American Journal of Pathology, Vol. 149, No. 3, September 1996

Changes in Neutrophil Actin and Shape during Sequestration Induced by Complement Fragments in Rabbits

Hideaki Motosugi, Lori Graham, Timothy W. Noblitt, Nicholas A. Doyle, William M. Quinlan, Yiming Li, and Claire M. Doerschuk

From the Herman B. Wells Center for Pediatric Research and the Section of Pulmonology and Intensive Care, Department of Pediatrics and the School of Dentistry, Indiana University Medical Center, Indianapolis, Indiana

Complement fragment-induced sequestration of neutrophils within the lungs may be mediated by stimulus-induced decreases in the deformability of neutrophils, prolonging their lung capillary transit times. As changes in deformability often occur through changes in cytoskeletal proteins, this study determined whether the distribution of actin within intracapillary neutrophils was altered by intravascular complement fragments and whether sequestered neutrophils were less deformed. Ultrathin cryosections of lung tissue from rabbits given an infusion of complement fragments or saline were immunolabeled with anti-actin antibodies. The number of gold particles/microvillus and the density of gold particles/μm^2 cytoplasm in the submembrane and the central region of intracapillary neutrophils was quantitated. Neutrophil shape was evaluated using laser confocal microscopy. In control rabbits, the ratio of submembrane/central gold was always greater than one and most neutrophils were elongated, 97% having shape factors > 1.10. The ratio of submembrane/central gold was greater in complement-treated rabbits (5.1 ± 0.9) than controls (2.6 ± 0.4; P < 0.026). The number of gold particles/microvillus was also increased in complement-treated rabbits (3.9 ± 0.5) compared with controls (2.3 ± 0.5; P < 0.045). Neutrophils were more often spherical when rabbits received complement fragments for 1.5 minutes than in control lungs or after 15-minute infusions. These data suggest that complement fragments induce a rapid redistribution of actin from the central to the submembrane region and the microvilli and result in more round neutrophils. This redistribution may decrease the deformability of neutrophils by altering the stiffness of the submembrane region and/or by preventing the microvilli from flattening. (Am J Pathol 1996, 149:963–973)

Intravascular inflammatory mediators induce sequestration of neutrophils within the microvasculature of all organs and particularly the lungs, as the lungs receive the entire cardiac output.[1–13] Within the pulmonary microvasculature, more than 95% of the sequestered neutrophils are localized within the capillary network whereas only 5% are in the arterioles and venules.[11,12] Infusion of complement fragments for just 10 minutes induces neutrophil sequestration and a mild increase in vascular permeability within the lungs of rabbits, and this injury requires neutrophils.[13]

Neutrophil sequestration is thought to be induced by an inflammatory mediator binding to specific receptors on neutrophils, resulting in signal transduction and neutrophil activation. This activation induces a number of changes within neutrophils that include alterations in the cytoskeletal proteins and increases in both the expression of adhesion molecules on the membrane and their avidity for their receptors.[2,3,7,14–26] Previous studies have shown that sequestration of neutrophils within the pulmonary capillaries induce by either formyl-methionyl-leucyl-phenylalanine (fMLP) or complement fragments does not require the CD11/CD18 adhesion com-

Supported by a research grant from the Riley Memorial Association and PHS HL48160. C. M. Doerschuk was a recipient of a Career Investigator Award from the American Lung Association.

Accepted for publication April 24, 1996.

Address reprint requests to Dr. Claire M. Doerschuk, Harvard School of Public Health, I-305, 665 Huntington Avenue, Boston, MA 02115.

plex.[12,27] However, once the neutrophils are sequestered within the pulmonary capillary bed, CD11/CD18-mediated adhesion is necessary to maintain this sequestration for more than 5 to 7 minutes.[12] Recent studies have suggested that L-selectin or P-selectin are also not required for this initial sequestration.[28,29] These studies suggest that the sequestration of neutrophils occurs through at least two sequential events.[12] The first occurs during the first 4 minutes after an inflammatory mediator enters the bloodstream and does not require either L-selectin, P-selectin, or CD11/CD18. After 5 to 7 minutes, CD11/CD18-mediated adhesion is necessary to maintain the sequestered neutrophils within the capillaries.

The mechanisms important in the initial process of sequestration may include a rapid stiffening of neutrophils induced by the inflammatory stimulus.[3,7,8,11,12,17–26] Neutrophils must deform into an oblong shape to pass through more than 60% of the capillary segments and virtually every capillary pathway.[30,31] If neutrophils became stiffer and could not deform, they would not be able to pass through the capillary bed. Many investigators have demonstrated that inflammatory stimuli, including C5a, interleukin-8, fMLP, and phorbol myristate acetate, rapidly induce stiffening of neutrophils, as measured by aspiration into micropipettes, the pressure or flow required to pass neutrophils through a filter containing 5-μm pores, or cell pokers that measure the pressure required to indent a neutrophil.[3,17–26] Worthen and colleagues[3] have suggested that stimulus-induced decreases in the deformability are also important *in vivo*, as pretreatment of isolated radiolabeled neutrophils with cytochalasin D reduced neutrophil sequestration within the lungs induced by fMLP. The mechanism through which this stiffening occurs has been suggested to be through the polymerization of globular (g) actin to filamentous (f) actin to form an actin-rich rim in the subcortical region beneath the neutrophil membrane.[3,17–26] However, direct observation of changes in the cytoskeleton *in vivo* has not yet been attempted.

The purpose of this study was to quantitate the distribution of actin within the central region, the submembrane shell, and the microvillar processes of neutrophils within the pulmonary capillaries and to compare this distribution in rabbits given infusions of complement fragments for 2 minutes with that in control rabbits that received no infusion. This study also tested the hypothesis that activated neutrophils are less able to deform than quiescent neutrophils by determining whether neutrophils were more spherical in rabbits given infusions of complement fragments for 1.5 or 15 minutes compared with control rabbits that received no infusion. The localization of actin within neutrophils in the pulmonary capillaries was evaluated using ultrastructural immunohistochemistry. The shape of neutrophils was quantitated in fixed lung tissue using laser scanning confocal microscopy.

