sim 9 \times 10^{-9}$ cm^2/s. Supplementing gels with heparin or fibronectin produced a significant decrease in μ, even at the lowest concentrations studied (1 μg/ml fibronectin or 0.4 μg/ml heparin). At least 100 μg/ml of laminin, or 20% of the total gel protein, was required to produce a similar decrease in μ. Scanning electron microscopy revealed two different gel morphologies: laminin or fibronectin appeared to coat the 150-nm collagen fibers, whereas heparin appeared to induce fiber bundle formation and, therefore, larger interstitial spaces. The decrease in μ observed in heparin-supplemented gels correlated with the increased mesh size of the fiber network, but the differences observed in μ for fibronectin- and laminin-supplemented gels did not correlate with either mesh size or the mechanical properties of the gel, as determined by rheological measurements. However, PMNs adhered to fibronectin-coated surfaces in greater numbers than to collagen- or laminin-coated surfaces, suggesting that changes in cell adhesion to protein fibers can also produce significant changes in cell motility within an ECM gel.

INTRODUCTION

Polymorphonuclear leukocytes (PMNs) transmigrate through the vessel wall and migrate through tissue toward an inflammatory site, making PMN migration across the basement membrane and through the tissue extracellular space critical elements of the inflammatory response. The molecular mechanisms of PMN attachment and transmigration across the vessel wall are well studied (McIntire, 1994; Springer, 1990). PMN migration through tissue parenchyma is probably influenced by tissue architecture and by composition of the local extracellular matrix (ECM), but little information on how these factors affect rates of migration is available.

PMN migration and chemotaxis are frequently studied by in vitro assays, such as the under-agarose, filter chamber, or direct visualization assays (reviewed in Haston and Wilkinson, 1988; Wilkinson et al., 1982). Direct visualization assays permit improved quantification of cell motility, but generally are more difficult and time-consuming to perform. The pattern of migration for individual PMNs is a persistent random walk (Fig. 1): PMNs tend to persist in their migration when observed for intervals shorter than the persistence time ($t < P$, where P is the persistence time), but change directions randomly when observed over longer time intervals. Freshly isolated human PMNs that are otherwise unstimulated migrate with a random motility coefficient (μ) ranging from 10^{-9} to 10^{-8} cm^2/s and a persistence time of $\sim$1 min (Parkhurst and Saltzman, 1992); this corresponds to a motile speed of $\sim$0.1–0.2 μm/s (Buettner et al., 1989; Lauffenburger et al., 1983).

Previous reports have demonstrated that PMN functions, such as adhesion, are influenced by the relative abundance of various ECM components. In one previous study, unstimulated PMNs adhered in greater numbers to fibronectin-coated surfaces than to gelatin- or laminin-coated surfaces (Bohnsack et al., 1990), whereas phorbol ester-stimulated PMNs adhered equally well to gelatin-, laminin-, and fibronectin-coated surfaces (Bohnsack et al., 1990). In another study, phorbol myristate acetate-stimulated PMNs adhered to collagen ($\sim$50%) in lower numbers than to laminin or fibronectin (70%) (Lundgren-Akerlund et al., 1993). In addition, laminin (Terranova et al., 1986), entactin (Senior et al., 1992), and fragments of fibronectin (Odekon et al., 1991) appear to act as chemotactic factors for PMNs, suggesting that local rates of migration within tissues are potentially regulated by several different ECM components. The differential effects of ECM molecules on PMN adhesion and motility have been defined principally by studies involving purified molecules in artificial systems (i.e., polystyrene culture plates or cellulose filters). These studies suggest that the composition of ECM will be an important determinant of cell motility, but only a few quantitative measurements are available that test this hypothesis directly (Parkhurst and Saltzman, 1992; Reid et al., 1990; Reid and Newman, 1991).

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A fraction of the work reported here was performed at Cornell University.

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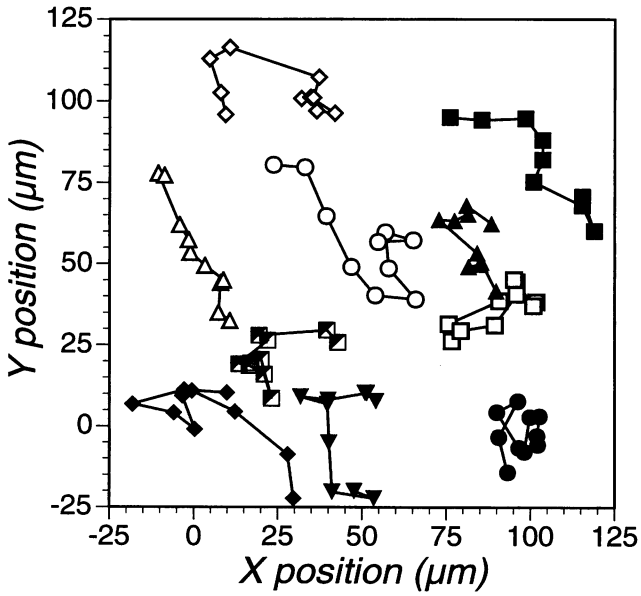

FIGURE 1 Pattern of movement of PMNs suspended in gels containing collagen type I. Each symbol represents the position of an individual cell, projected onto the two-dimensional microscopic field of view. The symbols for an individual cell show cell position at 90-s intervals over a 13.5-min total observation period. These paths were produced by a computer simulation for a cell population with $\mu = 4 \times 10^{-9}$ cm^2/s and P = 30 s; and these simulated cell paths are statistically indistinguishable from the paths produced by tracking PMN migration through a collagen gel (see Parkhurst and Saltzman, 1992, for details on computer simulation and comparison with measured cell paths).

In this report we measured the patterns and rates of random migration for PMNs suspended within reconstituted gels of ECM molecules. Reconstituted collagen type I gels provide a convenient matrix for the examination of cell motility (Brown, 1982; Grinnell, 1982; Islam et al., 1985), which we characterized for quantitative analysis of PMN migration in a previous report (Parkhurst and Saltzman, 1992). Here the migration of human PMNs was monitored within gels containing collagen type I with laminin, fibronectin, or heparin added during the reconstitution procedure. Rates of PMN migration were determined using a quantitative direct visualization method, in which the motile paths of 40–100 cells were tracked and compared with the extent of adhesion to the ECM-coated surfaces. Physical properties of the gels were also characterized by scanning electron microscopy (SEM) and oscillatory shear rheometry.

