

# MACROPHAGE DEFORMABILITY AND PHAGOCYTOSIS

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## ABSTRACT

The influence of several metabolic inhibitors and pharmacologic agents on macrophage deformation (induced by fluid shear stress) was examined in relationship to changes in ATP content and phagocytosis of latex beads. Two relatively specific inhibitors of glycolysis (iodoacetate [IA], and sodium fluoride [NaF]) and a sulfhydryl-binding agent (*N*-ethylmaleimide [NEM]) markedly inhibited phagocytosis and reduced cell deformability. A microtubule-disrupting agent (vinblastine) and a highly specific inhibitor of glycolysis (2-deoxyglucose) markedly inhibited phagocytosis without influencing cell deformability. An organomercurial sulfhydryl binding agent *p*-chloromercuribenzene (PCMBS) and a microfilament-disrupting agent (cytochalasin B) inhibited phagocytosis and increased cell deformability. The effects of these agents on phagocytosis and cell deformability bore no consistent relationship to alterations in cellular content of ATP.

The observation that 2-deoxyglucose, the most specific inhibitor of glycolysis examined, reduced ATP content to levels far lower (15% of control values) than those achieved by any other agent examined and inhibited phagocytosis without altering cell deformability, suggests that alterations in cell deformability induced by NaF, IA, NEM, PCMBS, and cytochalasin B are not due to inhibition of glycolysis per se, but instead result from direct or indirect effects of these agents on cell constituents, possibly contractile proteins, which are determinants of cell deformability.

The finding that cytochalasin B, NEM, PCMBS, and IA interfere with phagocytosis and alter cell deformability, together with evidence that these agents interact with isolated actin and myosin, suggests that contractile proteins are important both in phagocytosis and as determinants of cell deformability.

The observation that vinblastine, colchicine, and heavy water (D<sub>2</sub>O) did not alter cell deformability, even though vinblastine caused formation of intracellular crystals of microtubular protein, indicates that microtubules are not major determinants of cell deformability.

The observations that beads adhered normally to surfaces of cytochalasin B- and of PCMBS-treated cells and that shear-stress-induced deformation was increased whereas phagocytosis was markedly inhibited, suggest that deformation of cells around beads associated with ingestion depends on some form of cellular (contractile?) activity, whereas deformation of cells by fluid shear stress is a passive phenomenon.

**KEY WORDS** macrophage · phagocytosis · deformability · electron microscopy · metabolism

The ability of macrophages to spread on surfaces, to insinuate themselves through small openings in blood vessels, and to wrap around particles during phagocytosis depends on their ability to undergo marked shape change, i.e., to deform. It has long been recognized that changes in cell shape associated with such activities are accompanied by changes in cytoplasmic consistency, i.e., "sol-gel" transformation (15, 19), and more recent studies indicate that these changes in cytoplasmic consistency or viscosity reflect the physical state and association of contractile proteins which in turn are modulated by energy metabolism, cations, and temperature (10, 11, 26, 27, 34, 38, 39). Contractile proteins may therefore not only provide for cell contractility, but, in addition, the physical state of contractile proteins, i.e., extent of polymerization and interaction of actin and myosin and related proteins, may determine the physical properties of cells (27) and their capacity to passively deform around an object or change shape in response to external forces. While numerous studies have considered metabolic aspects of ingestion by phagocytes (6, 11, 21, 24, 33), very few investigators have examined the role of cellular deformability in phagocyte function (16, 17, 22).

Previous metabolic studies with inhibitors of anaerobic glycolysis indicate that the ingestion phase of phagocytosis is an energy-dependent process (1, 14, 24, 33). Yet, it is not clear whether depressed phagocytosis under these conditions is, in fact, a direct consequence of compromised energy metabolism or whether both phenomena are unrelated manifestations of cell injury. For example, iodoacetate (IA) is known to react with numerous protein sulfhydryl groups in cells (5, 29, 44), and it is conceivable that in addition to inhibiting glycolytic enzymes and limiting energy available for contractile activity, this reagent might interact with other membrane and/or cytoplasmic protein sulfhydryl groups altering cytoplasmic consistency and the ability of cells to deform in a passive manner.

The purpose of this study was to investigate the effects of selected metabolic inhibitors on macrophage deformability and to examine the interrelationships between cell deformability, phagocytosis, and ATP content of macrophages. A method

was devised for evaluating deformability of macrophages by subjecting them to a deforming fluid shear stress in a concentric cylinder viscometer. A new method was also devised for quantifying phagocytosis in electron micrographs. Marked changes in macrophage deformability were induced by several metabolic inhibitors; these alterations (both increased and decreased deformability) were consistently associated with impaired phagocytosis. Two agents (vinblastine and 2-deoxyglucose) had no effect on cell deformability but markedly impaired phagocytosis. No consistent relationship was observed between cell deformability, phagocytosis, and ATP content.

## MATERIALS AND METHODS

### *Animals*

Male guinea pigs ~500 g in weight were obtained from Eldridge Laboratory Animals (Barnhart, Mo.).

### *Chemicals*

The sources of reagents used in the experiments are listed below: sodium fluoride (NaF), potassium cyanide (KCN), and 2,4-dinitrophenol (2,4-DNP) from Fisher Scientific Co. (Fair Lawn, N. J.); IA as the sodium salt, *p*-chloromercuribenzenesulfonate (PCMBs), 2-deoxy-D-glucose (2DG), dithiothreitol (DTT), heavy water (D<sub>2</sub>O), and *N*-ethylmaleimide (NEM) from Sigma Chemical Co. (St. Louis, Mo.); vinblastine sulfate from Eli Lilly and Co. (Indianapolis, Ind.); cytochalasin B from Imperial Chemical Industries (Macclesfield, Cheshire, England); dimethyl sulfoxide (DMSO), trypan blue and triolein from Matheson, Coleman and Bell (Norwood, Ohio); and thioglycollate from Difco Laboratories (Detroit, Mich.).

### *Preparation of Cells*

Macrophages were obtained from guinea pig peritoneal exudates induced by triolein and thioglycollate (0.04 ml and 0.02 ml/gm body weight, respectively). 4 days after injection, the animals were exsanguinated and the peritoneal exudate was harvested in 100 ml of cold (4°C) phosphate-buffered saline (PBS) (136 mM NaCl, 4.9 mM KCl, 2 mM NaH<sub>2</sub>PO<sub>4</sub>, 13.1 mM Na<sub>2</sub>HPO<sub>4</sub>), 280 mosmol, pH 7.4, containing 4 mM sodium citrate as a chelating agent. (All PBS solutions contained 10 mM glucose, unless otherwise noted.) The cell suspensions were washed twice in the citrated medium, placed on a Ficoll-Hypaque gradient as described by Boyum (3), and centrifuged at 400 *g* for 10 min to remove red cells and polymorphonuclear leukocytes. The interface was removed, washed again, and suspended in PBS containing 0.5 mM Ca<sup>++</sup> and 0.5 mM Mg<sup>++</sup>. The final cell suspension contained 80% ± 5 (SD) macrophages, 14% ± 7 polymorphonuclear leukocytes, and 6% ± 4 lymphocytes.

## Concentric Cylinder Viscometer

The concentric cylinder (Couette) viscometer (Fig. 1) used to deform macrophages was constructed by the School of Engineering at Washington University (St. Louis, Mo.), where it is employed to study the effects of fluid shear stress on erythrocytes (17, 20, 36, 37). It consists of a stationary clear-glass outer cylinder and a rotating stainless steel inner cylinder 10.0 cm in diam and 5.0 cm high. The inner cylinder has a hollow core through which liquid from a circulating water bath (Forma Scientific, Inc., Marietta, Ohio) circulates for temperature control. The gap between the two cylinders is 0.206 cm. The temperature is monitored by thermistor probes (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted at the top and bottom of the gap and is maintained at 37°C during all experiments.

