

# Synchrony of Cell Spreading and Contraction Force as Phagocytes Engulf Large Pathogens

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**Abstract.** A simple micromechanical method has been used to directly measure the force of contraction in single mammalian phagocytes (blood granulocytes) during engulfment of large yeast pathogens. Both the time course of cell spreading over the yeast particle and increase in cell body contractile force were quantitated at three temperatures in the range of 23–35°C. The surprising feature of the phagocyte response was that engulfment and cell body contraction occurred in a serial sequence: i.e., the phagocyte spread rapidly over the particle at a steady rate with no detectable cell body contraction; when spreading stopped, contraction force in the cell body then rose steadily to a plateau level that remained stationary until the next se-

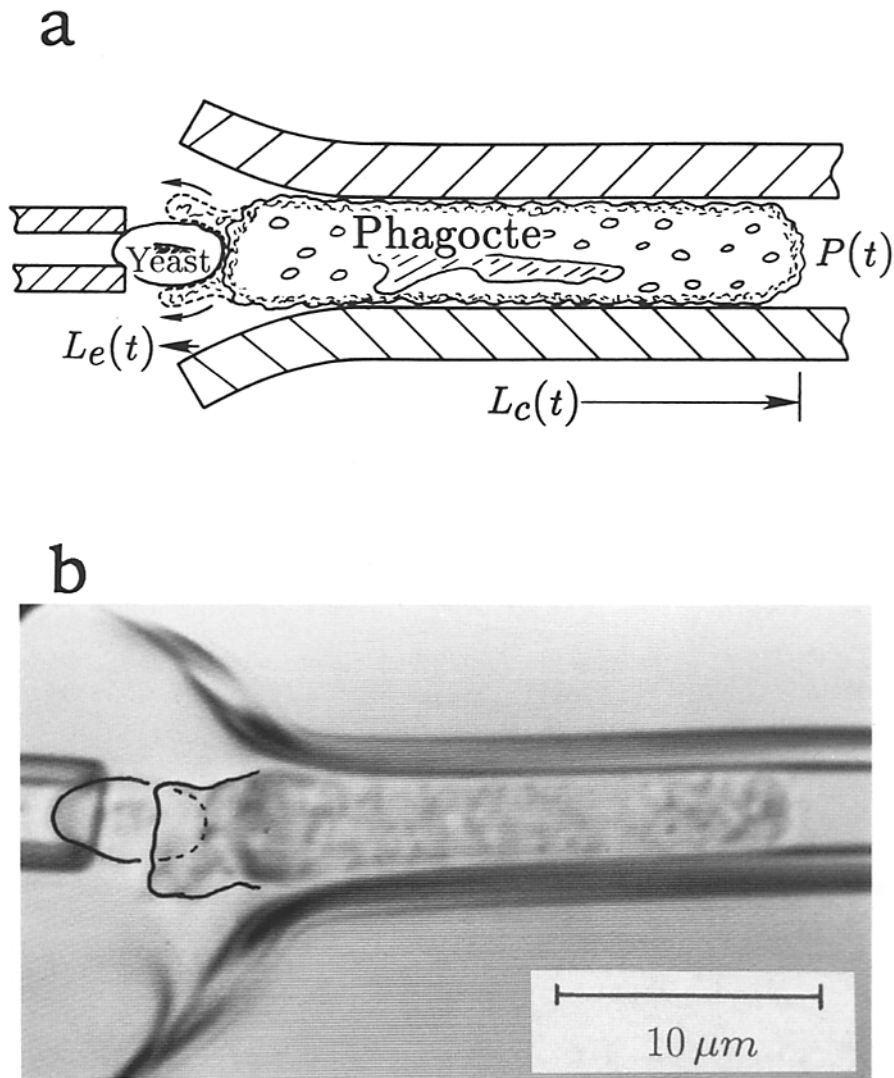
quence of spreading and contraction. Both spreading and contraction exhibited abrupt start/stop kinetics. Also impressive, the cell contraction force stimulated by phagocytosis was quite large ( $\sim 10^{-8}$  N)—two orders of magnitude larger than the force necessary to deform passive phagocytes to the same extent. If distributed uniformly over the cell cross section, the contraction force is equivalent to an average contractile stress of  $\sim 10^3$  N/m<sup>2</sup> (0.01 Atm). These physical measurements in situ set critical requirements for the mechanism of force generation in granulocytes, imply that a major increase in network cross-linking accompanies build-up in contractile force and that subsequent network dissolution is necessary for locomotion.