MATERIALS AND METHODS

Cell separation

Whole blood from healthy donors was collected by venipuncture into vials containing sodium heparin. PMNs were separated from whole blood samples and purified as previously described (Parkhurst and Saltzman, 1994). Briefly, heparinized whole blood was layered onto Ficoll-Hypaque medium (MPRM) (Mono-Poly Resolving Medium; ICN, Irvine, CA) and centrifuged at 1600 rpm for 30 min. The lower band of cells was collected, layered onto MPRM, and centrifuged at 2900 rpm for 15 min. The supernatant was collected and washed twice with Medium 199 (M199 with Hanks' balanced salts without sodium bicarbonate; Gibco, Grand Island, NY). Finally, the cells were resuspended in M199 at ~6.5×10^6 PMN/ml. The concentration of the cell suspension was estimated by using a 1:10 solution of trypan blue and counting the number of cells on the hemacytometer.

Collagen preparation

Solutions of type I collagen were prepared from rat tail tendons by a previously reported method (Parkhurst and Saltzman, 1992). Briefly, tendons from tails of rats were removed under 70% ethanol. The tendons were washed in fresh ethanol and placed in 0.02 M acetic acid (~100 ml solution/g wet tendon). Collagen from tendons was allowed to solubilize in acetic acid for at least 2 days at 4°C. The mixture was then centrifuged at 14,000 rpm for 30 min, and the supernatant was collected and centrifuged at 20,000 rpm for 30 min. The clear collagen-containing solution was collected and stored at 4°C. The concentration of collagen was determined by protein assay (microBCA, Pierce, Rockford, IL) and lyophilization.

Preparation and characterization of collagen gels

PMNs were encapsulated in type I collagen gels by a method similar to that previously reported (Parkhurst and Saltzman, 1992). First, the pH and osmolarity of the liquid collagen solution were raised to physiological levels (pH 7.2; osmolarity 300 mosM) by thoroughly mixing the following solutions in a microcentrifuge tube quickly and on ice: a predetermined amount of protein solution (fibronectin or heparin solutions at 1 mg/ml in M199; laminin solutions at 1 mg/ml in Tris-buffered saline) to obtain the desired concentration in a final gel volume of 250 μl, a predetermined amount of 0.1 M NaOH so that the final solution pH was 7.2, a predetermined amount of liquid collagen (1.44 mg/ml in 0.02 M acetic acid) to obtain the desired concentration, 1.5 μl 5% wt/vol sodium bicarbonate, 21.9 μl 10× concentration M199 (with Hanks' salts and L-glutamine, without sodium bicarbonate; Gibco), 10 μl 250 mM HEPES (Gibco), and a predetermined amount of highly purified water (18 MΩ resistance) to bring the final solution volume, including cells, to 250 μl. At this point, 31.3 μl of the PMN suspension was added, and the solution was gently but thoroughly mixed by repeat pipetting. This solution was drawn into a flat glass capillary tube (0.7 × 7.0 mm; Vitro Dynamics, Mountain Lakes, NJ), which was then sealed and incubated at 37°C for 7–10 min.

Scanning electron microscopy of gels

Collagen gel samples were fixed, dehydrated, and dried using standard techniques (Glauert, 1975); these procedures minimize, but do not eliminate, shrinkage of the collagen gel microstructure during preparation for electron microscopy (Parkhurst and Saltzman, 1992; Saltzman et al., 1992). Circular 12-mm glass coverslips were coated with polylysine to improve adhesion. Collagen gels (with and without laminin, fibronectin, or heparin) were prepared as described above. While the sample was in liquid form, a few drops were placed on the coverslips, which were then placed in a 37°C incubator for 15 min to allow the solution to gel. The resulting gels were fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS) (Sigma) for 1 h at room temperature, and then washed three times in PBS. Specimens were dehydrated through a series of graded ethanol solutions, and critical point dried. Coverslips and samples were sputter coated with a thin film of gold/palladium, and examined with an Amray scanning electron microscope, operated at 20 kV. Photographs produced by SEM were projected onto a screen. The diameter of the fibers was measured on the projected image; 10–20 different measurements were taken for each SEM. The measurements were averaged and normalized by the length of the projected image of the scale bar. Statistical significance of the differences between mean fiber diameters was determined by t-tests.

Measurement of cell motility in the gels

Our method for quantifying the rate of migration of a population of cells moving in three-dimensional gels was based on previous work by Dunn (1983) and Gail and Boone (1970); the experimental approach and assumptions underlying the data analysis were described previously (Parkhurst and Saltzman, 1992). Briefly, the capillary tube, containing PMNs suspended uniformly throughout a reconstituted ECM gel, was placed on the stage of an inverted light microscope (Diaphot; Nikon, Garden City, NY). An incubator surrounding the stage was preheated and maintained at 37°C throughout each experiment. Using a 20× phase-contrast objective, a field of view was selected in the middle of the tube (at least 100 μm from either tube wall). A computer-based image analysis system (Pantera with Data Translation image capture hardware) was used to monitor cell movements in the tube. A video camera (model NC-70; Dage-MTI, Wabash, MI) was attached to the microscope, and a continuous record of cell migration was produced by a time-lapse video recorder (JVC, BR-9000U; Opto-Systems, Jenkintown, PA) at 1/60th the normal video frame rate (i.e., 30 frames/min). Digitized still images of the field of view were also collected on the computer: one image was collected every 90 s for 15 min.