While the inner cylinder is slowly rotating, the gap is filled from below with cells suspended in buffer; fluid in both end regions is then removed and replaced by air. A few cells (<5%) in menisci at the end regions of the gap are probably not subjected to shear stress.

Fluid shear stress within the gap is produced by rotating the inner cylinder. Before the experiments, shear stress is determined by performing a torque measurement on the outer cylinder (40).

The angular speed of the inner cylinder is determined by reading on a digital voltmeter the output of a tachometer generator connected to an electric motor (Dynamatic, Kenosha, Wis.). The motor has a range of 65–3,300 rpm and is adjusted to give the desired shear stress.

## Deformation of Macrophages

To a 1.0-ml suspension of 40,000 cells per mm<sup>3</sup> in

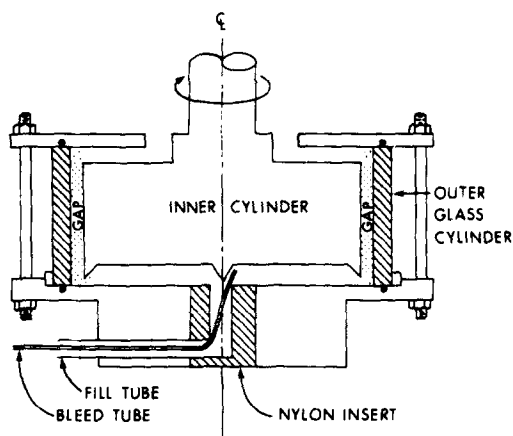


FIGURE 1 Schematic diagram of the viscometer. Cell suspensions are added through the fill tube and chased with air to confine the cells to the gap region. Glutaraldehyde is added from above during rotation to preserve the cells in their sheared shape. Cells are then recovered through the bleed tube.

siliconized test tubes, an equal volume of PBS or PBS containing a metabolic inhibitor was added. The cells were then incubated at 37°C in a rotating constant temperature device for 30 min (unless otherwise stated). After this, a 0.8-ml aliquot was extracted with perchloric acid for ATP analysis and kept on ice. The remaining cells were pelleted, resuspended in 0.4 ml of PBS, and added to 40 ml of PBS containing the appropriate inhibitor and 5.6–6.0% polyvinylpyrrolidone (mol wt 360,000, Sigma Chemical Co. St. Louis, Mo.) to give a relative viscosity of 50–60 centipoise. (This high relative viscosity was essential to deform white cells by fluid shear stress.)

Cells in high viscosity PBS were then placed in the concentric cylinder viscometer. Excess fluid was removed from the end regions, and the cells were subjected to a fluid shear stress of 1,000 dyn/cm<sup>2</sup> (~775 rpm) for 5 min at 37°C.

Experiments were terminated by adding 2.0 ml of 4% glutaraldehyde in PBS (containing red food dye) to the gap from above while the cells were still subjected to shear stress. To achieve rapid mixing of glutaraldehyde with the viscous suspension, it was necessary to increase the rate of rotation to ~925 rpm (~1,500 dyn/cm<sup>2</sup>) for ~2 s. Fixation was continued for 2 min at 1,000 dyn/cm<sup>2</sup>. Under these conditions, control macrophages developed a uniaxially elongated shape which was preserved by fixation.<sup>1</sup>

After fixation, macrophages were removed from the gap, washed in distilled water three times, settled on glass slides, and dried by warm air from a hair dryer. They were stained with Feulgen-light green and observed with a microscope. From photomicrographs of cells at a final magnification of × 1065, deformability was quantified by measuring the greatest width and, perpendicular to this, the greatest length of individual cells using a computerized planimeter (Hewlett-Packard model 9100B Calculator, 9107A digitizer, and 9101 extended memory, Hewlett-Packard Co., McMinnville, Oreg.).

An index of deformability was obtained by computing the mean and standard deviation of length-to-width ratios for 75 cells from each experiment. No correction was made for the <5% of cells in end-region menisci not subjected to shear stress. Significance was determined by the *t* test (31).

## Phagocytosis

Macrophages were suspended at a concentration of 10,000 cells/mm<sup>2</sup>. To 0.8 ml of suspension in siliconized test tubes, 0.1 ml of PBS or 0.1 ml of PBS containing an inhibitor was added. After incubation at 37.5° in a rotat-

<sup>1</sup> In preliminary experiments, it was found that deformation of cells briefly subjected (up to 1 min) to 1,500 dyn/cm<sup>2</sup> of shear stress was comparable to that of cells subjected to 1,000 dyn/cm<sup>2</sup> for 5 min. On the other hand, when cells were subjected to 1,500 dyn/cm<sup>2</sup> for 5 min, a high percentage underwent fragmentation.

ing constant temperature device, 0.05 ml of a 3.3% (wt/vol) suspension of 0.5- $\mu\text{m}$  polystyrene latex beads (Dow Chemical Co., Midland, Mich.) in 0.9% saline was added, to provide 3,250 beads per cell. After a 6-min incubation, cells were fixed overnight (~18 h) by addition of 1 ml of 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4), and postfixed for 30 min in 1% osmium tetroxide in cacodylate buffer. After three washes in 0.1 M sodium acetate, the cells were stained with uranyl acetate (30) and suspended in 70% ethanol. They were then drawn into capillary tubes and pelleted by centrifugation. The pellets were removed, serially dehydrated in graded ethanol, and embedded in Epon 812. Silver sections of blocks were cut on a Porter-Blum MT2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) with a diamond knife, mounted on copper grids, stained with uranyl acetate (4) and lead citrate (28), and examined with a Philips EM 200 electron microscope. Photographs of cross sections of 20 cells were enlarged 2.5 times from the negative, to give a final magnification of approx.  $\times 7235$ .

Adsorption and phagocytosis of beads were assessed by counting the number of beads adsorbed to the plasma

membrane and the number of intracytoplasmic beads in each cell cross section (Fig. 2). The cross-sectioned circumference and cytoplasmic area (less nucleus) of individual cells were subsequently measured by tracing the cell boundaries with a computerized planimeter. Data were expressed as number of beads adsorbed per micron of cell circumference and numbers of beads ingested per square micron of cell cytoplasm.

#### ATP Analysis

Perchloric acid cell extracts were neutralized with 2 M  $\text{KHCO}_3$  and preserved at  $-60^\circ\text{C}$  until measurements were performed. Adenosine triphosphate was quantified by the fluorometric hexokinase-glucose-6-phosphate dehydrogenase method (9). Measurements were made in a Farrand model A3 fluorometer (Farrand Optical Co., Inc., Valhalla, N. Y.).

Cell protein was measured by the technique of Lowry et al. (18), and ATP content relative to total protein was determined. Changes in ATP content after treatment with metabolic inhibitors were expressed as a percentage of control values.