**P**ATHOGEN ingestion by phagocytes is an essential component of immune defense in animals and represents a subclass of general amoeboid motions. Extensive evidence exists for the motional characteristics of locomoting cells (Abercrombie et al., 1970; Abercrombie, 1982; Trinkaus, 1984; Lackie, 1986; Lee et al., 1990; Theriot and Mitchison, 1991) and temporal characteristics of phagocytosis (Rabinovitch, 1967; MacFarlane and Herzberg, 1984; Evans, 1989). However, little is known about the coordination between contractile forces in the cell cytostructure and cell spreading which is essential for understanding the physical mechanism of ingestion. Here, we report the first simultaneous measurements of pathogen engulfment and contractile force in single blood phagocytes (human granulocytes) after local stimulation of the cell by a large yeast particle. A unique feature of the experimental approach is that contractile force is measured isometrically (at constant cell body length) which minimizes internal dissipation of forces by cytoplasmic motion. Significant findings from these studies include: (a) both pathogen engulfment and build-up in con-

tractile force  $f$  are constant speed processes with abrupt start/stop kinetics; (b) cell body contraction follows pathogen engulfment and the cell structure is progressively stiffened; (c) at full contraction, cell body stresses  $\sigma_c$  reach levels of 0.01 Atm ( $10^3$  Nm<sup>2</sup>) which are two orders of magnitude greater than the passive cell turgor pressure. When related to microscopic mechanisms proposed for cell locomotion (Kron and Spudich, 1986; Ishijima et al., 1991), the measurements of contractile force set limits for the minimum number of actin-myosin “motors” ( $>10^4$ /cell) as well as the minimum shear strength of the network structure. Even more critical, the time period  $\Delta t_c$  required to reach a fully contracted-stiff state (where contractile force is maximal) can be compared with test tube measurements of “time to contraction” for in vitro actin networks (Janson et al., 1991) to predict molecular characteristics of network structure in situ (e.g., cross-link density and filament/motor ratio). The implication of this comparison is that the initial actin structure in unstimulated phagocytes must be sparsely connected but with a significant number of potential myosin motors. Because of the enormous increase in cell contraction force, the network structure must progressively tighten either by pulling out slack in long actin filaments or by steady assembly of network cross-links. Most likely, there is major increase in cross-link density because of the observation that only a slight spontaneous recoil occurs when the applied force is set to zero.

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**Figure 1.** Micromechanical method to simultaneously observe pathogen engulfment and measure cell body contraction force. The large pathogen (yeast particle) is maneuvered to touch the fully aspirated phagocyte (blood granulocyte). Then, the suction pressure  $P$  is adjusted to maintain constant cell body length  $L_c$  after stimulation and throughout subsequent activation. The length  $L_c$  is measured from the yeast contact point to the cell cap inside the pipet. The length  $L_e$  is the extent of lamella advance over the yeast measured from the contact point. (a) Schematic. (b) Videomicrograph of a blood granulocyte engulfing a yeast particle. The contour line has been superimposed to outline the yeast-granulocyte interface.