The digitized images and videotape were viewed, and the positions of 3–20 cells were determined on each digitized image by tracing the cell outline and calculating the center of mass. This procedure produced an x, y record of cell position on each frame (Fig. 1). In previous experiments, we repeatedly traced and calculated the position of representative cells; based on this procedure, we estimate that the error in our estimate of cell position is less than 1 μm. To examine a large population of cells obtained from a variety of donors, seven to nine individual experiments were evaluated together. For each cell, the squared displacement was calculated for every possible time interval; this information was accumulated for a given population of cells. The persistence time (P) and random motility coefficient (μ) were determined by comparing the entire set of measured squared displacements versus time to a commonly used model for cell migration (see Parkhurst and Saltzman, 1992, for details):

$$D^2(t) = 4\mu(t - P + Pe^{-t/P}), \tag{1}$$

where $D^2(t)$ is the theoretical mean squared displacement projected within the horizontal plane of focus over a time interval of length t for the persistent random-walk model. We neglected positioning error in this analysis, because our expected error is much less than $(2\mu P)^{1/2}$ (see Dickinson and Tranquillo, 1993, for details on the possible effects of positioning error in this analysis). The data were fit to Eq. 1, using a modified Gauss-Newton iterative nonlinear regression technique. An optimized, generalized least-squares regression technique is available (Dickinson and Tranquillo, 1993), and we have used this improved procedure in preliminary analysis of some of our data. However, we found that ordinary nonlinear least-squares regression yields reliable parameter estimates, provided that at least 40 cell paths are analyzed, so the results presented here were obtained by the simpler regression technique that we described previously (Parkhurst and Saltzman, 1992).

Cell adhesion to ECM-coated surfaces

PMNs at a concentration of 2×10^6 cells/ml were labeled with calcein acetoxymethyl ester (calcein AM, 1 mM; Molecular Probes) diluted in PBS or Hoescht 33342 dye (Sigma) diluted in PBS. The wells of a 24-well tissue culture plate (Falcon 3047; Becton Dickinson) were coated with 500 μl/well of 20 μg/ml collagen, fibronectin, or laminin and incubated overnight at 4°C. Each well was washed three times with PBS, and 500 μl of the cell suspension was placed in each well. The cells were settled to the bottom of the wells by centrifugation for 1 min at 500 rpm, and incubated for 20 min at 37°C. Unbound PMNs were removed by inverting the plate onto a paper towel and gently rinsing the wells three times with PBS, or centrifuging upside down for 10 min at 1000 rpm. The adherent PMNs were solubilized with 0.1% sodium dodecyl sulfate in 50 mM Tris (pH 8.5), and the fluorescence intensity was measured in a fluorometer (SLM Aminco 8000C; Hitachi). The number of adherent PMNs was determined by comparison to solutions containing known numbers of cells from the same preparation, which were lysed by the same procedure. To determine the independence of the means, a t-test assuming unequal variances was performed.

Rheological measurements

Gel response to oscillatory shear was measured with a rheometer (Rheometrics; RFS2) in cone and plate geometry. The instrument was preheated to 37°C and maintained at constant temperature throughout the experiment. Both cone and plate were washed with deionized H_2O and allowed to dry before each sample was tested. Each 1.5-ml sample (containing collagen with and without fibronectin, laminin, or heparin) was prepared as described above and placed on the plate. The cone was lowered onto the solution and was left undisturbed for 30 min to permit gelation. In preliminary experiments, this period was discovered to be sufficient for production of a reproducible gel. A humid environment was created around the sample to prevent evaporation during the measurement. After gelation, the sample was subjected to oscillatory shear by rotating the plate at 2% strain with a frequency ranging from 0.1 to 100 rad/s.

In these measurements, a sinusoidally varying shear field of frequency ω was imposed on the gel by oscillating the plate; the amplitude of the resulting torque and the phase angle between the imposed shear and resulting torque on the cone were measured. The stress response (τ) was also oscillatory, but shifted from the imposed shear by an angle ϕ:

$$\tau = \tau_{max}\cos(\omega t - \phi), \tag{2}$$

where τ_{max} is the maximum value of the shear response. This stress can be decomposed into in-phase and out-of-phase terms:

$$\begin{aligned}\tau &= -\eta'\gamma_{max}\omega\cos(\omega t) - \eta''\gamma_{max}\omega\sin(\omega t)\\ &= -G''\gamma_{max}\cos(\omega t) - G'\gamma_{max}\sin(\omega t),\end{aligned} \tag{3}$$

where γ_{max} is maximum strain, η' and η'' are the two dynamic viscosity coefficients, and G' and G'' are the storage and loss moduli, respectively. The value of G', related to the stress in phase with strain, provides information about the elasticity of the gel. The loss modulus, G'', related to the stress out of phase with the strain, is a measure of the dissipated energy in the system (Prud'homme, 1989).

RESULTS

Cell-populated, reconstituted collagen type I gels were formed within glass capillary tubes. As we have described previously (Parkhurst and Saltzman, 1992; Saltzman et al., 1992), the procedure is reproducible and versatile. When observed by SEM, reconstituted gels containing 0.1–2.0 mg/ml collagen appear to contain a random array of protein fibers (Figs. 2 *a* and 3 *a*). In previous experiments, we found that fiber diameter remained constant (~150 nm) with increasing collagen concentration, but fiber density increased in proportion to collagen concentration (Saltzman et al., 1992). For this report we developed techniques for producing stable gels of varying composition by the addition of other ECM components to collagen type I. The addition of laminin, fibronectin, or heparin to the collagen gel produced characteristic gel structures, as observed in carefully dehydrated samples by SEM (Figs. 2 and 3). Addition of either laminin or fibronectin to the collagen gel appeared to produce similar gel structures: as the concentration of laminin or fibronectin increased from 1 to 100 μg/ml, the protein