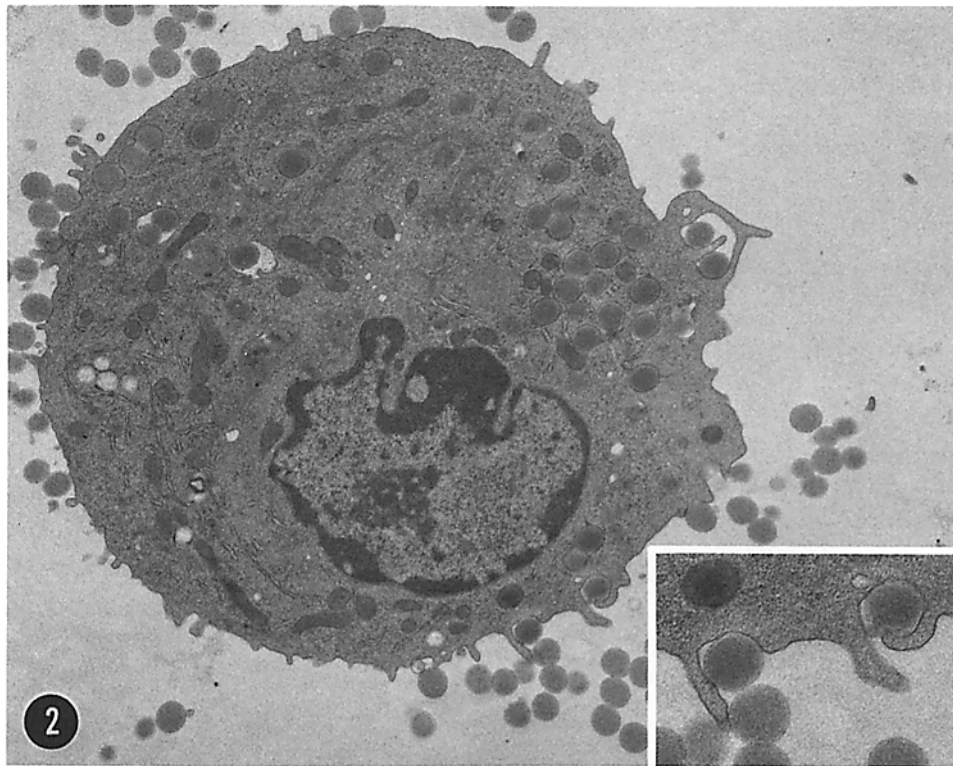


FIGURE 2 Micrograph of a typical macrophage after exposure to 0.5- $\mu\text{m}$  latex beads for 6 min. Many beads are either adherent to the membrane or have been ingested. The circumference and area of the cross section can be measured with the computerized planimeter to quantify adsorption and ingestion of beads (see text for details).  $\times 7,235$ . Inset  $\times 16,640$ .

### Viability

Viability of shear-stressed macrophages was assessed by exclusion of trypan blue and by testing their ability to phagocytose. The ability to exclude an impermeable dye, trypan blue, was tested by incubating 0.5 ml of a cell suspension with 0.1 ml of 0.4% trypan blue for 10 min at 37°C. After incubation, 100 cells were examined under a microscope for evidence of dye uptake.

Ability to phagocytose was checked by incubating 0.9 ml of sheared (unfixed) cells with 0.05 ml of 3.3% suspension of 0.5- $\mu\text{m}$  polystyrene latex beads for 10 min. The cells were then fixed in glutaraldehyde and processed for electron microscope evaluation. The ability to ingest beads and the preservation of cellular integrity and ultrastructure were verified with the electron microscope.

## RESULTS

### *Effects of Fluid Shear Stress on Macrophages*

Suspension of cells in a high viscosity medium was necessary to deform macrophages. Cells suspended in PBS alone tended to fragment as they started to deform (at  $\sim 3,000$  dyn/cm<sup>2</sup> for 40 s). In polyvinylpyrrolidone (PVP)-PBS, cells underwent deformation at  $\sim 1,000$  dyn/cm<sup>2</sup> and no appreciable fragmentation was observed up to shear stresses of  $\sim 1,500$  dyn/cm<sup>2</sup>.

The viability of macrophages subjected to 1,000 dyn/cm<sup>2</sup> fluid shear stress for 5 min, as assessed by the trypan blue exclusion technique, did not differ from that of control cells, e.g., 95% of cells ex-

cluded the dye. Sheared macrophages also retained their phagocytic capacity as judged by their ability to ingest beads. In addition, no morphologic alterations were evident in these cells by electron microscopy.

When sheared in PVP-PBS, control macrophages showed a consistent uniaxial elongation (Fig. 3). The mean length-to-width ratio varied from  $1.520 \pm 0.376$  SD to  $1.858 \pm 0.678$  and always showed a highly significant increase ( $P < 0.001$ ) over nonsheared controls ( $1.111 \pm 0.143$  to  $1.187 \pm 0.250$ ).

A histogram plot of ratios for individual cells from a typical experiment at 1,000 dyn/cm<sup>2</sup> for 5 min at 37°C is shown in Fig. 4. Most nonsheared cells ( $\sim 90\%$ ) were roughly spherical with ratios of  $< 1.30$ . On the other hand, length-to-width ratios of cells subjected to shear stress varied between 1.0 and 3.0, with  $\sim 280\%$  of cells having ratios in excess of 1.3. Thus,  $\sim 20\%$  of cells resist deformation under the conditions of these experiments.

The amount of shear-induced deformation is, then, the difference between the mean ratios of sheared cells versus nonsheared cells (sheared cells - nonsheared cells).

### *Deformation in the Viscometer*

Three known inhibitors of glycolysis were examined for their effects on macrophage deformability (Table I). NaF caused a significant decrease in deformability at all concentrations used (Fig. 5a). This decreased deformability was dose related,

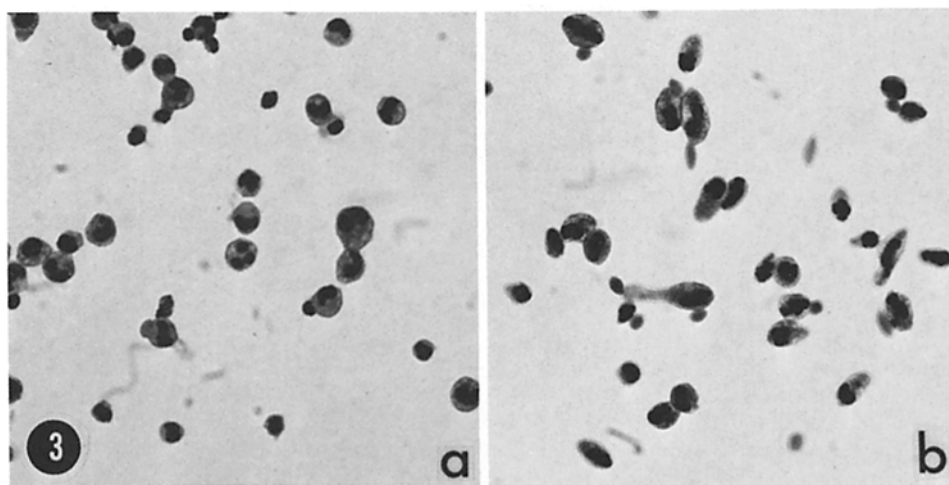


FIGURE 3 Light micrographs of macrophages.  $\times 426$ . (a) Normal nonsheared cells with an almost spherical shape. (b) Macrophages sheared at 1,000 dyn/cm<sup>2</sup> for 5 min at 37°C demonstrate typical elongation. Note that occasional red cells exhibit comparable shape changes.