## Materials and Methods

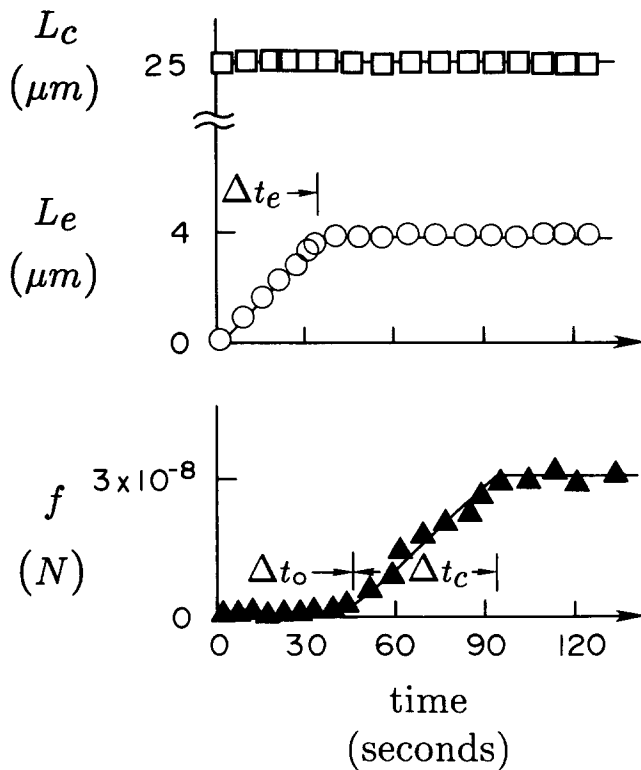
### Micromechanical Test

A simple micropipet technique was devised to measure contraction force in a single phagocyte (blood granulocyte) as the cell engulfed a large yeast pathogen at one end. The method is illustrated by the schematic diagram and video micrograph image in Fig. 1. The tests were performed with the cell body length  $L_c$  held constant by pipet suction force (i.e., isometric) and without adhesion of the cell to the glass wall. Natural coating of the glass by proteins in the plasma containing buffer was sufficient to prevent adhesion (as evidenced by the easy sliding of the phagocyte out of the pipet under very small positive pressure). Pipets were fabricated with a special "bell shape" entrance so that no axial force was applied to the phagocyte by the glass face of the pipet. Passive cells were chosen before aspiration based on spherical shape without active projections. For the passive cells, only suction pressures of  $10^{-4}$  Atm ( $10 \text{ N/m}^2$ ) were required to slowly extend the cell to a full cylindrical length of  $\sim 25 \mu\text{m}$  inside a pipet of  $\sim 3.5 \mu\text{m}$  caliber (Evans and Yeung, 1989). Once inside the pipet, the passive cell could easily be moved up and down the bore with small  $\pm$  suction pressures. After aspiration, the rear end of the phagocyte was positioned at the pipet entrance; then, a zymosan (yeast) particle was maneuvered with a second pipet to touch the exposed end. Within a brief period, the phagocyte began to spread over the yeast particle; the extent of engulfment was limited by the presence of the yeast holding pipet. Throughout the test, the suction pressure  $P$  applied to the phagocyte was adjusted as necessary to maintain

a constant cell body length  $L_c$  (measured from the yeast contact to the cell cap). If the applied suction pressure was too large, the cell body would narrow (neck down) near the pipet entrance which would allow the overall cell length to increase. On the other hand, if the pressure was too low, contraction would cause expansion of the cell region outside the pipet entrance which would reduce the overall cell length. Spreading of the cell over the yeast particle had little effect on the cell body length because  $<10\%$  of the cell volume was needed to produce the lamella surrounding the yeast. At various times in the test, the large pipet was translated back and forth axially to verify that the phagocyte was not adherent to the glass wall. Since the phagocyte was free to slip inside the pipet, the isometric length was maintained solely by the pipet suction. The actual force experienced by the cell body was the suction force ( $f = \pi R_p^2 P$ ) depreciated by the ratio ( $d_a/R_p$ ) of the annular fluid gap  $d_a$  to the pipet radius  $R_p$ . The gap dimension could not be resolved optically but was estimated from observation of flow of small particles upstream of the cell plug. The estimate showed that  $>90\%$  of the suction force was applied to the cell body. Because of the cylindrical geometry, the axial force had to be essentially the same at any cross section of the cell body.