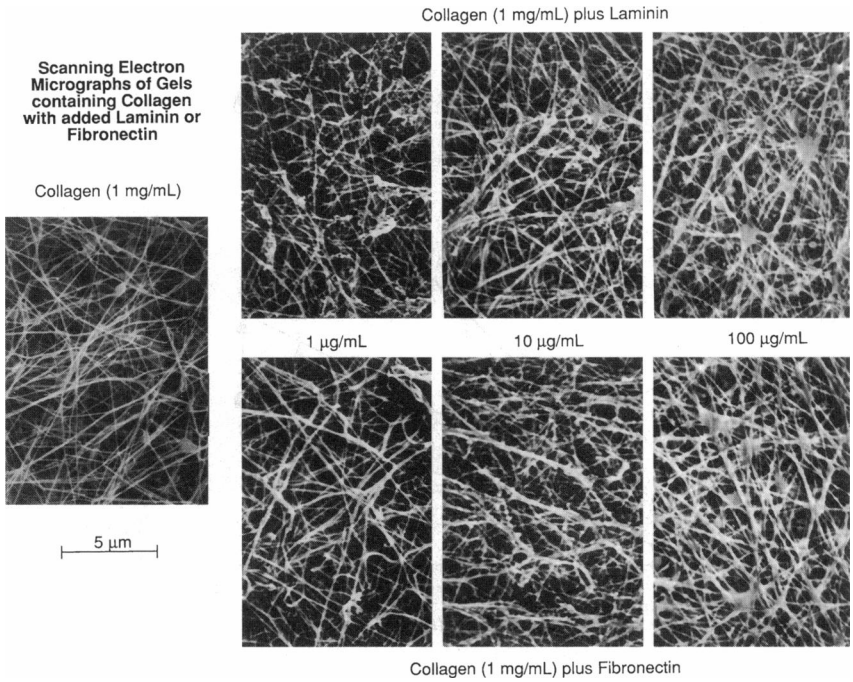

FIGURE 2 Scanning electron micrographs of gels containing (*a*) collagen, (*b*) collagen with 1 μg/ml laminin, (*c*) collagen with 10 μg/ml laminin, (*d*) collagen with 100 μg/ml laminin, (*e*) collagen with 1 μg/ml fibronectin, (*f*) collagen with 10 μg/ml fibronectin, and (*g*) collagen with 100 μg/ml fibronectin. Reprinted from Baldwin et al., *International Journal of Developmental Neuroscience 14:*351–364, (1996), with kind permission from Elsevier Science Ltd.

fibers became progressively thicker and were more difficult to bring into focus (Fig. 2), suggesting that laminin or fibronectin was coating the collagen fibers. The addition of small amounts of laminin or fibronectin (1 μg/ml) had little effect on the fiber diameter, whereas larger amounts (10 to 100 μg/ml) of the added protein had a more pronounced effect: the diameter of the fibers increased from ~150 nm for plain collagen gels to ~210 nm for 10 μg/ml added protein and ~240 nm for 100 μg/ml added protein (Table 1). Heparin, on the other hand, appeared to induce reorganization of the collagen fibers into thicker bundles, resulting in an overall structure with a larger mesh size (Fig. 3): the addition of small amounts of heparin (0.4 μg/ml) had little effect, but larger amounts of heparin increased the fiber diameter substantially, up to 262 nm (Table 1).

Rates of PMN migration within collagen gels containing laminin, fibronectin, or heparin were quantified by comparing cell movements within each gel (Fig. 1) to a mathematical model of persistent random walk (Eq. 1, Fig. 4). Because experimental measurements were collected every 90 s and the average persistence time for PMNs in these mea-

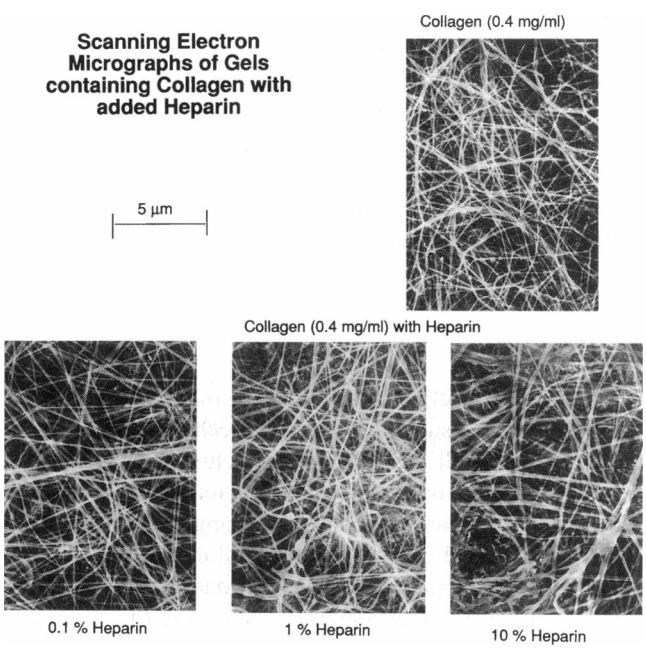

FIGURE 3 Scanning electron micrographs of gels containing (*a*) collagen, (*b*) collagen with 0.4 μg/ml heparin, (*C*) collagen with 4 μg/ml heparin, and (*d*) collagen with 40 μg/ml heparin.

TABLE 1 Fiber diameter and interfiber spacing or mesh size (δ) for different gel compositions containing 0.4 mg/ml collagen

Gel	d_f (nm)	δ (μm)
Collagen only	152 ± 25	7.2
With 1 μg/ml laminin	162 ± 29	7.2
With 10 μg/ml laminin	208 ± 40	7.1
With 100 μg/ml laminin	236 ± 34	7.0
With 1 μg/ml fibronectin	158 ± 45	7.2
With 10 μg/ml fibronectin	215 ± 66	7.1
With 100 μg/ml fibronectin	238 ± 51	7.0
With 0.4 μg/ml heparin	164 ± 9.2	7.8
With 4 μg/ml heparin	182 ± 10	8.6
With 40 μg/ml heparin	262 ± 18	12.4

Fiber diameters (d_f, mean ± standard deviation from 10–20 separate measurements) for 10 and 100 μg/ml laminin or fibronectin and 4 and 40 μg/ml heparin were found to be statistically different from the plain collagen gels by *t*-test, assuming unequal variances ($p < 0.03$).