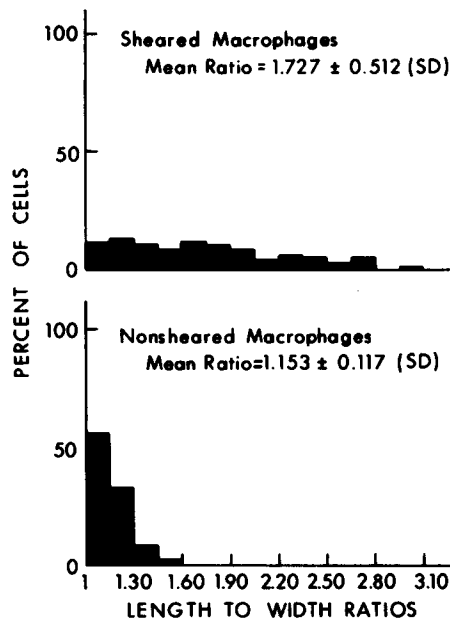


FIGURE 4 Distribution of cell length-to-width ratios from a representative experiment in which macrophages were subjected to 1,000 dyn/cm<sup>2</sup> fluid shear stress for 5 min at 37°C.

with the cells becoming less deformable as the duration of incubation and the concentration of NaF were increased. For example, there was a significant difference ( $t = 6.473$ ,  $P < 0.001$ ) in deformability of cells incubated in 20 mM NaF for 30 min vs. 20 mM for 60 min ( $1.421 \pm 0.455$  SD and  $1.173 \pm 0.161$ , respectively); there was also a significant decrease ( $t = 5.079$ ,  $P < 0.001$ ) in deformability of cells incubated in 5 mM NaF for 60 min vs. 10 mM NaF for 60 min ( $1.329 \pm 0.340$  and  $1.173 \pm 0.161$ , respectively). At 0.5 mM and 1.0 mM IA, cells retained an almost spherical shape after shearing. Cells incubated with 2-deoxyglucose at concentrations ranging from 5 mM 2DG:5 mM glucose to 10 mM 2DG:0 mM glucose for 30 min demonstrated no change in deformability from controls.

Two sulfhydryl-binding reagents were tested for effects on macrophage deformability during fluid shear stress (Table II). NEM profoundly decreased deformability equally at all concentrations used, i.e., the cells remained virtually spherical, identical to nonsheared control cells, after shearing (Fig. 5*b*). Incubation of cells in 0.01 mM PCMBs (an organomercurial) for 30 min did not

TABLE I  
Effect of Inhibitors of Glycolysis on Macrophage Deformability and ATP Content

|                        | Deformation               |                   |                       |
|------------------------|---------------------------|-------------------|-----------------------|
|                        | Inhibitor                 | Control*          | ATP                   |
| Na                     |                           |                   |                       |
| 20 mM, 30 min          | $1.421 \pm 0.455$ ‡§ (4)¶ | $1.694 \pm 0.579$ | $48.9 \pm 5.94$ ¶ (2) |
| 5 mM, 60 min           | $1.329 \pm 0.340$ § (2)   | $1.716 \pm 0.665$ | $31.9 \pm 0.57$ (2)   |
| 10 mM, 60 min          | $1.213 \pm 0.247$ § (2)   | "                 | $51.9 \pm 8.49$ (2)   |
| 20 mM, 60 min          | $1.173 \pm 0.161$ § (2)   | "                 | $66.1 \pm 13.65$ (2)  |
| Ia, 30 min             |                           |                   |                       |
| 0.1 mM                 | $1.540 \pm 0.484$ § (4)   | $1.705 \pm 0.562$ | $29.4 \pm 3.12$ (4)   |
| 0.5 mM                 | $1.265 \pm 0.264$ § (4)   | $1.695 \pm 0.572$ | $32.7 \pm 9.00$ (3)   |
| 1.0 mM                 | $1.246 \pm 0.219$ § (5)   | $1.689 \pm 0.594$ | $42.0 \pm 11.47$ (4)  |
| 2-deoxyglucose, 30 min |                           |                   |                       |
| 5 mM 2DG:5 mM Glu      | $1.475 \pm 0.441$ ** (3)  | $1.515 \pm 0.399$ | $56.8 \pm 3.30$ (3)   |
| 9 mM 2DG:1 mM Glu      | $1.453 \pm 0.366$ ** (3)  | $1.498 \pm 0.330$ | $22.5 \pm 2.33$ (2)   |
| 10 mM 2DG:0 mM Glu     | $1.567 \pm 0.491$ ** (2)  | $1.550 \pm 0.451$ | $14.4 \pm 5.16$ (2)   |
| 0 mM 2DG:0 mM Glu      | $1.602 \pm 0.493$ ** (2)  | "                 | $69.3 \pm 4.38$ (2)   |
| 0 mM 2DG:1 mM Glu      | $1.541 \pm 0.412$ ** (2)  | $1.534 \pm 0.447$ | $90.5 \pm 0.50$ (2)   |

\* Controls were incubated in buffer alone before shearing.

‡ Composite mean and standard deviation of length-to-width ratios of cells subjected to fluid shear stress of 1,000 dyn/cm<sup>2</sup> for 5 min at 37°C.

§ Probability that the difference observed was due to chance alone is  $P < 0.001$ .

¶ Number of experiments performed. 75 cells for each experiment were measured.

¶ Mean  $\pm$  standard deviation of ATP content of inhibitor-treated cells expressed as percentage of controls. Number of measurements in parentheses.

\*\* Probability that the difference observed was due to chance alone is  $P > 0.1$ .