Materials and Methods

Animals

New Zealand White rabbits weighing 2.4 to 3.1 kg were used in this study.

Complement Fragments

Rabbit plasma was incubated with zymosan A yeast (5 mg/ml; Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37°C.[11,12] The complement-activated plasma was then centrifuged twice at 800 × *g* for 15 minutes. Plasma containing complement fragments was used within 1 hour.

Protocol A: Immunohistochemical Localization of Actin within Neutrophils

Rabbits were anesthetized with ketamine hydrochloride (80 to 100 mg/kg i.m.) and acepromazine maleate (8 to 10 mg/kg i.m.) and placed supine. Catheters were inserted in the aorta through the right internal carotid artery and in the marginal ear vein. A tracheostomy was performed and the animals breathed room air spontaneously. After a blood sample was taken from the arterial line to determine the circulating leukocyte counts and differential, complement fragments were infused at a rate of 0.3 ml/kg/minute for 2 minutes (n = 4). Control rabbits received no infusion (n = 4). After a blood sample was obtained from the arterial line, the heart was stopped by intra-arterial injection of saturated potassium chloride.

The chest was rapidly opened, the base of the heart was tied, and 0.025% glutaraldehyde in phosphate-buffered saline (PBS) was instilled through the trachea at 30 cm H_2O pressure. The lungs were removed and allowed to fix for 1 hour at 4°C. The lung tissue was then sectioned into 1 × 1 × 1 mm cubes and fixed for an additional 60 minutes. After rinsing in PBS, the tissue sections were infiltrated with 2.5 mol/L sucrose overnight at 4°C. Each tissue fragment was then placed on an aluminum specimen pin and frozen in liquid nitrogen. Sections measuring

90 to 110 nm were cut using a cryo-ultramicrotome and collected on formvar-coated copper grids.

Actin was localized using immunogold labeling techniques. The grids were inverted on 40-μl droplets of PBS containing 5% fetal calf serum (FCS). The grids were incubated with the primary mouse monoclonal anti-actin antibody (Boehringer Mannheim, Indianapolis, IN) at a concentration of 0.01 mg/ml. This anti-actin antibody recognizes all actin isoforms in both the globular and the filamentous form.[32] To assess nonspecific gold labeling, additional cryosections were incubated with nonimmune mouse IgG (Sigma) at the same concentration. After washing in PBS/FCS, the sections were incubated with a colloidal gold-labeled anti-murine antibody (1:26 dilution; Sigma). After post-fixing in 1% glutaraldehyde, the sections were embedded and contrasted with 1.8% methylcellulose containing 0.3% uranyl acetate in distilled water.

The tissue sections were examined using a Phillips CM 10 transmission electron microscope. Neutrophils were identified by their segmented nuclear lobes and cytoplasmic granules. All neutrophils were photographed at ×27,000 to ×32,000 magnification. In each animal, at least five neutrophils were identified.

The density of gold particles was measured in the submembrane region, which was designated as a rim of cytoplasm extending 0 to 0.5 μm below the plasma membrane, and the central region of cytoplasm between the nuclear lobes. This density was determined by counting the number of gold particles on a measured surface area of cytoplasm and was expressed as gold particles/μm^2 cytoplasm in each region. The change in the density of actin in the submembrane compared with the central regions was quantitated for each neutrophil by calculating the ratio:

$$\text{submembrane/central actin density} = \frac{\text{number of gold particles}/\mu m^2 \text{ submembrane rim}}{\text{number of gold particles}/\mu m^2 \text{ central cytoplasm}}.$$

The number of gold particles in each microvillar process was also counted and expressed as number of gold particles/microvillar process. For each neutrophil, the average value of gold particles/microvillar process was calculated.

Protocol B: Neutrophil Shape

New Zealand White rabbits were anesthetized as described in protocol A. A tracheostomy was performed and the animals breathed room air spontaneously. Catheters were placed in the aorta through the left carotid artery and in the marginal ear vein. After blood was sampled to determine the circulating leukocyte counts and differential, infusion of complement fragments was begun at a rate of 0.3/kg/minute for either 1.5 (n = 5) or 15 (n = 5) minutes. Control animals received infusions of saline (n = 5). After either 1.5 or 15 minutes, blood was sampled for circulating leukocyte counts, and the heart was stopped by intra-arterial injection of saturated potassium chloride. The chest was rapidly opened, the base of the heart was tied, and 4% paraformaldehyde was instilled through the trachea at a pressure of 30 cm H_2O. The heart and lungs were removed from the thoracic cavity and fixed overnight. Tissue blocks were removed from the posterior portion of the left middle lobe, embedded in paraffin, and sectioned at 25 μm.

The day before observation using the laser confocal microscope, the sections were deparaffinized and incubated with 0.1% Triton X-100 in PBS for 30 minutes. They were stained for 30 minutes with a mixture of ethidium bromide (Molecular Probes, Eugene, OR) in 100 μg/ml PBS to identify nuclei[33] and fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) in 50 μg/ml PBS to identify the cytoplasm.[25,26]

The sections were examined using a laser scanning confocal microscopy (Odyssey Microscope, Noran Instruments, Middleton, WI). Neutrophils were identified using an excitation wavelength of 529 nm and an emission wavelength of >590 nm to visualize ethidium-bromide-labeled nuclei. Neutrophils that were located within longitudinally sectioned capillaries were examined, and measurements were made using excitation and emission wavelengths of 488 and 520 to 540 nm, respectively, to visualize FITC-phalloidin. The focal plane, which measured <0.2 μm in depth (z dimension), through the largest cross-section (x-y plane) of the neutrophil was used for measurements. The major axis was taken to be the axis parallel to the capillary walls. The minor axis was the axis perpendicular to the major axis midpoint of the major axis. The shape factor was calculated as the major axis/minor axis.[30] This shape factor measured 1.0 if the neutrophil were spherical and increased as the neutrophil deformed into elongated shapes.