### Phagocytes

Blood granulocytes were obtained freshly from small finger prick blood samples suspended in a large excess of plasma containing buffer. The blood type compatible plasma was separated from whole blood collected in sodium citrate anticoagulant, filtered to remove bacteria or other con-

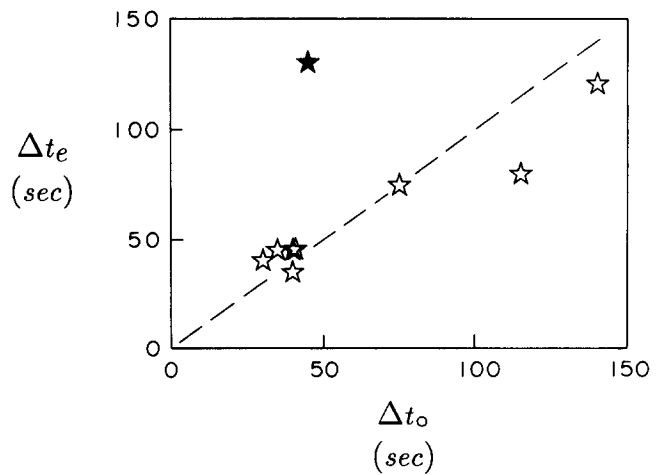


**Figure 2.** An example of yeast engulfment  $L_e$ , phagocyte length  $L_c$ , and pipet suction force  $f = \pi R_p^2 P$  versus time measured in a single phagocytosis test at 23°C. Note: both the speed of engulfment and rise in the suction force (adjusted to keep the cell length constant) were steady then ceased abruptly. In most cases, spreading and cell body contraction appeared to be serial as shown (i.e.,  $\Delta t_e \approx \Delta t_o$ ). The time period  $\Delta t_e$  was the duration of the spreading phase and  $\Delta t_o$  was the phase lag between onset of spreading and onset of cell body contraction. Impressively, the level of contraction force ( $\sim 10^{-8}$  N) was two orders of magnitude greater than the initial suction force ( $\sim 10^{-10}$  N) required for aspiration of the passive phagocyte into the pipet.

taminants, and then diluted with an isotonic buffer to a concentration of 20% plasma. A small amount of dilute cell suspension was placed in one microchamber on a microscope stage. In an adjacent chamber on the stage, zymosan (yeast) particles were suspended in buffer. Single yeast particles were selected and transferred to the granulocyte chamber for each phagocytosis test. Separation of granulocytes and yeast particles helped to maintain the granulocytes in a passive state before the phagocytosis test. The yeast particles were opsonized by complement proteins present in the plasma. (Citrate plasma concentrations of 20% or more have been shown to be sufficient for complete opsonization of yeast particles [Evans, 1989].) The microscope chambers were maintained at the appropriate temperature by specially designed heat exchanger.