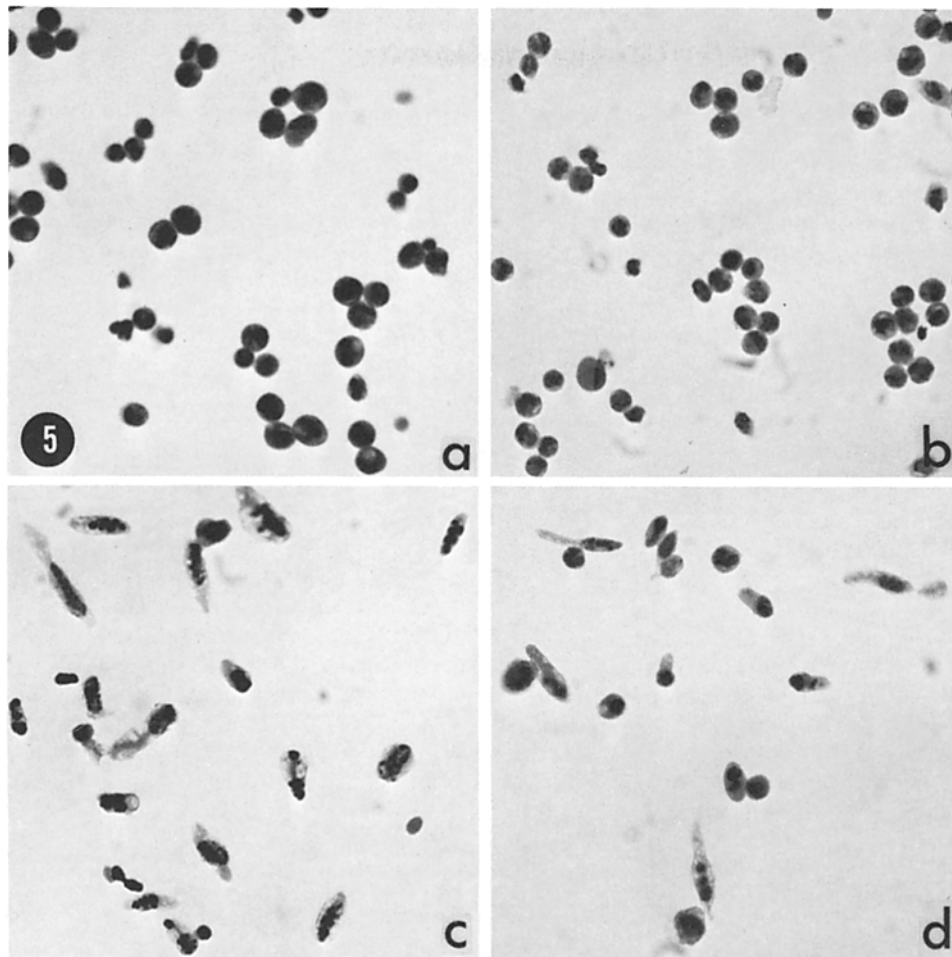


FIGURE 5 Light micrographs of macrophages treated with metabolic inhibitors before shearing.  $\times 426$ . (a) NaF, 20 mM for 30 min. These cells remained almost spherical after shearing. (b) NEM, 0.1 mM for 30 min. These cells, too, are undeformed. (c) PCMBS, 0.1 mM for 30 min. Besides a marked increase in deformability, these cells consistently demonstrated nuclear and cytoplasmic swelling. (d) Cytochalasin B, 10  $\mu\text{g}/\text{ml}$  for 30 min. These cells were elongated to a greater extent than normal.

affect their deformability. However, incubations in 0.1 and 1.0 mM PCMBS for 30 min both resulted in increased deformability (Fig. 5c). PCMBS is reputed to penetrate cells slowly (13, 41), but, in order to minimize intracellular penetration of the reagent still further, cells were incubated in 0.1 mM PCMBS for only 10 min. Even with this shorter exposure to the agent, deformability was significantly increased. Distinct morphologic changes were evident in PCMBS cells examined by light microscopy (Fig. 5c); the intensity of cytoplasmic staining was decreased and nuclei appeared swollen.

Cytochalasin B at a concentration of 10  $\mu\text{g}/\text{ml}$

for 30 min was the only other reagent tested which increased cell deformability (Table III, Fig. 5d). At 1  $\mu\text{g}/\text{ml}$ , cytochalasin B was without effect. There was no significant difference ( $t = 1.613$ ,  $P > 0.1$ ) in deformability ratios of control cells incubated with or without 1% DMSO.

Colchicine, a microtubule-disrupting agent, was evaluated at  $10^{-4}$  and at  $10^{-6}$  M in a single experiment; deformability was not significantly changed at either concentration ( $t = 1.789$ ,  $P > 0.05$  at  $10^{-4}$  M), although ATP content was appreciably reduced at the higher concentration. To obtain independent morphologic evidence of an effect on microtubules, cells were incubated with vinblas-

TABLE II  
Effects of Sulfhydryl-Binding Reagents on Macrophage Deformability and ATP Content

|                 | Deformation          |               |                   |
|-----------------|----------------------|---------------|-------------------|
|                 | Reagent              | Control*      | ATP               |
| <b>NEM</b>      |                      |               |                   |
| 0.1 mM, 30 min  | 1.191 ± 0.166‡§ (2)¶ | 1.695 ± 0.546 | 109.1 ± 7.42¶ (2) |
| 0.5 mM, 30 min  | 1.138 ± 0.156§ (3)   | 1.668 ± 0.551 | 85.6 ± 8.49 (2)   |
| 1.0 mM, 30 min  | 1.152 ± 0.146§ (4)   | 1.698 ± 0.604 | 101.6 ± 1.56 (2)  |
| <b>PCMBS</b>    |                      |               |                   |
| 0.01 mM, 30 min | 1.776 ± 0.638** (2)  | 1.719 ± 0.547 | 107.6 ± 9.97 (2)  |
| 0.10 mM, 30 min | 2.132 ± 0.841§ (3)   | 1.662 ± 0.540 | 106.6 ± 7.05 (3)  |
| 1.00 mM, 30 min | 1.985 ± 0.691§ (2)   | 1.585 ± 0.405 | 105.9 ± 7.78 (3)  |
| 0.10 mM, 30 min | 1.938 ± 0.620§ (2)   | "             | 118.6 ± 12.09 (2) |

\* Control values are for 30-min incubations in buffer before shearing.

‡ Composite mean and standard deviations of length-to-width ratios of cells subjected to a fluid shear stress of 1,000 dyn/cm<sup>2</sup> for 5 min at 37°C.

§ Probability that the difference observed was due to chance alone is  $P < 0.001$ .

¶ Number of experiments performed. 75 cells for each experiment were measured.

¶ Mean and standard deviation of ATP content of inhibitor-treated cells expressed as percentage of controls.

\*\* Probability that the difference observed was due to chance alone is  $P > 0.1$ .

tine at 10<sup>-5</sup> M for 60 min, and an aliquot was taken for electron microscope examination. Typical vinblastine-induced crystalline structures (2) associated with microtubule disruption and precipitation of microtubular protein were readily found in cells examined by electron microscopy (Fig. 6), but there was no change in deformability (Table III). D<sub>2</sub>O, which is reported to stabilize microtubules, likewise had no effect on deformability.

Two inhibitors of oxidative metabolism, KCN and 2,4-DNP, were without effect on deformability at the concentrations used, and the reducing agent DTT had no effect at 2 mM (Table IV).

#### ATP Content

ATP content of control cells from 14 experiments was 6.38 ± 0.65 (SD) μ mol of ATP/g cell protein. In Tables I-IV, ATP content of cells is expressed as a percent of control values in order to facilitate direct comparison of data from different experiments with slightly different control values.

All three inhibitors of glycolysis depressed ATP levels relative to controls (Table I). However, increasing concentrations of NaF and IA were associated with increasing levels of ATP (and decreased deformability). The decrease in ATP content by IA was most marked at 0.1 mM (compared to ATP levels with 10 mM IA,  $t = 2.12$ ,  $P < 0.05$ ), the lowest dose level employed, whereas deformability was decreased at 1.0 mM much more than at 0.1 mM IA. Depression of ATP by

2DG was also dose dependent, with 100 mM 2DG:0 mM glucose yielding the lowest relative ATP content of any inhibitor assayed.

Neither NEM nor PCMBS caused any consistent change in ATP content from control values (Table II), although ATP levels tended to increase slightly with PCMBS.

All reagents considered to interact with either microfilaments or microtubules (cytochalasin B, colchicine, vinblastine, and heavy water) generally produced small-to-moderate decreases in ATP content (Table III). ATP content of cytochalasin B-treated cells was expressed as percent of DMSO controls. (There was no difference between DMSO controls and controls in buffer only.)

Inhibitors of oxidative metabolism, KCN and 2,4-DNP, and the reducing agent DTT caused slight-to-moderate decreases in ATP content (Table IV).