Histograms describing the frequency distribution of shape factors were constructed, and cumulative frequency distributions were calculated for each group.

Table 1. *Circulating Neutrophil Counts before and during Infusion ($\times 10^6$/ml blood)*

Protocol 1	Time relative to infusion: Before	2 minutes
Complement fragments	1.75 ± 0.44	0.04 ± 0.01
Saline	1.65 ± 0.41	1.58 ± 0.42

Protocol 2	Before	1.5 minutes	15 minutes
Complement fragments			
1.5 minutes	1.98 ± 0.23	0.049 ± 0.004*	
15 minutes	1.76 ± 0.20	0.046 ± 0.002*	0.042 ± 0.007*
Saline	1.18 ± 0.16	1.25 ± 0.15	1.31 ± 0.20

*Significantly different from neutrophil counts before infusion of complement fragments; $P < 0.05$.

The surface area of the cross section of each neutrophil was estimated by assuming the shape to be an ellipse and using the formula:

$$\text{surface area} = \pi \frac{ab}{4},$$

where a = major axis and b = minor axis. The volume was also estimated using the formula for a rotated ellipse:

$$\text{volume} = \pi \frac{abc}{6},$$

where a = major axis, b = minor axis, and b and c are the axes in the plane perpendicular to the vessel wall so that $b = c$.

The hypothesis for these studies is that activated neutrophils cannot deform and are therefore more spherical than neutrophils in the pulmonary capillaries of normal rabbits. However, the lungs normally contain a large number of marginated neutrophils that will already be deformed before infusion of complement fragments,[4,5,8,12,30,34] and these neutrophils will lead to underestimates of the effect of complement fragments on inhibition of neutrophil deformation. Therefore, the number of neutrophils that might be expected to be spherical was estimated by calculating the total number of circulating neutrophils available to sequester in the lungs immediately upon infusion of complement fragments. This number was compared with the total number of neutrophils in the capillary bed before the infusion. The number available to sequester was estimated to be the total number of neutrophils/ml of blood before the infusion multiplied by the blood volume of the rabbit (60 ml/kg[34]). The total number of marginated neutrophils in the lungs of normal rabbits was taken to be 18×10^8 to 21×10^8.[11,34] The percentage of neutrophils that might be expected to have a spherical shape was then equal to

$$\frac{\text{number of circulating neutrophils}}{\text{number of circulating neutrophils} + \text{number of marginated neutrophils}}$$

where

$$\text{number of circulating neutrophils} = \frac{\text{number of circulating neutrophils}}{\text{ml blood}} \times \text{body weight} \times 60 \text{ ml blood/kg}.$$

This predicted percent increase in spherical cells was then compared with the observed increase obtained using the cumulative frequency distribution of shape factors.

Statistics

The circulating neutrophil counts, ratio of submembrane/central actin densities, average number of gold particles/microvillus process for each neutrophil, length of major and minor axes, surface area, volume, and shape factor were compared using either Student *t*-tests or analyses of variance, as appropriate. When an analysis of variance was found to be significant, multiple contrasts with a Bonferroni correction were used to determine which groups were significantly different.[35] Cumulative frequency distributions were calculated and compared using the Kolmogorov-Smirnov test.[36]

Results

Infusion of complement fragments induced neutropenia by 1.5 minutes that persisted through the 15-minute infusion (Table 1).

Table 2. *Distribution of Actin within Neutrophils*

	Actin densities in submembrane/ central regions	Gold particles/ microvillus
Complement fragments	5.1 ± 0.9*	3.9 ± 0.5*
Saline	2.6 ± 0.4	2.3 ± 0.5

The ratio of the density of actin in the submembrane region to the central region of neutrophils was expressed as the ratio of gold particles/μm^2 submembrane cytoplasm to gold particles/μm^2 central cytoplasm. The second column shows the average number of gold particles/microvillar process for each neutrophil.

*Significantly greater than in neutrophils in pulmonary microvasculature of saline-treated rabbits. In the saline-treated group, 26 neutrophils from five rabbits were evaluated. The effect of complement fragments was examined in 28 neutrophils from five rabbits.

Protocol A: Immunohistochemical Localization of Actin within Neutrophils

The distribution of actin within the cytoplasm of neutrophils in the pulmonary capillaries is described in Table 2 and Figures 1 to 4. Electron micrographs of immunogold-labeled actin within intra-capillary neutrophils in the lungs of rabbits given either complement fragments or saline are shown in Figure 1. The ratio of submembrane/central actin density, as quantitated by the ratio of gold particles/μm^2 cytoplasm in the submembrane region to that in the central region, was 2.6 ± 0.4 in the control animals, indicating that the density of actin was twice as great in the cyto-