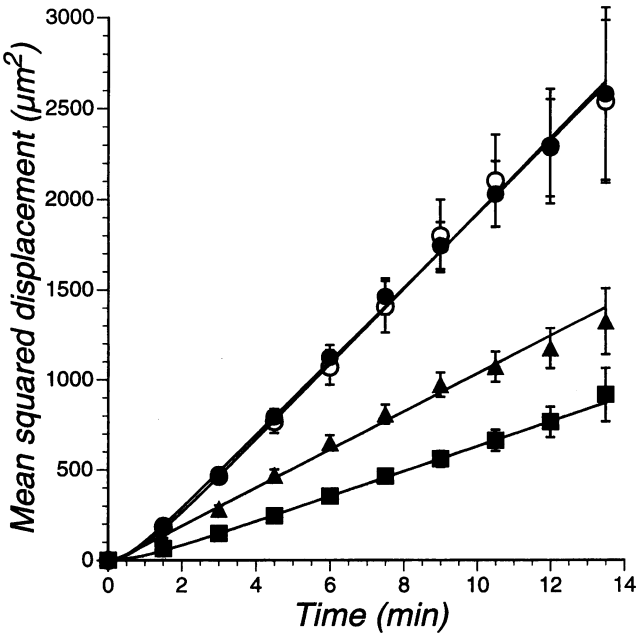

FIGURE 4 Mean squared displacement versus time for PMNs migrating through gels containing 0.4 mg/ml collagen (○), 0.4 mg/ml collagen plus 10 μg/ml fibronectin (■), 0.4 mg/ml collagen with 10 μg/ml laminin (●), and 0.4 mg/ml collagen with 4 μg/ml heparin (▲). Symbols represent mean squared displacement; error bars represent standard error of the mean; and the solid lines represent Eq. 1, with μ and P adjusted to best fit the data.

surements was 44 s (comparable to the average persistence time obtained in a previous study, 51 s; Parkhurst and Saltzman, 1992), there was considerable uncertainty in the estimation of P by this procedure (Table 2). This is consistent with previous reports (Parkhurst and Saltzman, 1992, 1994), which also suggest that changes in persistence time during random cell migration are more subtle (and probably less important for the overall dispersion of a cell population)

TABLE 2 Random motility coefficient (μ) and persistence time (P) for human PMNs migrating in gels of extracellular matrix

Gel	N_{cells}	P (s)	μ (10^{-9} cm²/s)
Collagen (0.4 mg/ml)*	72	56 ± 24	10 ± 0.6
With 1 μg/ml laminin	96	25 ± 41	10 ± 1.1
With 10 μg/ml laminin	70	36 ± 18	8.5 ± 0.4
With 100 μg/ml laminin	56	40 ± 24	5.6 ± 0.4
Collagen (0.4 mg/ml)	67	78 ± 20	8.2 ± 0.4
With 1 μg/ml fibronectin	79	50 ± 20	4.5 ± 0.2
With 10 μg/ml fibronectin	62	55 ± 21	2.9 ± 0.2
With 100 μg/ml fibronectin	46	25 ± 23	2.2 ± 0.1
Collagen (0.4 mg/ml)	42	48 ± 26	8.7 ± 0.6
With 0.4 μg/ml heparin	50	66 ± 23	5.5 ± 0.3
With 4 μg/ml heparin	49	13 ± 16	4.4 ± 0.2
With 40 μg/ml heparin	55	35 ± 19	3.7 ± 0.2

The best estimates for P and μ are provided with 95% confidence limits. *Gels containing collagen differ slightly in ionic compositions. In these gels, prepared as in the laminin-containing gels, a small volume of Tris-buffered saline was used in place of M199 (see Materials and Methods).

than changes in the random motility coefficient, μ. The random motility coefficients, which were approximately equal to the slope of the mean squared displacement versus time curves (Fig. 4), were estimated with reasonable precision by this technique (Table 2).

As previously reported, μ varied with collagen concentration in the gel, with optimal motility occurring at an intermediate concentration (Fig. 5 *a*). For gels with 0.4 mg/ml collagen, μ varied significantly with the addition of small amounts of laminin, fibronectin, or heparin (Fig. 5 *b*). All of the random motility coefficients decreased with increasing ECM concentration, but the magnitude of the decrease varied for each ECM component. PMN motility was unchanged with the addition of 1 μg/ml laminin, whereas at 10 μg/ml laminin the random motility coefficient decreased only moderately (~15%). The addition of either heparin or fibronectin led to significant decreases (at least 40%) in the random motility coefficient, even at the lowest concentration tested.

The extent of PMN adhesion to collagen-, laminin-, or fibronectin-coated plastic surfaces was also determined (Fig. 6). Cells adhered in equal numbers to collagen- or laminin-coated surfaces under these conditions; approximately 1.7 times as many cells adhered to fibronectin-coated surfaces. Although substantial variability was observed, the number of cells adhering to fibronectin-coated surfaces was greater than the number of cells adhering to either laminin- or collagen-coated surfaces ($p < 0.05$).

For all gels, both G' and G'' remained relatively constant over the range of frequencies studied (0.1–100 rad/s) (Fig. 7). To study the difference in gel response, G' and G'' were examined at a single frequency and plotted as a function of gel composition (Fig. 8). Laminin- and fibronectin-added gels produced similar responses to shear: the addition of small amounts of laminin or fibronectin (1, 10 μg/ml) caused both moduli to decrease, whereas larger amounts (100 μg/ml) resulted in a substantial increase over the plain collagen gels (Fig. 8 *a*). Heparin-added gels produced a different response: for 0.4 μg/ml and 4 μg/ml heparin-added gels, there was a significant increase in G' and G'' over plain collagen gels at all frequencies examined (Fig. 8 *b*).

DISCUSSION

The physical structure and properties of reconstituted collagen gels were studied using two techniques: SEM and rheology. With SEM, the microstructure of the gel was visualized; as we have described previously, the plain collagen gels appear to contain entangled networks of ~150-nm fibers (Fig. 2 *a*). Whereas adding a small amount (1 μg/ml) of laminin (Fig. 2 *b*) or fibronectin (Fig. 2 *e*) had little effect on the fiber diameter, increasing the concentration to 10 μg/ml or 100 μg/ml produced a significant increase in fiber diameter, ~30% and ~40%, respectively (Table 1). The addition of laminin and fibronectin to the gels also altered the physical appearance of the collagen