#### Phagocytosis

Data from representative experiments documenting effects of metabolic inhibitors on adsorption and ingestion of 0.5-μm polystyrene latex beads are shown in Tables V-VII. NaF markedly decreased bead ingestion at all concentrations used, without affecting bead adhesion (Table V). IA decreased ingestion by ~50% at 0.1 mM and almost completely inhibited ingestion at 0.5 mM (Table V). Although adhesion of latex beads at 0.1 mM IA was significantly increased over that of



controls, this effect was not observed in a second experiment (not shown), nor was it evident in incubations at 0.5 mM IA. 2DG caused a dose-dependent decrease in phagocytosis, with a significant decrease of ~50% at 9 mM 2DG:1 mM glucose (Table V); 10 mM 2DG:0 mM glucose

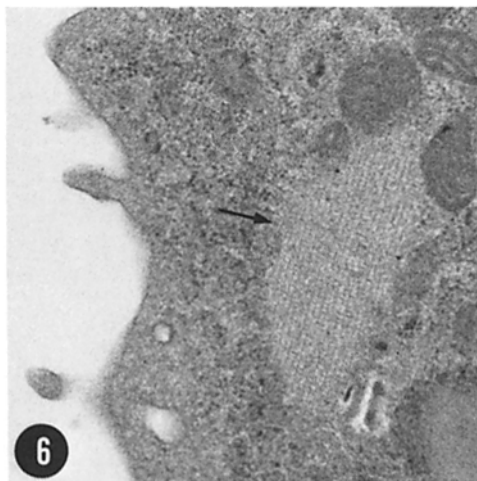


FIGURE 6 This electron micrograph of a portion of a vinblastine-treated macrophage demonstrates a typical crystalline structure (arrow) associated with microtubule disruption by the agent. Many such structures were present in the cells.  $\times 31,600$ .

further depressed ingestion to ~25% of the control value. However, even this concentration of 2DG did not diminish ingestion as effectively as the higher concentrations of NaF and IA. Adhesion was not affected by 2DG.

NEM markedly depressed ingestion of beads even at the lowest concentration used (0.1 mM, Table VI). There was an increase in bead adhesion in this experiment, but this was not confirmed in other experiments (not shown). PCMBS at 0.01 mM for 30 min and at 0.1 mM for 10 min depressed ingestion of beads to ~50% of control values; 0.1 mM PCMBS for 30 min further decreased ingestion to 16% of the control value (Table VI). PCMBS had no effect on bead adhesion at any concentration used. During electron microscope evaluation of phagocytosis, degenerative changes, i.e., cytoplasmic and nuclear swelling, were noted only in PCMBS-treated macrophages (Fig. 7). This swelling was documented by an increase in cell area after incubation with 0.1 and 1.0 mM PCMBS for 30 min. The mean area of 40 control cells was  $31.92 \pm 8.33 \mu\text{m}^2$  ( $\bar{x} \pm \text{SD}$ ). After PCMBS treatment at 1.0 mM for 30 min, the area was  $39.88 \pm 11.59 \mu\text{m}^2$  ( $t = 3.520$ ,  $P < 0.001$ ), and with 0.1 mM for 30 min it was  $40.13 \pm 15.42 \mu\text{m}^2$  ( $t = 2.964$ ,  $P < 0.005$ ). PCMBS at 0.01 mM for 30 min and at 0.1 mM for

TABLE III  
Effects of Agents which Interact with Microfilaments and Microtubules on Cell Deformability and ATP Content

|                                     | Deformation                                 |                   |                             |
|-------------------------------------|---|-------------------|-----------------------------|
|                                     | Reagent                                     | Control*          | ATP                         |
| Cytochalasin B                      |   |                   |                             |
| 1 $\mu\text{g}/\text{ml}$ , 30 min  | $1.673 \pm 0.701 \ddagger \S (5) \parallel$ | $1.709 \pm 0.512$ | $85.0 \pm 19.05 \nabla (4)$ |
| 10 $\mu\text{g}/\text{ml}$ , 30 min | $1.907 \pm 0.712^{**} (5)$                  | "                 | $75.0 \pm 17.34 (4)$        |
| Vinblastine                         |   |                   |                             |
| $10^{-5}$ M, 60 min                 | $1.635 \pm 0.575 \S (2)$                    | $1.716 \pm 0.665$ | $81.5 \pm 16.90 (2)$        |
| Colchicine                          |   |                   |                             |
| $10^{-6}$ M, 60 min                 | $1.661 \pm 0.559 \S (1)$                    | "                 | $83.4 (1)$                  |
| $10^{-4}$ M, 60 min                 | $1.563 \pm 0.460 \S (1)$                    | "                 | $60.2 (1)$                  |
| D <sub>2</sub> O                    |   |                   |                             |
| 100%, 60 min                        | $1.800 \pm 0.627 \S (2)$                    | "                 | $75.9 \pm 9.90 (2)$         |

\* Controls were incubated in buffer alone before shearing, except in cytochalasin B experiments in which the buffer contained 1% DMSO.

‡ Composite mean and standard deviation of length-to-width ratios of cells subjected to a fluid shear stress of 1,000 dyn/cm<sup>2</sup> for 5 min at 37°C.

§ Probability that the difference observed was due to chance alone is  $P > 0.1$ .

|| Number of experiments performed. 75 cells for each experiment were measured.

∇ Mean and standard deviation of ATP content of inhibitor-treated cells expressed as a percentage of controls. For cytochalasin B the controls contained 1% DMSO. Number of measurements performed in parentheses.

\*\* Probability that the difference observed was due to chance alone is  $P < 0.001$ .

TABLE IV  
Effects of a Reducing Agent and Inhibitors of Oxidative Metabolism on Macrophage Deformability and ATP Content

|                | Deformation          |               | ATP              |
|----------------|----------------------|---------------|------------------|
|                | Reagent              | Control*      |                  |
| DTT            |                      |               |                  |
| 2 mM, 30 min   | 1.570 ± 0.415‡§ (2)¶ | 1.558 ± 0.424 | 79.2 ± 9.55¶ (2) |
| KCN            |                      |               |                  |
| 1.0 mM, 30 min | 1.500 ± 0.454§ (2)   | "             | 80.1 ± 0.35 (2)  |
| 2,4-DNP        |                      |               |                  |
| 0.1 mM, 30 min | 1.485 ± 0.570§ (2)   | "             | 65.4 ± 3.46 (2)  |

\* Control values are for 30-min incubations in buffer before shearing.

‡ Composite mean and standard deviation of length-to-width ratios of cells subjected to a fluid shear stress of 1,000 dyn/cm<sup>2</sup> for 5 min at 37°C.

§ Not significantly different ( $P > 0.1$ ) from control values.

¶ Number of experiments performed. 75 cells for each experiment were measured.

¶ Mean and standard deviation of ATP content of inhibitor-treated cells expressed as percentage of controls. Number of measurements in parentheses.

TABLE V  
Phagocytosis and Adhesion of Beads\* by Macrophages after Treatment with Inhibitors of Glycolysis

| Inhibitor              | Adhesion       | $P$ ‡  | Ingestion      | $P$    |
|------------------------|----------------|--------|----------------|--------|
| NaF                    |                |        |                |        |
| Control§               | 0.238 ± 0.166¶ |        | 0.921 ± 0.482¶ |        |
| 20 mM, 30 min          | 0.334 ± 0.153  | >0.1   | 0.124 ± 0.153  | <0.001 |
| Control                | 0.174 ± 0.097  |        | 0.639 ± 0.457  |        |
| 5 mM, 60 min           | 0.133 ± 0.067  | >0.1   | 0.024 ± 0.059  | <0.001 |
| 20 mM, 60 min          | 0.174 ± 0.103  | >0.1   | 0.000 ± 0.000  | <0.001 |
| Ia, 30 min             |                |        |                |        |
| Control                | 0.238 ± 0.166  |        | 0.921 ± 0.482  |        |
| 0.1 mM                 | 0.421 ± 0.202  | <0.005 | 0.456 ± 0.455  | <0.005 |
| 0.5 mM                 | 0.302 ± 0.182  | >0.1   | 0.074 ± 0.142  | <0.001 |
| 2-deoxyglucose, 30 min |                |        |                |        |
| Control                | 0.174 ± 0.097  |        | 0.639 ± 0.457  |        |
| 5 mM 2DG:5 mM Glu      | 0.173 ± 0.148  | >0.1   | 0.498 ± 0.399  | >0.1   |
| 9 mM 2DG:1 mM Glu      | 0.244 ± 0.137  | >0.1   | 0.303 ± 0.288  | <0.01  |
| 10 mM 2DG:0 mM Glu     | 0.190 ± 0.123  | >0.1   | 0.176 ± 0.208  | <0.001 |

\* After incubation with inhibitors for the time shown in the table, cells were allowed to ingest 0.5- $\mu$ m beads for 6 min (see text for further details).