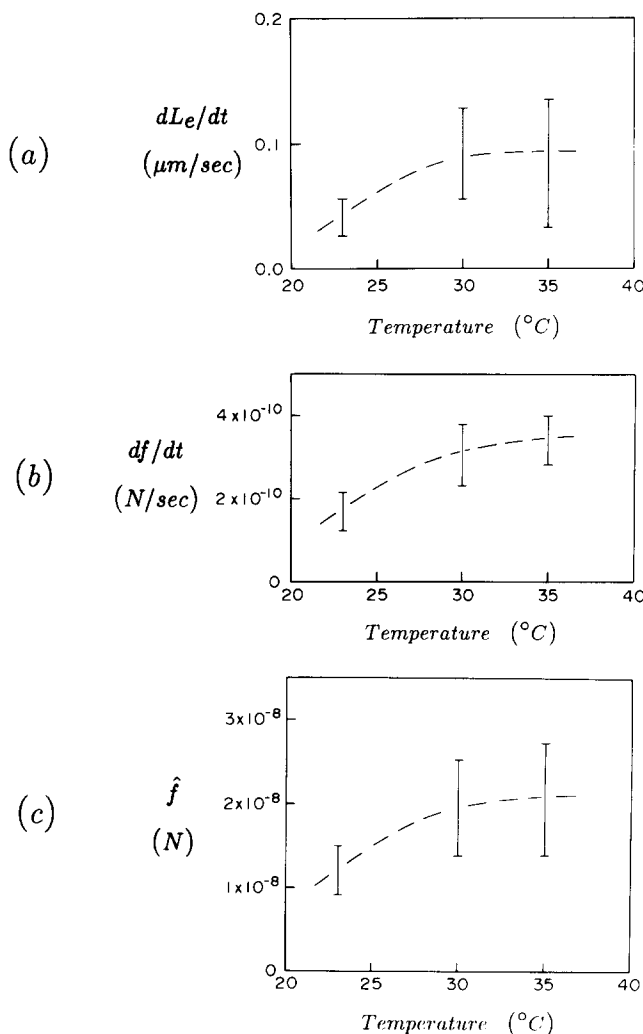
## Results

Using the micromechanical method illustrated in Fig. 1, the extent  $L_e$  of yeast particle engulfment and phagocyte contractile force  $f$  (at constant cell body length  $L_c$ ) were measured simultaneously in each phagocytosis test. Data from an initial sequence of spreading and isometric contraction is shown in Fig. 2 for a test at 23°C. As seen in Fig. 2, the behavior was alternate phases of engulfment and contraction. The correlation between the spreading period  $\Delta t_e$  and the onset  $\Delta t_o$  of contraction is plotted in Fig. 3 for cells



**Figure 3.** Correlation between the onset  $\Delta t_o$  of contraction in the cell body and the interruption  $\Delta t_e$  of spreading over the yeast surface for the initial phase of activity after stimulation at 23°C. Except for one phagocyte (data point in black), the response was serial-consistent with  $\Delta t_o \approx \Delta t_e$  (straight line).

tested at 23°C. A perfectly serial sequence is expected to be characterized by  $\Delta t_o \approx \Delta t_e$  which is consistent with the majority of the data at 23°C (Fig. 3). Occasionally contraction appeared to start before spreading ceased especially at higher temperatures where cells were labile and often activated. At long times and especially at elevated temperatures (>30°C), phagocyte contraction became erratic and the cell cycled through phases of force generation and relaxation (all at constant length) often with intermittent increments in yeast engulfment. The serial behavior of spreading and cell body contraction was observed at all temperatures. However, setting the temperature to 23°C was especially useful because most of the granulocytes were in passive states before stimulation by the yeast. Also, the process of activation was slower which allowed the first sequence of spreading and contraction to be more easily isolated. During the initial activation process, both the speed  $dL_e/dt$  of engulfment and rise in cell force per unit time  $df/dt$  were steady and ceased abruptly as shown in Fig. 2. These rates were found to increase with temperature as shown in Fig. 4, *a* and *b*. On the other hand, as demonstrated previously (Evans, 1989), the rate of engulfment became exceedingly slow (with commensurate protraction of spreading time  $\Delta t_e$ ) for temperatures below 15°C. The maximum contraction force  $\hat{f}$  also increased with temperature (Fig. 4 *c*). Interestingly, the time period  $\Delta t_c$  required to reach maximum contraction (given directly by the maximum force divided by rate— $\Delta t_c = \hat{f}/(df/dt)$ ) was relatively independent of temperature as shown in Fig. 5. Also observed during contraction, the cell body became progressively transformed from a soft deformable sphere in the passive state to a very stiff structure that persisted until contraction subsided. This feature was exposed clearly when the suction pressure was rapidly reduced to zero and the cell was removed from the pipet as shown in Fig. 6. Only a small spontaneous recoil ( $\sim 10\%$  of overall length) occurred as demonstrated by comparison of Fig. 6, *a* and *b*. The quick recoil was followed by a long period ( $\sim 1$  min) of extensional shortening where the structure softened as contraction subsided (Fig. 6 *c*). Eventually, extensional recovery