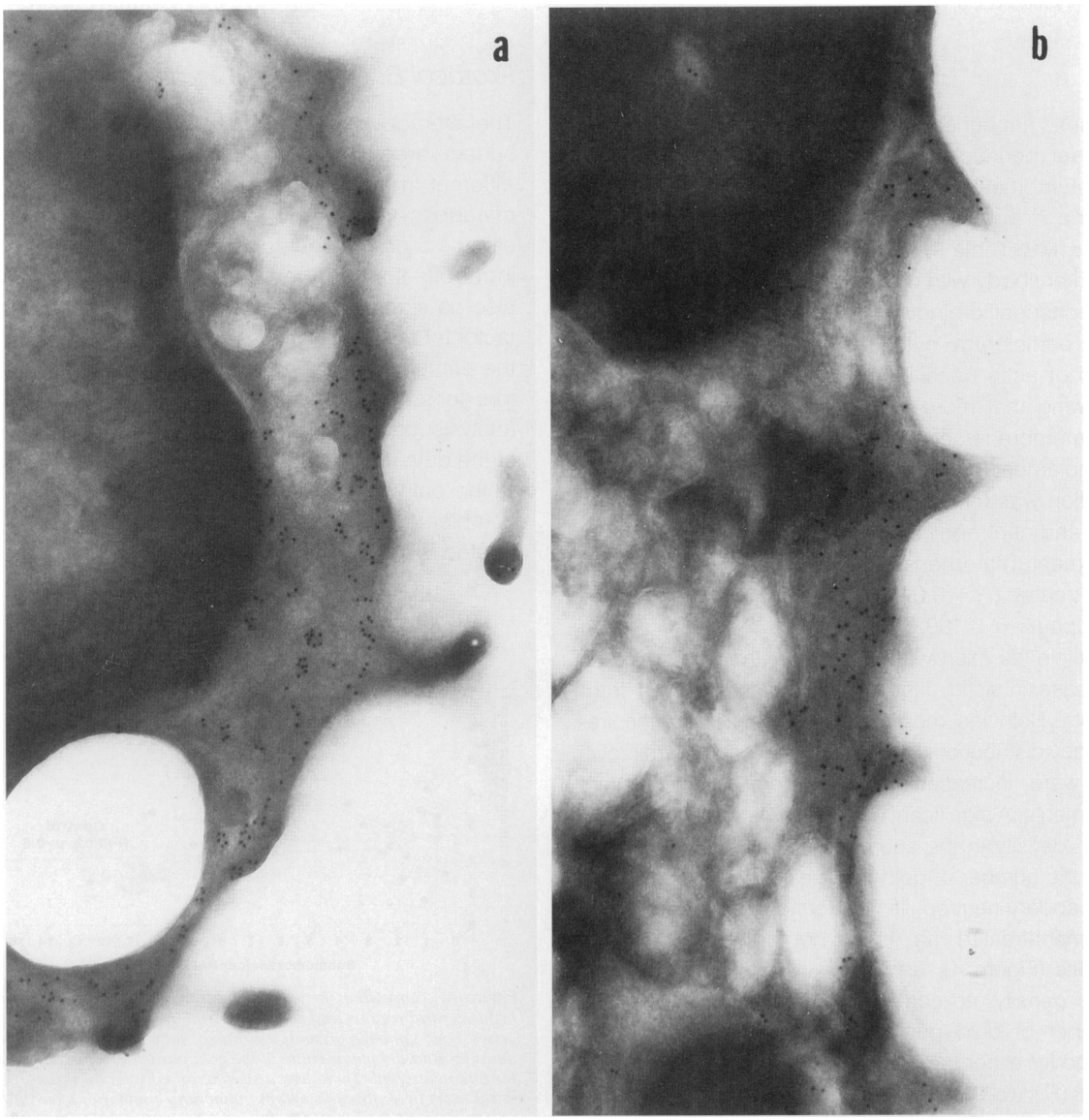

Figure 1. *Electron micrographs showing ultrastructural localization of actin within intracapillary neutrophils in the lungs of rabbits that received infusion of saline* (a) *or complement fragments* (b) *for 2 minutes. Magnification, ×47,000 (colloidal gold particle = 10 nm).*

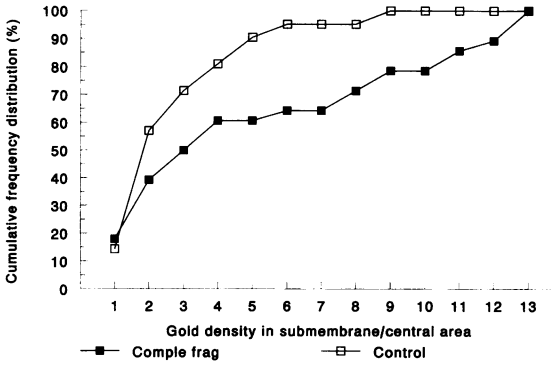

Figure 2. *Cumulative frequency distribution of the ratio of actin densities in the submembrane rim to the central region of neutrophils within pulmonary capillaries. More neutrophils showed higher values of submembrane/central actin density when rabbits were treated with complement fragments for 2 minutes* (■) *compared with those in control rabbits* (□; P < *0.01*).

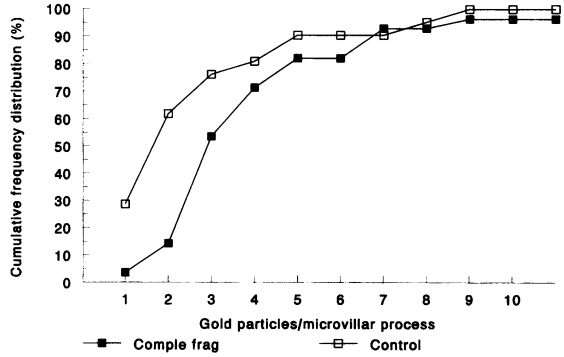

Figure 3. *Cumulative frequency distribution of the actin density in microvillar processes in neutrophils within pulmonary capillaries. The number of gold particles/microvillar process, a measure of actin concentration within these processes, was increased when rabbits received infusions of complement fragments for 2 minutes* (■) *compared with those in control rabbits* (□).

plasmic rim beneath the plasma membrane as in the central, perinuclear region before activation. This ratio of submembrane/central actin density increased after a 2-minute infusion of complement fragments to 5.1 ± 0.9 (Table 2). Control grids replacing the anti-actin antibody with nonimmune IgG showed virtually no nonspecific binding of gold particles (range, 0 to 0.05 particle/μm^2 cytoplasm). Figure 2 describes the ratio of actin densities as a frequency distribution, showing that more neutrophils have high ratios of submembrane/central actin density after exposure to complement fragments than in control lungs. No correlation was found between the ratio of actin densities and neutrophil shape for either the complement fragment-treated animals ($R = 0.150$; $P > 0.5$), saline-treated ($R = 0.0144$; $P > 0.05$), or the combined groups ($R = 0.109$; $P > 0.05$).