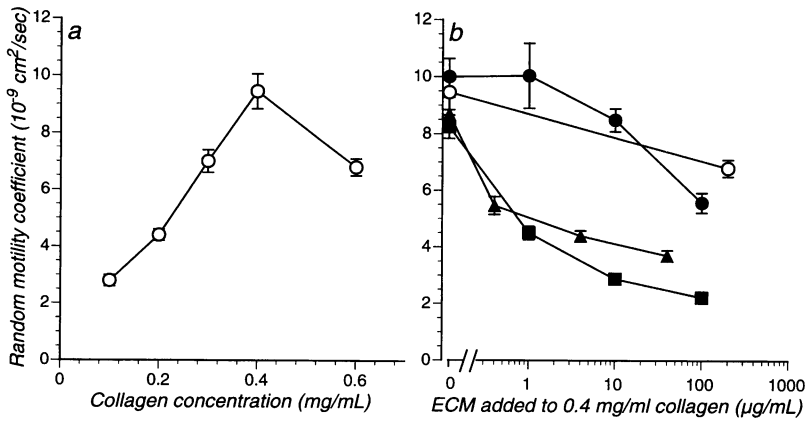

FIGURE 5 (*a*) Random motility coefficients for neutrophil migration through gels with varying collagen concentration. (*b*) Random motility coefficients for neutrophil migration through 0.4 mg/ml collagen gels containing collagen only (○), collagen with laminin (●), collagen with fibronectin (■), and collagen with heparin (▲). Symbols indicate best estimate of μ, obtained by comparing experimental data (mean squared displacement versus time) to Eq. 1. The error bars indicate 95% confidence limits.

fibers; as the concentration of fibronectin or laminin increased, the fibers appeared to become coated with the added protein. Adding heparin to the gels also increased fiber diameter, although the fibers appeared to be aggregated rather than coated (Fig. 3). This observation is consistent with previous reports that heparin (McPherson et al., 1988) and another glycosaminoglycan (GAG), dermatan sulfate (Uldbjerg and Danielsen, 1988), aggregate collagen fibrils.

Because preparation of samples for SEM required dehydration, which potentially changes the structure of these highly hydrated samples, we examined the rheology of these fibrillar gel networks as an indirect indicator of gel structure. The response to the applied shear was similar for laminin- and fibronectin-added gels (Fig. 8). The addition of small amounts (1 or 10 μg/ml) of laminin or fibronectin caused a decrease in the storage (G') and loss (G'') moduli, whereas larger amounts (100 μg/ml) caused an increase (Fig. 8 *a*). We believe that this response is due to the coating of protein on the collagen fibers. At low concentrations, an uneven or thin coating inhibits the formation of tight entanglement points and allows more water to penetrate between the fibers. Fewer entanglements decrease the elasticity of the gel, explaining the observed decrease in G'; enhanced hydration between the fibers decreases the gel viscosity, explaining the observed decrease in G''. As larger quantities of fibronectin or laminin (100 μg/ml) were added, the collagen fibers become thickly and evenly loaded with protein, causing enhanced entanglement and increased viscosity. To determine the thickness of the coating as a function of protein addition, we estimated the amount of laminin or fibronectin required to form a monolayer coverage on the fibers by first determining the fiber surface area per volume in 0.4 mg/ml collagen gels (79 cm^2/cm^3, which we estimate as $4V_f/d_f$; see below). The projected surface area of adsorbed laminin or fibronectin molecules depends

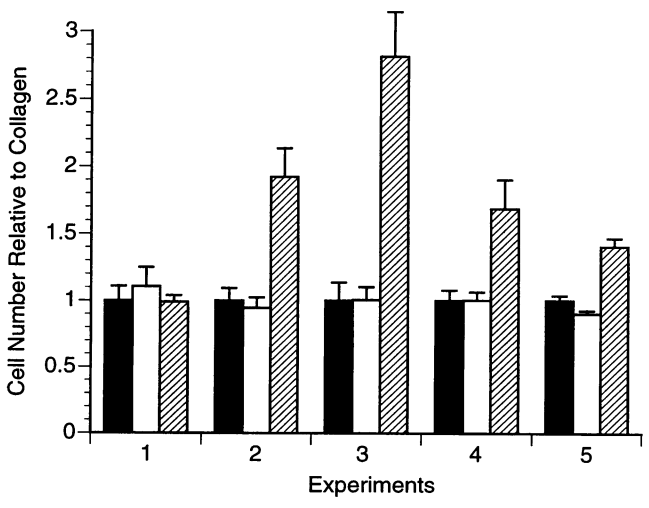

FIGURE 6 Number of PMNs adhering to fibronectin-coated (▨) or laminin-coated (□) surfaces relative to collagen-coated (■) surfaces. Each experiment represents blood cells from different donors and three to six wells of each protein on independently coated plates. The error bars represent the standard deviation among the wells in each experiment.

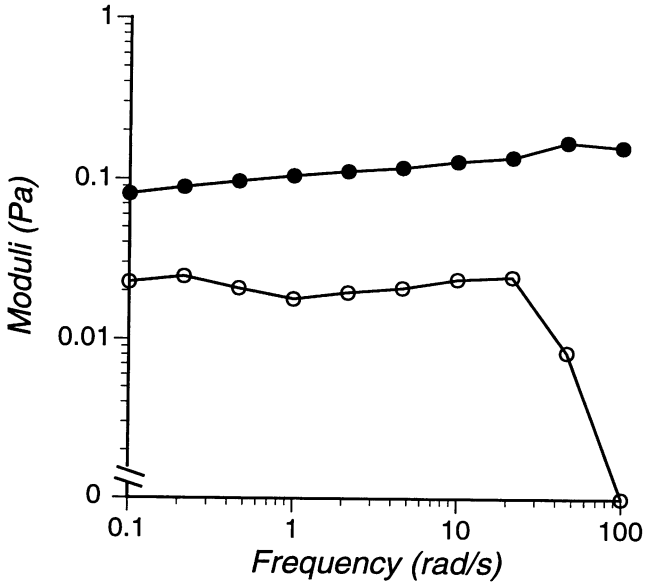

FIGURE 7 Oscillatory shear measurement on 0.4 mg/ml collagen gel with a frequency scan from 0.1 to 100 rad/s and 2% strain, G' (○) and G'' (●). Gel breakdown appears to begin at frequencies higher than ~20 rad/s, as shown in the drop of G''.