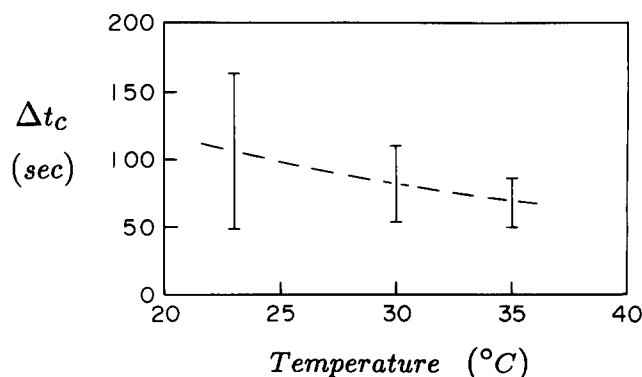


**Figure 4.** In the spreading and contraction phases, the engulfment length  $L_e$  and cell contraction force  $f$  increased steadily with time. The following figures demonstrate the dependencies of rate of engulfment  $dL_e/dt$ , rate of force increase  $df/dt$ , and maximum force  $\hat{f}$  on temperature. (a) The speed of engulfment of the yeast determined from the length versus time. (b) The rate of increase of cell contraction force determined from the pipet suction versus time. (c) Measurements of maximum contraction force. The brackets represent averages  $\pm$  SD from data measured in 10 tests at each temperature. Note: the rate of engulfment became exceedingly slow when the temperature was lowered to 15°C or below.

allowed the cell to “locomote” over the yeast and often onto the yeast-holding pipet.

## Discussion

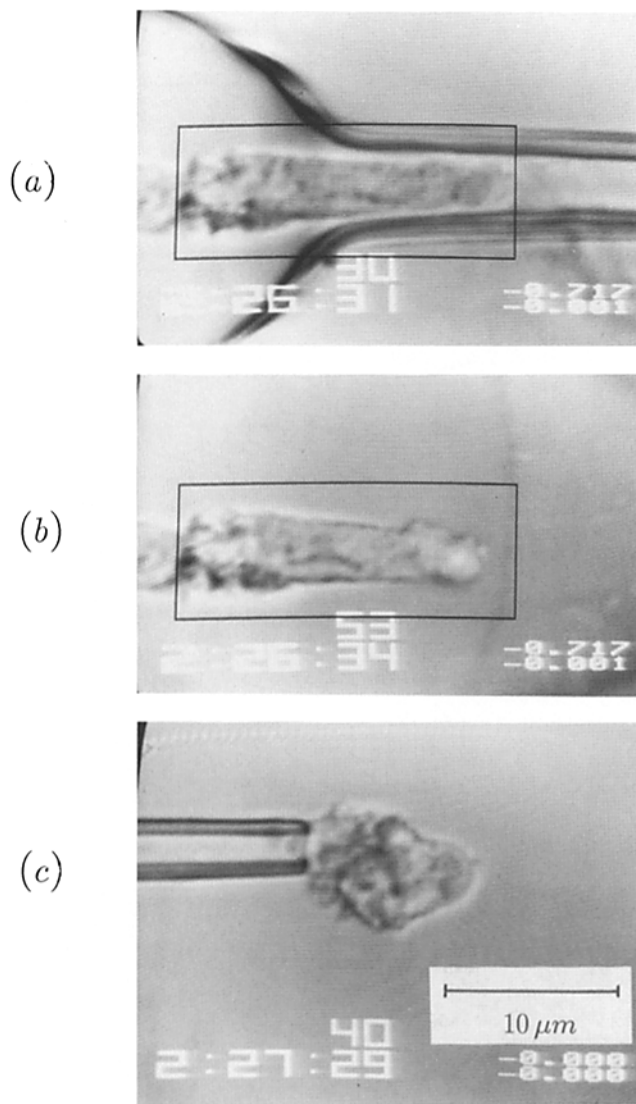
The observation that pathogen engulfment and cell body contraction occur in series demonstrates that the spreading process in engulfment is localized to the vicinity of the pathogen contact. This feature was also seen in previous experiments where yeast particles were “fed” to passive granulocytes (Evans, 1989). The cell bodies were observed to remain spherical and soft through most of the spreading process. The local characteristics of lamella spreading without cell body contraction (and the observation of material ad-



**Figure 5.** The time period required to reach maximum contraction force measured in the initial contraction phase at three temperatures. The brackets represent averages  $\pm$  SD for 10 tests at each temperature.