‡ Probability that the difference observed is due to chance alone.

§ All controls are for 30-min incubations in buffer before phagocytosis.

¶ Mean and standard deviation of the number of beads adsorbed per micron of cell circumference for 20 cells.

¶ Mean and standard deviation of the number of beads ingested per square micron of cell cytoplasm for 20 cells.

10 min did not affect mean cell area ( $30.07 \pm 7.67 \mu\text{m}^2$  and  $34.34 \pm 9.86 \mu\text{m}^2$ , respectively).

Cytochalasin B depressed bead ingestion at both 1 and 10  $\mu\text{g}/\text{ml}$  for 30 min (Table VII). Again, dose dependency was evident, with ingestion of beads reduced to 44% of the control value at 1  $\mu\text{g}/\text{ml}$  and 7% of control values at 10  $\mu\text{g}/\text{ml}$ . Bead adhesion was not affected at these concentrations. DMSO at 1% had no effect on either ingestion or adhesion of beads. Vinblastine at  $10^{-5}$

M for 60 min decreased ingestion to 25% of normal (Table VII); the increased bead adhesion was not observed in a second experiment.

## DISCUSSION

### Methodology

Fixation of macrophages subjected to fluid shear stress in a concentric cylinder viscometer provides a new means for evaluating macrophage

TABLE VI  
Phagocytosis and Adhesion of Beads by Macrophages after Treatment with Sulfhydryl-Binding Reagents

| Inhibitor       | Adhesion       | P*    | Ingestion     | P      |
|-----------------|----------------|-------|---------------|--------|
| <b>NEM</b>      |                |       |               |        |
| Control‡        | 0.238 ± 0.166§ |       | 0.921 ± 0.482 |        |
| 0.1 mM, 30 min  | 0.420 ± 0.250  | <0.01 | 0.003 ± 0.009 | <0.001 |
| <b>PCMBS</b>    |                |       |               |        |
| Control         | 0.174 ± 0.097  |       | 0.639 ± 0.457 |        |
| 0.01 mM, 30 min | 0.172 ± 0.121  | >0.1  | 0.315 ± 0.285 | <0.025 |
| 0.1 mM, 10 min  | 0.230 ± 0.119  | >0.1  | 0.317 ± 0.327 | <0.025 |
| 0.1 mM, 30 min  | 0.202 ± 0.116  | >0.1  | 0.101 ± 0.135 | <0.001 |

\* Probability that the difference observed is due to chance alone.

‡ All controls are for 30-min incubations in buffer before phagocytosis.

§ Mean and standard deviation of the number of beads ingested per micron of cell circumference for 20 cells.

|| Mean and standard deviation of the number of beads ingested per square micron of cell cytoplasm for 20 cells.

TABLE VII  
Phagocytosis and Adhesion of Beads by Macrophages after Treatment with Cytochalasin B and Vinblastine

| Inhibitor                  | Adhesion       | P*   | Ingestion     | P      |
|----------------------------|----------------|------|---------------|--------|
| <b>Cytochalasin B</b>      |                |      |               |        |
| Control, 30 min‡           | 0.206 ± 0.101§ |      | 0.574 ± 0.395 |        |
| 1 µg/ml, 30 min            | 0.287 ± 0.154  | >0.1 | 0.250 ± 0.237 | <0.005 |
| 10 µg/ml, 30 min           | 0.189 ± 0.110  | >0.1 | 0.041 ± 0.082 | <0.001 |
| 1% DMSO, 30 min            | 0.221 ± 0.143  | >0.1 | 0.380 ± 0.297 | >0.1   |
| <b>Vinblastine</b>         |                |      |               |        |
| Control, 60 min            | 0.178 ± 0.143  |      | 0.571 ± 0.422 |        |
| 10 <sup>-5</sup> M, 60 min | 0.248 ± 0.109  | >0.1 | 0.124 ± 0.143 | <0.001 |

\* Probability that the difference observed is due to chance alone.

‡ All controls were incubated in buffer for 30 min before phagocytosis.

§ Mean and standard deviation of the number of beads ingested per micron of cell circumference for 20 cells.

|| Mean and standard deviation of the number of beads ingested per square micron of cell cytoplasm for 20 cells.

deformability. Previous studies of leukocyte deformability have utilized cell elastimetry on individual polymorphonuclear leukocytes (16, 17, 22). Deformation of cells by fluid shear stress offers several advantages when compared to cell elastimetry. It eliminates contact between deforming apparatus and cells; since both adhesion and contact of cells with foreign surfaces may affect cell deformation, and since both phenomena can be altered by chemical probes, it is desirable to minimize such interactions. In addition, deformation of cells in a viscometer followed by glutaraldehyde fixation permits subsequent staining with positive identification of cell types and morphologic measurements on large numbers of cells, as well as further ultrastructural studies of deformed cells.

Cell deformation induced by fluid shear stress is the consequence of external forces acting on the cell as a whole. The forces and mechanisms involved in this deformation may differ somewhat

from those involved in deformation of cells around small objects of phagocytosis and spreading of cells on surfaces; the latter deformations are more focal in nature and involve forces of interaction only between that portion of the cell in contact with the particle or surface concerned and the particle surface itself. Although we would expect both types of deformation to be influenced by physical properties and functions of cytoplasmic constituents as well as by plasma membrane and subjacent organelles including microfilaments, it would appear (see discussion below) that under the conditions of these experiments shear stress-induced deformation is a passive phenomenon, whereas cell deformation around beads requires active cooperation of the cell.

To correlate deformability with macrophage function, we investigated the effects of metabolic inhibitors on phagocytosis by using a new quantitative, morphologic technique which permits unequivocal differentiation and documentation of in-