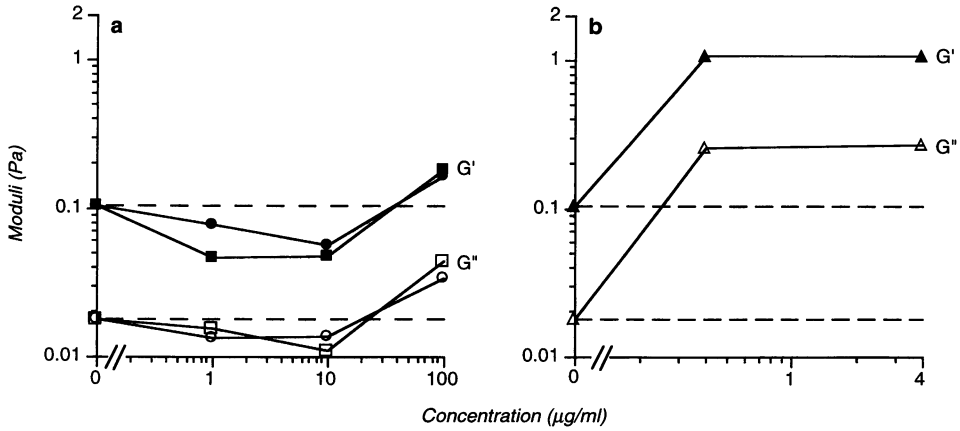

FIGURE 8 (*a*) Storage and loss moduli at 2% strain and 1 rad/s for 0.4 mg/ml collagen gel with varying concentrations of fibronectin (G', ■; and G'', □) and laminin (G', ●; and G'', ○). The moduli for gels with 1 μg/ml fibronectin showed slight variability among the three trials done, and a representative trial was selected. (*b*) Storage and loss moduli at 2% strain and 1 rad/s for 0.4 mg/ml collagen gel with varying composition of heparin. ▲, G'; △, G''.

on their orientation at the fiber surface; laminin and fibronectin molecules have projected surface areas in the range of 430-8200 and 160-1600 nm^2, respectively (Engel et al., 1981). Therefore, monolayer coverage of the collagen fibers requires 1.4–26 μg/ml of laminin or 3.7–37 μg/ml of fibronectin. This simple calculation is consistent with the idea that the change in rheological behavior occurs when the fibers become uniformly coated with laminin or fibronectin, which happens at concentrations slightly greater than 10 μg/ml.

The moduli G' and G'' did not vary significantly with heparin concentration (Fig. 8 *b*). But moduli in heparin-supplemented gels were significantly higher than the moduli for both plain collagen gels and gels with 100 μg/ml added laminin or fibronectin. This increase in the moduli could be due to the aggregation of the fibers: as they aggregate and align, the strength of individual fiber bundles should increase, resulting in increased moduli.

The observations from SEM and rheological measurement led us to propose two models of ECM gel structure: one for collagen fibers that are coated by an additional ECM component (laminin or fibronectin) and one for collagen fibers that are aggregated by an additional ECM component (heparin) (Fig. 9). Previously we used a model for randomly arranged fibers (Fanti and Glandt, 1990) to predict the mesh size or interfiber spacing, δ, in collagen gels (Saltzman et al., 1994):

$$\delta = d_f\left[\left(\frac{\ln(2)}{V_f}\right)^{1/2} - 1\right], \qquad (4)$$

where d_f is the diameter of the fibers and V_f is the volume fraction. For plain collagen gels containing 0.4 mg/ml of collagen, assuming a protein-specific volume of 0.74 cm^3/g (Lehninger, 1975), this model predicted an interfiber spacing of 7.2 μm (Table 1), less than the diameter of a human PMN (~13 μm; Kapff and Jandl, 1981). Mesh sizes for other collagen concentrations can be easily calculated: mesh size varies inversely with the square root of concentration, reaching 14 μm at 0.1 mg/ml. This mesh size is consistent with our observation that cells do not remain suspended in gels with concentrations lower than 0.1 mg/ml (Parkhurst and Saltzman, 1992). Predicted mesh sizes are larger than the apparent mesh size observed in micrographs (Figs. 2 and 3), but it is exceedingly difficult to measure spacings between fibers in a three-dimensional array from a projected two-dimensional SEM image. Therefore we have used an approach that depends on fiber diameters (which can be reliably measured from SEM images) and reasonable models of fiber geometry.

Coated collagen fibers (Fig. 9 *a*) should have an average mesh size that is reduced by two times the thickness of the coating:

$$\delta_{coated} = \delta - 2\delta_{coating}. \qquad (5)$$

This simple expression was used to estimate interfiber spacing for fibronectin- and laminin-added gels; $\delta_{coating}$ was estimated from the observed increase in d_f with the addition of laminin or fibronectin (Table 1). Aggregated fibers, on the other hand, should have an average mesh size that is

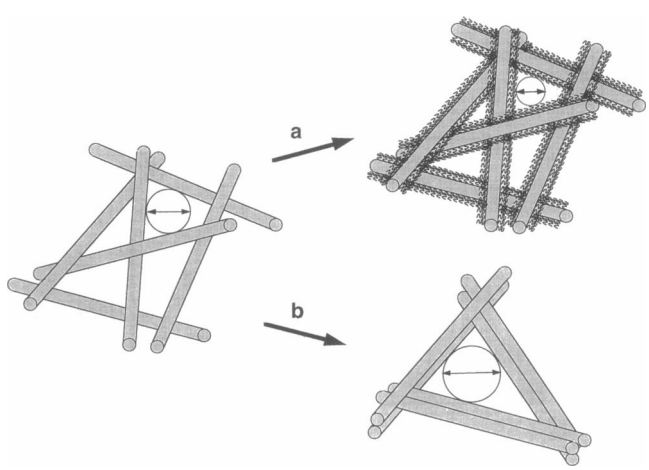

FIGURE 9 Models of fiber coating and aggregation. As fibers become coated, the fiber arrangement remains constant, but the diameter of the fibers increases. With aggregation, the fiber diameter increases, and the interfiber spacing or mesh size becomes progressively larger. A typical mesh size, or interfiber spacing, in the gel is indicated by the open circle.

determined by the thickness of fiber aggregates (Fig. 9 *b*):

$$\delta_{agg} = (d_f)_{agg}\left[\left(\frac{\ln(2)}{V_f}\right)^{1/2} - 1\right]. \quad (6)$$

This expression was used to estimate mesh size for heparin-added gels (Table 1).