vance at the front of the lamella) support the concept that lamellae spread by rapid polymerization of actin along the lead boundary (Evans and Dembo, 1990; Evans, 1993). This concept has also been supported directly by elegant experiments with caged fluorescent actin in rapidly locomoting fish scale keratocytes (Theriot and Mitchison, 1991). Indeed, the rates of advance observed for both granulocyte engulfment of yeast and locomotion of keratocytes are of comparable magnitude. The theoretical limit to the rate of spreading by boundary polymerization is the rate of actin monomer addition which implies speeds up to  $0.5 \mu\text{m}/\text{s}$  for rate constants of  $10 \mu\text{M}^{-1}\text{s}^{-1}$  (Pollard and Cooper, 1986)—given actin concentrations of  $10 \mu\text{M}$  and monomer dimension of 5 nm. Also consistent with this mechanism of spreading, the rates of engulfment measured here for highly extended granulocytes (length  $\sim 25\text{--}30 \mu\text{m}$ ) are the same as those found in the previous study with unstressed granulocytes (spherical diameter  $\sim 9 \mu\text{m}$ ). Hence, the rate of lamella advance is independent of passive cell extension. This feature is opposite to suggestions in the past that the rate of “spreading may be inversely related to the degree of spreading of the cell as a whole” (Chen, 1979). In the early work of Chen (1979), it was found that fully extended fibroblasts were restrained from spreading until adhesive attachments at the trailing edge of the cell were physically disrupted. Then, there was a rapid recoil (shortening of the cell) followed by spreading. The response appears similar to the behavior described here for granulocytes. However, initial extension of the granulocyte body clearly did not effect the spreading process. Most likely, it is the restraining force associated with adhesive attachments for fibroblasts that opposes contraction until a cell is released. It is interesting to note that when the fibroblasts were released, the recoil (cell shortening) was much greater than observed for granulocytes which indicates fewer cross-links in the network structure.

The fluid-like structure of unstimulated granulocytes (Evans and Yeung, 1989) shows that there is little (if any) macroscopic gel structure in passive granulocytes. On the other hand, at full contraction in these phagocytosis tests, granulocyte bodies became very stiff and exhibited only a slight spontaneous recoil when the suction force opposing contraction was nulled. Thus, contractile stresses were quickly opposed by network rigidity exposing a highly cross-linked network structure. The cell body length shortened ap-



**Figure 6.** (a) Videomicrograph of a granulocyte held at maximum contractile force. (b) Videomicrograph of the same granulocyte less than 3 s after pipet suction has been nulled which demonstrates a small spontaneous recoil driven by the contractile force. (c) Videomicrograph of the granulocyte about 1 min later which shows the extensional recovery and softening of the cell structure as contraction subsides.

precipally only when the contraction subsided indicating dissolution of network cross-link structure. The implication is that network cross-link density increases significantly as contractile force builds up but the capability for shortening is significantly impeded. Increase in cross-linking appears to be required for transmission of contractile force but extensional shortening (clearly necessary for cell locomotion) appears to require dissolution of the network structure. Hence, maximal gelation leads to maximal force but minimal length contraction. On the other hand, solation of network structure allows maximal contraction but diminishes contractile force. The concept of gelation - force correspondence has been promoted for many years (Stendahl and Stossel, 1980; Stossel, 1982; Taylor and Fehcheimer, 1982); however, there has been disagreement over whether

or not solation is required for extensional shortening (Janson et al., 1991).

Gelation of actin filaments into networks is clearly an important process in cell contraction. As such, there have been extensive studies of gelation and contraction of actin assemblies in vitro. Recently, actin network contraction has been monitored both by macroscopic observation of whole networks in test tubes and by microscopic images of local network regions (Janson et al., 1991). Both measures yielded the same "time to contraction" which demonstrated that the rate of condensation of the network was uniform and intensive— independent of sample size. Consequently, viscous hydrodynamic resistance from squeezing solvent out of the network must not have been a significant factor in the contraction of these network assemblies. Most likely, the time to contraction represented the submicroscopic tightening of network filaments in opposition to contractile forces where the rate was limited by energetic dissipation in the tightening mechanism (e.g., myosin motor movement along actin filaments and the kinetics of ATP hydrolysis). Based on this reasoning, we postulate that the times for network contraction in vitro can be compared to the time periods  $\Delta t_c$  required to reach maximum force in our isometric tests of granulocyte contractions. It was found from the in vitro measurements (Janson et al., 1991) that contraction times increase as the actin:myosin ratio is increased (i.e., as the number of motors per filament is decreased) and as the actin:filamin (Actin Binding Protein) ratio is increased (i.e., as the density of cross-links is reduced). By comparison, time periods for contraction of 60–70 s observed in our granulocyte experiments indicate that the initial actin structure must be a very loose weakly cross-linked assembly with large numbers of potential motors. In the in vitro studies (Janson et al., 1991), the actin assemblies that exhibited fast contraction times ( $\sim 60$  s) were essentially fluid-like materials which is again consistent with the deformability of passive granulocytes before activation.