Within the microvillar processes, there was also an increase in actin after infusion of complement fragments. Both the mean value (Table 2) and the frequency distribution (Figure 3) of gold particles/microvillus were increased after infusion of complement fragments. A significant correlation between the ratio of actin density in the submembrane to central regions and the number of gold particles/microvillus in the intra-capillary neutrophils was found in the complement fragment-treated rabbits but not in the saline-treated rabbits (Figure 4). Similar to changes in the ratio of actin density, no correlation was found between the number of gold particles/microvillus and neutrophil shape for either the complement fragment-treated animals ($R = 0.299$; $0.25 > P > 0.10$), saline-treated ($R = 0.335$; $0.25 > P > 0.10$), or the combined groups ($R = 0.0389$; $P > 0.05$).

Protocol B: Neutrophil Shape

The average length of the major and minor axis, the surface area, and the volume were not significantly different in neutrophils within pulmonary capillaries of rabbits treated with complement fragments for either 1.5 or 15 minutes when compared with those in rabbits that received saline (Table 3). There was also no significant difference in the average shape factor for any of the groups (Table 3). Figure 5 shows the cumulative frequency distribution of shape factors for each neutrophil in the three groups. This analysis makes no assumptions about the normality of the data. More spherical neutrophils were present in the pulmonary capillaries of rabbits treated with complement fragments for 1.5 minutes than those treated with either saline or complement fragments

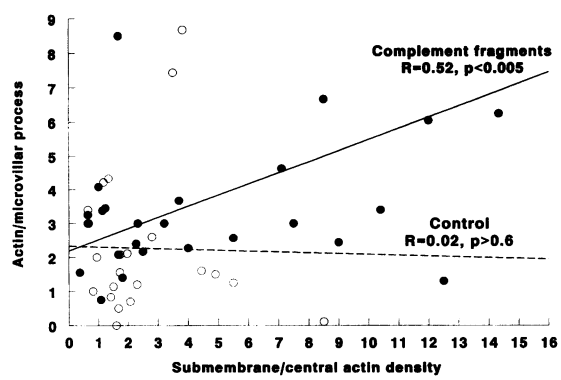

Figure 4. *Correlation between the actin densities in the submembrane/central regions and the concentration of actin/microvillar processes. Each symbol represents the ratio of submembrane/central actin densities and the mean value of the gold particles/microvillar process for one neutrophil. There was a significant correlation between these parameters in neutrophils within pulmonary capillaries after infusion of complement fragments* (●; R = *0.521*, P < *0.005*). *However, no correlation was observed in neutrophils within the capillaries of rabbits given infusion of saline* (○; R = *0.020*, P > *0.6*).

Table 3. *Size and Shape of Neutrophils in Pulmonary Capillaries*

	Major axis (μm)	Minor axis (μm)	Surface area (μm^2)	Volume (μm^3)	Shape factor	% neutrophils with: Shape factor <1.40	% neutrophils with: Shape factor >2.00	Diameter (μm) of neutrophils with shape factor <1.05
Saline (n = 62)	7.85 ± 1.13	5.41 ± 0.70	33.4 ± 6.7	122.8 ± 42.1	1.47 ± 0.29	44	6.4	6.34 (n=1)
Complement fragments								
1.5 minutes (n = 58)	7.47 ± 1.49	5.37 ± 0.69	31.7 ± 8.1	115.9 ± 42.0	1.41 ± 0.31	60	5.2	6.44 (n=2)
15 minutes (n = 60)	8.01 ± 1.46	5.22 ± 0.83	32.8 ± 8.0	117.2 ± 44.6	1.58 ± 0.45	43	15.0	6.26 (n=1)

The ratio of submembrane/central actin densities, average number of gold particles/microvillus process for each neutrophil, length of major and minor axes, surface area, volume, and shape factor were compared using analyses of variance. When an analysis of variance was found to be significant, multiple contrasts with a Bonferroni correction were used to determine which groups were significantly different.[35] Data are expressed as mean ± SD.

for 15 minutes. In particular, the number of neutrophils having a shape factor of less than 1.4 was 60% after a 1.5-minute infusion of complement fragments, 43% after 15 minutes, and 44% in the animals treated with saline infusion, indicating that there was an increase in the fraction of spherical neutrophils of 16% when rabbits received complement fragments for 1.5 minutes (Table 3). In contrast, more neutrophils were deformed and flattened in animals that received complement fragments for 15 minutes, as these animals had 8.6% more neutrophils that showed shape factors greater than 2.0 (Table 3).

Previous studies have estimated the number of neutrophils within the normal pulmonary microvasculature of rabbits weighing 3.4 kg to be 18×10^8 to 21×10^8. The increase due to sequestration of all circulating neutrophils was calculated as the number of circulating neutrophils/ml of blood (Table 1) multiplied by the blood volume/kg body weight and was calculated to be 3.06×10^8. Therefore, the percentage of the total sequestered neutrophils that were delivered to the lungs during the 1.5-minute infusion and might be expected to be spherical was 12.7 to 14.5%. This predicted value of spherical neutrophils corresponds closely with the observed value of 16% (Table 3).

These data were also used to estimate the diameter of spherical neutrophils and to determine the fraction of neutrophils that deform as they pass through the pulmonary capillary bed of normal animals. Only a total of four neutrophils, two in the lungs of rabbits that received complement fragments for 1.5 minutes and one each in the lungs that received saline or complement fragments for 15 minutes, were nearly spherical with shape factors less than 1.05. The mean diameter of these four nearly spherical neutrophils measured 6.37 ± 0.19 μm. In the rabbits that received only saline, 98% of the neutrophils had shape factors greater than 1.05, 97% had shape factors greater than 1.10, and 92% had shape factors greater than 1.15.