Along with the structural changes that occur in the collagen gel with the addition of heparin, PMN motility was substantially reduced (Fig. 5). We found that the addition of as little as 0.4 μg/ml of heparin to 0.4 mg/ml collagen gels caused a significant decrease in the random motility coefficient. The addition of heparin appeared to aggregate the collagen fibers into larger bundles (Fig. 3), causing both the fiber diameter and interfiber spacing to increase with increasing heparin concentration (Table 1). Again, the predicted mesh sizes are consistent with our observation that many cells appeared to be falling toward the bottom face of the capillary tube containing the 40 μg/ml heparin gels (but not the 0.4 or 4 μg/ml gels). This phenomenon must be due to the presence of interfiber spacings (12 μm, Table 1) as large as a cell diameter (13 μm). Therefore, PMN motility in heparin/collagen gels appears to depend primarily on interfiber spacing: cells move more slowly as the spacing increases, up to the point where the mesh size is approximately equal to a cell diameter, so the gel can no longer support motility. In fact, mesh size appears to be an important determinant of random motility in collagen gels: increasing mesh size by either lowering collagen concentration (which influences fiber density) or adding heparin (which influences fiber diameter) produced similar changes in μ (Fig. 10). (At the lowest heparin concentration, motility is significantly reduced, although mesh size is only slightly increased. Although this may represent a high sensitivity to small changes in the mesh size, it might also reflect differences in mechanical properties of gels with different fiber diameters. Gels containing small amounts of heparin have much higher G' and G'' than gels containing collagen alone (Fig. 8).)

If neutrophil motility were dependent only upon the structure of the gel, we would expect to observe similar behavior in laminin/collagen and fibronectin/collagen gels, which responded identically to oscillatory stress (Fig. 8 *a*) and were visually similar by SEM (Fig. 2, *b–g*). Instead, we found that the motility of PMNs in gels with laminin was significantly different from that in gels with fibronectin. Addition of small amounts of fibronectin (1 μg/ml) decreased the motility by ~50%, whereas it required 100 times as much laminin to cause a similar decrease (Fig. 5 *b*). These observations are consistent with previous reports: the migration of neutrophils through laminin-coated filters was faster than migration through fibronectin-coated filters (Suchard, 1993), and the addition of up to 100 μg/ml laminin to collagen gels similar to the ones tested here had no detectable effect on the migration of PC12 cells, whereas as little as 1 μg/ml fibronectin inhibited migration (Baldwin et al., 1996).

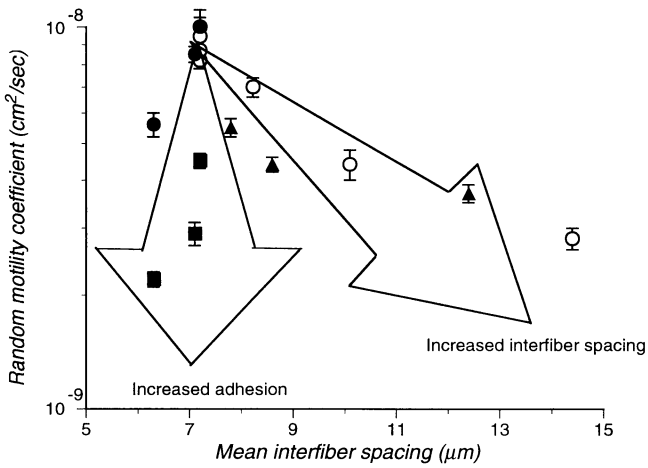

FIGURE 10 Mesh size and adhesion influence PMN migration within ECM gels. The random motility coefficients, μ, from Fig. 5 are plotted as a function of mesh size, which was estimated for each gel as described in the text (see also Table 1). In some cases, such as pure collagen (○) and collagen/heparin (▲) gels, μ decreases predictably with increasing mesh size. In other cases, such as collagen/fibronectin (■) and collagen/laminin (●), μ decreases without substantial changes in the mesh size, suggesting that cell interaction with the protein fibers also influences migration. Here we label that influence as "adhesion," but recognize that this could represent the effect of intracellular biochemical signals secondary to binding of ECM at the cell surface.

An increase in the concentration of collagen does not affect the motility coefficient as dramatically as the addition of comparable quantities of fibronectin or laminin (Fig. 5); therefore PMN motility is dependent upon gel composition, and not upon the total protein concentration in the gels. This observation suggests that the interaction of PMNs with fibers composed of specific proteins also influences motility. PMNs adhered in greater numbers (~1.7 times) to fibronectin-coated surfaces than to laminin- or collagen-coated surfaces (Fig. 6). Adhesion to fibronectin was variable, but statistically different, from adhesion to laminin or collagen. The extent of adhesion observed here is consistent with previous studies of unstimulated PMNs: ~17% to fibronectin, ~15% to laminin, ~10% to collagen type IV (Dri et al., 1991); ~15% to gelatin, ~17% to fibronectin, <10% to laminin (Bohnsack et al., 1990). This differential adhesion can explain the changes in motility: PMNs adhere to fibronectin more readily than to laminin or collagen, causing the cells to cling to the fibronectin-coated fibers rather than migrate through them. Because the gels are structurally indistinguishable, adhesion must be extremely important, with small differences in adhesion producing large changes in motility (compare 10 μg/ml laminin- and fibronectin-supplemented gels in Fig. 5 *b*, for example). It is possible that cell interactions with laminin and fibronectin generate intracellular biochemical signals that influence cell motility within the gel (Clark and Brugge, 1995); our emphasis on adhesive mechanisms is due to the well-known cell adhesion characteristics of these molecules and previous observations of the correlation between adhesion and

motility in other systems (DiMilla et al., 1993; Wu et al., 1994).

Our results suggest two mechanisms by which ECM molecules can influence PMN motility through collagen gels. The addition of an ECM component can 1) modify the fibrous structure of the gel or 2) alter adhesive interactions between the cell and the fibers of the gel. Changes in mesh size or adhesion produce changes in μ of comparable magnitude (Fig. 10), so both may be important in the regulation of cell motility within the extracellular spaces of tissues. Our results, which were obtained with reconstituted gel systems, suggest that rates of PMN invasion into tissues may vary substantially with the local composition of ECM in the tissue.

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