Measurements of contractile force provide important in situ data that can be compared with models proposed for force generation in actin networks. The popular model is the myosin molecular motor where myosin molecules are thought to ratchet actin filaments past one another in the network (Huxley, 1957; Southwick and Stossel, 1983; Kron and Spudich, 1986). When restrained, forces on the order of  $10^{-12}$  N can be produced by a single motor molecule (Huxley, 1957; Ishijima et al., 1991). Forces of cell contraction ( $\sim 10^{-8}$  N, Fig. 4 c) found in our isometric tests indicate that more than  $10^4$  motors would be necessary to fulfill the task. (Interestingly, the level of cell contraction force [ $\sim 10^{-8}$  N] stimulated by phagocytosis is comparable to traction forces produced by fibroblasts cultured on elastic substrates [Harris et al., 1980].) Based on cell volume, the myosin concentration in a granulocyte would have to be  $>0.1$   $\mu$ M and the mean actin filament length between motor sites would be on the order of 300–400 nm. The minimal concentrations implied for myosin (0.1  $\mu$ M) and actin (10  $\mu$ M) are well within the range of data published for granulocytes (Stossel, 1982; Southwick and Stossel, 1983). At full contraction, the rate of ATP hydrolysis in the cell would reach  $5 \times 10^{-19}$  mol  $s^{-1}$  based on 30 ATP/s per actomyosin (Ishijima et al., 1991). This requirement for ATP hydrolysis is easily accommodated by the  $Mg^{2+}$  ATPase activity (10

nmol s<sup>-1</sup> mg<sup>-1</sup>) reported for myosin plus F-actin purified from rabbit phagocytes (Stossel et al., 1980).

The level of contractile force sets minimal requirements for shear strength of the network structure. Assuming that the network is uniformly distributed over the cylindrical cross section in the pipet (i.e.,  $f \sim \pi R_p^2 \sigma_c$ ), the contractile forces imply stress levels of  $\sigma_c \sim 10^3$  N/m<sup>2</sup> (0.01 Atm). A puzzling feature of actin networks in shear viscometers (Janmey, 1991) is that rupture occurs at much lower stresses ( $\sim 10^2$  N/m<sup>2</sup>) than the stress deduced from cell contractile forces. However, if the contractile force is distributed over  $10^4$  filament connections between motors, the strength of a single filament would only have to exceed  $10^{-12}$  N which is significantly lower than  $10^{-10}$  N reported for rupture of single actin filaments (Kishino and Yanagida, 1988).

Finally, there was a significant general feature observed in all of these phagocytosis tests: i.e., rates of particle ingestion and rise in contractile force were steady with abrupt start/stop kinetics. Steady rates imply that processes of network assembly and contraction in phagocytes operate far from equilibrium (Evans, 1993). The reasoning is that processes near equilibrium tend to slow down and deviate from linear behavior because of either chemical—or diffusive—kinetic limitations. Hence, chemical reactions for assembly, contraction, and disassembly of network structure obey simply “mass action” dynamics which proceed at fixed rates limited only by local concentrations of constituents. Furthermore, the abrupt start/stop kinetics indicate that signals governing the sequences of assembly and contraction are very fast and essentially decouple from the chemistry of network polymerization and force build-up. Small molecules (e.g., Ca<sup>++</sup>) with large diffusivities ( $D \sim 10^{-6}$  cm<sup>2</sup>/s) could easily communicate signals over distances of 10 μm within very short times ( $\sim 1$  s). These special features permit significant reduction in the complexity of physical models for cell motion (Evans, 1993).

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