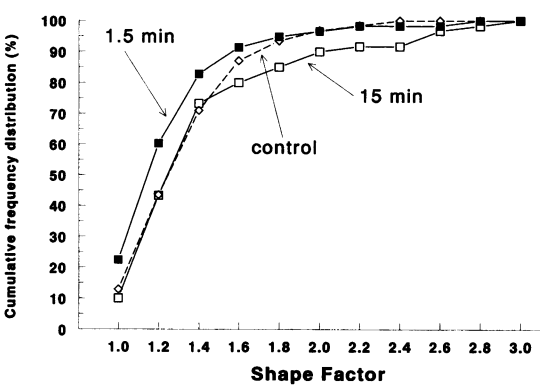

Figure 5. *Cumulative frequency distributions of shape factors for neutrophils within pulmonary capillaries. More neutrophils in the lungs of rabbits given infusion of complement fragments for 1.5 minutes had shape factors that were close to 1 than animals treated with complement fragments for 15 minutes or control animals given infusion of saline. In contrast, infusion of complement fragments for 15 minutes resulted in a small increase in the number of neutrophils that were more elongated.* ■, *rabbits given infusion of complement fragments for 1.5 minutes;* □, *rabbits given infusion of complement fragments for 15 minutes;* ◆, *rabbits given infusion of saline. Cumulative frequency distributions were compared using the Kolmogorov-Smirnov Test.*[36]

Discussion

Previous studies have demonstrated that intravascular complement fragments induce a rapid and virtually complete sequestration of neutrophils within the pulmonary capillary bed[2,5,6,11,12] and that this sequestration does not require L-selectin, P-selectin, or CD11/CD18-mediated adhesion between neutrophils and endothelial cells.[12,28,29] The present study addresses the hypothesis that sequestration of neutrophils induced by circulating inflammatory mediators occurs through changes in the biomechanical properties of neutrophils that result in decreased deformability by evaluating the subcellular distribution of a major cytoskeletal protein as well as

changes in neutrophil shape that are important in their transit. The data show that the ratio of submembrane/central actin density and the gold particles/microvillus in intra-capillary neutrophils is increased after a 2-minute exposure to complement fragments, suggesting that actin rapidly redistributes from the central regions of the cells into the submembrane regions as well as into the microvillar processes when intravascular neutrophils are activated. These changes are associated with a greater number of spherical neutrophils within the pulmonary capillary bed.

Other investigators have demonstrated using a variety of techniques that binding of inflammatory mediators to receptors on neutrophils induces a rapid stiffening of these cells that is accompanied by a decrease in their ability to deform.[3,17–26] The mechanism for this stiffening has been suggested to involve the polymerization of g-actin to f-actin, increasing the rigidity of the submembrane cytoskeletal network.[3,17–26] Although our study did not evaluate the polymerization of actin, the observed increase in immunoreactive actin likely resulted in increased formation of microfilaments. However, it is possible that increasing the viscosity of the submembrane rim of cytoplasm by increasing the concentration of actin monomers could also result in increased stiffening without requiring polymerization. Increases in the viscoelastic properties of the submembrane rim may similarly decrease the neutrophil's ability to deform.

The observation that the concentration of actin in the microvillar processes increases rapidly after intravascular activation of neutrophils suggests an alternative or additional hypothesis. Normally, neutrophils contain 140 to 200% more plasma membrane than is necessary to surround the cytoplasm when neutrophils are spherical.[37,38] This excess membrane is thought to be required for neutrophils to change their shape, as the smallest surface area to volume ratio is that of a sphere, and excess surface area is required to accommodate changes in shape. An increase in the concentration of actin within the microvillar processes may prevent them from flattening and contributing their excess membrane for shape changes, decreasing the neutrophils' ability to deform. Alternatively, this increased concentration of actin may actually facilitate contraction of microvillar processes and their incorporation into the body of the cell, particularly if the monomers assembled into microfilaments. In fact, these studies may suggest the hypothesis that the microvillar processes rapidly stiffen, inhibiting deformation immediately after activation, whereas at later time points this accumulation of actin facilitates their flattening and subsequent changes in neutrophil shape. This hypothesis is supported by data demonstrating that stimulus-induced decreases in deformability are transient, and neutrophils regain their ability to deform within 15 to 30 minutes despite continued exposure to the inciting stimulus. However, direct measurements of microvillar stiffness have not been made to our knowledge.

Previous studies have measured the diameter of spherical unstimulated neutrophils using electron microscopy and a statistical method suitable for calculating the frequency distribution of diameters for a polydispersed system of spherical shapes.[30] Laser scanning confocal microscopy permits optical sectioning through the entire thickness of neutrophils in capillaries and selection of the largest plane. However, its resolution is less than that of electron microscopy, and it does not allow identification of plasma membrane and microvillar processes. When the measurements of diameter from these two very different techniques are compared, the value for the diameter of spherical neutrophils obtained using confocal microscopic measurements of neutrophils with shape factors close to 1 was 6.37 $\pm$ 0.19 μm and compared very closely with values of 6.4 $\pm$ 0.6 μm calculated from ultrastructural measurements.[30]

Both confocal microscopy and electron microscopic studies showed that only a small fraction of the neutrophils present within the capillary bed of control, unstimulated lungs were spherical. However, using confocal microscopy, the average shape factor and its variability was smaller (1.47 $\pm$ 0.29) than that obtained using electron microscopy (1.9 $\pm$ 1.0[30]). One possible explanation for this discrepancy is related to the fact that confocal microscopy permits selection of the largest cross section whereas electron microscopy results in cross sections that may deviate considerably from the central one, even when only neutrophils in longitudinally sliced capillary segments are included. Shape factors measured on more peripheral cross sections require the assumption that neutrophils change shape only along the axis that is parallel to the capillary segment and that the two axes perpendicular to the vessel wall change similarly. One possible interpretation of the smaller shape factors and variability in values is that neutrophils change shape in two directions, lengthening as well as flattening to pass through capillary segments.

A shape factor of 1.4 was selected as the threshold value for comparison between groups for two reasons. First, previous studies have shown that all neutrophils in large-vessel blood have shape factors less than 1.4[30] and that 94% of leukocytes in pulmo-

nary arterioles have shape factors less than 1.25.[39] Second, previous studies have shown that neutrophils can deform from a diameter of 6.4 μm to 5.1 to 5.2 μm as quickly as red blood cells without a measurable delay in pulmonary transit time,[31] and this change in diameter alters shape from a shape factor of 1 to approximately 1.4. When this threshold value of shape was applied to the data presented here, only a small increase in the more spherical neutrophils was observed after infusion of complement fragments for 1.5 minutes. In fact, only an additional 16% of neutrophils had a more spherical shape than those observed in control animals (Table 3). However, calculations to determine what increase we would expect to find if every circulating neutrophil sequestered as a spherical neutrophil showed that only a 12.7 to 14.5% increase could be expected, very close to the 16% increase observed. We interpret these data to indicate that neutrophils sequestered by infusion of complement fragments maintain their spherical shape for longer than predicted from the frequency distribution of neutrophil shape in normal pulmonary capillaries.

In contrast, neutrophils that were sequestered in the lungs after a 15-minute infusion of complement fragments had elongated shapes more frequently than control animals (Figure 5). Although this increase was small, it is interesting in light of data showing that, by 15 minutes, CD11/CD18-mediated adhesion is critical for maintaining the sequestered neutrophils within the pulmonary capillaries.[12] This flattening may be mediated through increased neutrophil-endothelial cell adhesion, and later time points may demonstrate this elongation more dramatically.

More of the intra-capillary neutrophils in the rabbits given short exposures to complement fragments display redistribution of actin to the submembrane region and the microvillar processes than display changes in shape, and there was no correlation between neutrophil shape and either the ratio of submembrane/central actin density or the number of gold particles/microvillar process. These observations may be due to the ability of neutrophils that are already within the lungs before the infusion of complement fragments to undergo redistribution of actin despite previous deformation. If, in fact, the changes in cytoskeletal portions mediate the stimulus-induced stiffening of neutrophils, these studies imply that redistribution of actin will not cause a deformed, elongated neutrophil to regain its spherical shape but rather will only inhibit a spherical neutrophil from undergoing deformation.

Finally, we and others have suggested that stimulus-induced changes in neutrophil volume may also increase the difficulty neutrophils encounter in passing through the pulmonary capillary bed and contribute to their sequestration.[3,4,8,12,40] However, the calculated surface area and volume of neutrophils showed no significant differences after either 1.5- or 15-minute exposures to complement fragments. Although these calculations are likely to underestimate both surface area and volume, it is unlikely that the magnitude of changes in volume will be sufficient to induce sequestration.

In summary, these studies demonstrate a rapid redistribution of actin from the central regions of neutrophils to the submembrane rim of cytoplasm and to the microvillar processes when neutrophils are activated *in vivo* by intravascular inflammatory mediators. These changes, as well as the increased polymerization of actin within the periphery of the cytoplasm observed by others,[22–26] are likely to alter the biomechanical properties of neutrophils, increasing their stiffness and viscosity and decreasing their ability to deform. Measurements of shape suggest that the majority of the newly sequestered cells are more spherical and less able to deform compared with those present within the normal pulmonary microvasculature. Although some of these arguments are indirect, these studies suggest that changes in the cytoskeleton that induce alterations in the biomechanical properties of neutrophils may alter neutrophil transit and result in sequestration of neutrophils during the inflammatory process.

Acknowledgments

We thank members of the Section of Hematology in the Department of Pathology for performing complete blood counts, Taffy Hooser and Loretta Collier for preparation of histological sections, and Barbara Fletcher for preparation of the manuscript.

References

1. Windsor ACJ, Mullen PG, Fowler AA, Sugarman HJ: Role of the neutrophil in adult respiratory distress syndrome. Br J Surg 1993, 50:10–17
2. Till GO, Johnson KJ, Kunkel R, Ward PA: Intravascular activation of complement and acute lung injury. J Clin Invest 1982, 69:1126–1135
3. Worthen GS, Schwab B, Elson EL, Downey GP: Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. Science 1989, 245:183–185
4. Lien DC, Henson PM, Capen RL, Henson JE, Hanson

WL, Wagner WW Jr, Worthen GS: Neutrophil kinetics in the pulmonary microcirculation during acute inflammation. Lab Invest 1991, 65:145–159
5. Rosolia DL, McKenna PJ, Gee MH, Albertine KH: Infusion of zymosan-activated plasma affects neutrophils in peripheral blood and bone marrow in sheep. J Leukocyte Biol 1992, 52:201–215
6. Albertine KH, Rosolia DL, Schuhl RA, Peters SP, Gee MH: Physical and cytochemical properties of neutrophils activated *in situ* in the lung during ZAP infusion in sheep. J Appl Physiol 1993, 74:1361–1373
7. Erzurum SR, Downey GP, Doherty DE, Schwab B III, Elson EL, Worthen GS: Mechanisms of lipopolysaccharide-induced neutrophil retention: relative contributions of adhesive and cellular mechanical properties. J Immunol 1992, 149:154–162
8. Selby C, MacNee W: Factors affecting neutrophil transit during acute pulmonary inflammation. Exp Lung Res 1993, 19:407–428
9. Wortel CH, Doerschuk CM: Neutrophils and neutrophil-endothelial cell adhesion in ARDS. New Horizons 1993, 1:631–637
10. Roberts PJ, Yong KL, Khwaja A, Johnson BV, Pizzey AR, Carver JE, Addison IE, Lunch DC: Pentoxifylline at clinically achievable levels inhibits FMLP-induced neutrophil responses but not priming, upregulation of cell-adhesion molecules, or emigration induced by GM-CSF. Eur J Haematol 1993, 150:1–10
11. Doerschuk CM, Allard MF, Hogg JC: Neutrophil kinetics in rabbits during infusion of zymosan-activated plasma. J Appl Physiol 1989, 67:88–95
12. Doerschuk CM: The role of CD18-mediated adhesion in neutrophil sequestration induced by infusion of activated plasma in rabbits. Am J Respir Cell Mol Biol 1992, 7:140–148
13. Gie RP, Doerschuk CM, English D, Coxson HO, Hogg JC: Neutrophil-associated lung injury following the infusion of activated plasma. J Appl Physiol 1991, 70: 1271–1278
14. Tonnesen MG, Smedly LA, Henson PM: Neutrophil-endothelial cell interactions: modulation of neutrophil adhesiveness induced by complement fragments C5a and C5a des arg and formyl-methionyl-leucyl-phenylalanine *in vitro*. J Clin Invest 1984, 74:1581–1592
15. Smith CW, Martin SD, Rothlein R, Toman C, Anderson DC: Cooperative interaction of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transmigration of human neutrophils *in vitro*. J Clin Invest 1989, 83:2008–2017
16. Mulligan MS, Varani J, Warren JS, Till GO, Smith CW, Anderson DC, Todd RF III, Ward PA: Roles of β_2 integrins of rat neutrophils in complement- and oxygen radical-mediated acute inflammatory injury. J Immunol 1992, 148:1847–1857
17. Westlin SF, Kiely J-M, Gimbrone MA Jr: Interleukin-8 induces changes in human neutrophil actin conformation and distribution: relationship to inhibition of adhesion to cytokine-activated endothelium. J Leukocyte Biol 1992, 52:43–51
18. Inano H, English D, Doerschuk CM: Effect of zymosan-activated plasma on the deformability of rabbit polymorphonuclear leukocytes and the role of the cytoskeleton. J Appl Physiol 1992, 73:1370–1376
19. Downey GP, Worthen GS: Neutrophil retention in model capillaries: deformability, geometry, and hydrodynamic forces. J Appl Physiol 1988, 65:1861–1871
20. Nash GB, Jones JG, Mikita J, Christopher B, Dormandy JA: Effects of preparative procedures and of cell activation on flow of white cells through micropore filters. Br J Haematol 1988, 70:171–176
21. Kawaoka EJ, Miller ME, Cheung ATW: Chemotactic factor-induced effects upon deformability of human polymorphonuclear leukocytes. J Clin Immunol 1981, 1:41–44
22. Wallace PJ, Westo RP, Packman CH, Lichtman MA: Chemotactic peptide-induced changes in neutrophil actin conformation. J Cell Biol 1984, 99:1060–1065
23. Howard TH, Oresajo CO: The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils. J Cell Biol 1985, 101:1078–1085
24. Frank RS: Time-dependent alterations in the deformability of human neutrophils in response to chemotactic activation. Blood 1990, 12:2606–2612
25. Packman CH, Lichtman MA: Activation of neutrophils: measurement of actin conformational changes by flow cytometry. Blood Cells 1990, 16:193–207
26. Pecsvarady Z, Fisher TC, Fabok A, Coates TD, Meiselman HJ: Kinetics of granulocyte deformability following exposure to chemotactic stimuli. Blood Cells 1992, 18: 333–352
27. Lundberg C, Wright SD: Relation of the CD11/CD18 family of leukocyte antigens to the transient neutropenia caused by chemoattractants. Blood 1990, 76: 1240–1245
28. Doyle NA, Quinlan WM, Graham L, Doerschuk CM: The role of LECAM-1 in neutrophil sequestration induced by infusion of complement fragments. Am Rev Respir Dis 1992, 145:A187
29. Kubo H, Doyle NA, Doerschuk CM: The role of P-selectin in complement fragment-induced neutrophil sequestration. Am J Respir Crit Care Med 1995, 151: A455
30. Doerschuk CM, Beyers N, Coxson HO, Wiggs B, Hogg JC: Comparison of neutrophil and capillary diameters and their relation to neutrophil sequestration in the lung. J Appl Physiol 1993, 74:3040–3045
31. Wiggs BR, English D, Quinlan WM, Doyle NA, Hogg JC, Doerschuk CM: The contributions of capillary pathway size and neutrophil deformability to neutrophil transit through rabbit lungs. J Appl Physiol 1994, 77:463–470
32. Lessard JL: Two monoclonal antibodies to actin: one muscle selective and one generally reactive. Cell Motil Cytoskeleton 1988, 10:349–362
33. Franklin WA, Locker JD: Ethidium bromide: a nucleic

acid stain for tissue sections. J Histochem Cytochem 1981, 29:572–576
34. Doerschuk CM, Allard MF, Martin BA, MacKenzie A, Hogg JC: Marginated pool of neutrophils in lungs of rabbits. J Appl Physiol 1987, 63:1806–1815
35. Holland BS, Copehaver MD: An improved sequentially rejective Bonferroni test procedure. Biometrics 1987, 43:417–423
36. Conover W: Practical Nonparametric Statistics, ed 2. Toronto, Wiley, 1989, pp 344–384
37. Bagge U: Granulocyte rheology. Blood Cells 1976, 2:481–490
38. Schmid-Schonbein GW, Shih YY, Chien S: Morphometry of human leukocytes. Blood 1980, 56:866–875
39. Gebb SA, Graham JA, Hanger CC, Godbey PS, Capen RL, Doerschuk CM, Wagner WW Jr: Sites of leukocyte sequestration in the pulmonary microcirculation. J Appl Physiol 1995, 79:493–497
40. Hogg JC: Neutrophil kinetics and lung injury. Physiol Rev 1987, 67:1249–1295