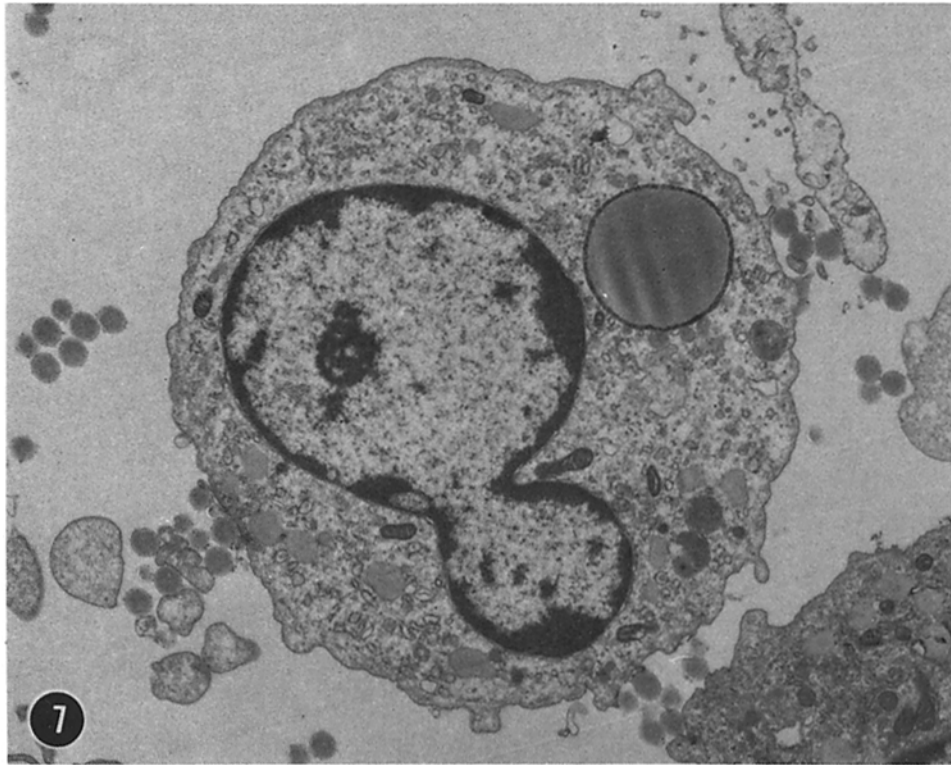


FIGURE 7 Electron micrograph of a macrophage treated with 0.1 mM PCMS for 30 min at 37°C and incubated with 0.5- $\mu$ m beads for 6 min. Both the nucleus and the cytoplasm appear swollen, and no beads have been phagocytosed.  $\times 7,235$ .

gested vs. adherent beads. A potential theoretical concern about the technique is that some beads adsorbed to the cell surface may have the appearance of, and be counted as, ingested beads in tangential sections near the cell surface. However, the observation that macrophages treated with some inhibitors showed virtually no ingestion, while adhesion of beads was not affected, indicates that any such tangential sectioning artifacts are inconsequential.

It can be seen in Tables V, VI, and VII that the coefficients of variation (standard deviation divided by the mean) of the numbers of beads adsorbed and ingested by control cells approached 50%. How much of this variation is due to non-random distribution of (a) beads adsorbed to cell surfaces and of (b) ingested beads inside cells, and how much is due to cell-to-cell variation was not ascertained and would in fact be difficult to determine even with serial-sectioning techniques. By performing bead counts on 20 cells from each group as was done in the present experiments, standard errors of the mean approach 10-15% of

the mean, and ~50% inhibitions of phagocytosis are statistically significant at confidence levels ranging from  $P < 0.025$  to  $P < 0.005$ . Clearly, smaller differences would achieve statistical significance if more cells are counted.

#### Experimental Results

These observations demonstrate that (a) ~80% of normal macrophages are readily deformed, i.e., undergo shape change, when subjected to fluid shear stress, and (b) metabolic inhibitors and pharmacologic agents which impair phagocytosis differ greatly in their effects on cell deformability. It is not clear why ~20% of macrophages resist deformation under the conditions employed. NaF, IA, and NEM markedly reduced cell deformability as well as phagocytosis; cytochalasin B and PCMS increased deformability while inhibiting phagocytosis; and 2-deoxyglucose and vinblastine inhibited phagocytosis without altering deformability characteristics of the cells. While all agents which altered cell deformability interfered with phagocytosis, the converse was not true; both 2-

deoxyglucose and vinblastine inhibited phagocytosis without influencing deformability.

The most specific inhibitor of glycolysis examined, 2-deoxyglucose, reduced ATP content to levels far lower (15% of control values) than those achieved by any other agent examined and inhibited phagocytosis without altering cell deformability. Similarly, colchicine, 2,4-DNP, D<sub>2</sub>O, and KCN reduced ATP content by 40, 35, 25, and 20%, respectively, without altering cell deformability. These observations suggest that alterations in deformability induced by NaF, IA, NEM, PCMBS, and cytochalasin B are not the consequence of decreased glucose transport (46), inhibition of glycolysis, or impaired energy metabolism per se, but rather are due to interaction of these agents with cellular constituents, possibly contractile (and/or associated) proteins, which are direct or indirect determinants of cell deformability. This interpretation is supported by many observations indicating that virtually all of these agents interact with and/or affect the association, the physical properties, and/or the function of isolated contractile proteins, i.e., actin and myosin (1, 25, 32, 37). If phagocytosis also depends on normally functioning contractile proteins as circumstantial evidence indicates (35), interaction of these agents with actin and myosin might be predicted to inhibit phagocytosis (as was observed) as well as alter cell deformability.

Indeed, the effects of these agents on isolated contractile proteins are consistent with the changes in deformability and inhibition of phagocytosis produced in intact cells in the present experiments. Agents such as cytochalasin B and organomercurials which interfere with association of actin and myosin (without blocking ATPase activity) (1, 25) and with polymerization of actin (35) are associated with decreased viscosity of these isolated proteins and, in the present experiments, with increased deformability of intact cells. Agents such as NEM and iodoacetamide which block myosin ATPase activity and ATP-induced disassociation of actomyosin (1, 25) are associated with increased viscosity of these proteins and, in the current experiments, with decreased deformability of intact cells. The tendency for ATP content of intact cells to increase (and for deformability of cells to decrease) with increasing concentrations of NaF and IA in the present experiments (Table I) has not been reported previously to our knowledge, but is consistent with the well-known facts that inhibition of glycolysis by these agents is only

relatively specific even at low concentrations (42, 43) and that inhibition of ATPase activity by NaF is dose dependent (12). Although NaF is known to interfere with the activity of many enzymes, including Na-K-ATPases (8) and "nonspecific" ATPases (12), it is not clear whether it interacts with and affects association of contractile proteins in the same way as IA and NEM.

While alterations in deformability induced by IA, NaF, NEM, PCMBS, and cytochalasin B attest to direct or indirect interaction of these agents with contractile proteins in intact cells, failure of 2DG and vinblastine to alter cell deformability cannot be construed as evidence that inhibition of phagocytosis by these agents is not mediated by altered function of contractile proteins. If cell deformability is related to actomyosin content per se, it is conceivable that 2DG and vinblastine may not alter the total amount of actin and myosin in association with each other, which would be consistent with maintenance of normal deformability as was observed, but may decrease rates of association and disassociation of actin and myosin (i.e. contractile activity) and thereby interfere with phagocytosis. In addition to its well-known interaction with microtubule proteins, vinblastine also precipitates actin (7, 45). Furthermore, vinblastine inhibits Mg-ATPase activity of neurostenin which appears to be the central nervous system equivalent of actomyosin (23). These effects could limit rates of association of actin with myosin and of disassociation of actomyosin, respectively, without altering total actomyosin content which would be consistent with the normal deformability characteristics observed.

The observation that vinblastine produced characteristic crystals of microtubular protein but did not alter cell deformability suggests that microtubules are not important determinants of cell deformability. Failure of heavy water and of colchicine to affect cell deformability are also consistent with this interpretation. Furthermore, in light of the fact that vinblastine can interact with actin, we would suggest that inhibition of phagocytosis by vinblastine may in fact be mediated by its actions on contractile proteins rather than by its effects on microtubules.

Of all the agents tested which interfered with phagocytosis, 2-deoxyglucose is the only one for which there are no reports of a direct or indirect interaction with isolated contractile proteins. Thus, inhibition of phagocytosis by 2-deoxyglucose is presumably a consequence of impaired

energy metabolism and depletion of cellular ATP with decreased rates of actomyosin formation and disassociation. It is of interest that, in the heart, 2DG selectively inhibits contractility without affecting membrane electrical characteristics (43).

The observation that phagocytosis was markedly inhibited in cells with increased deformability after treatment with cytochalasin B and PCMB, even though bead adhesion to the cell surface was unaffected, indicates that under these experimental conditions the force of adhesion between cell and bead is insufficient to deform the cell around the bead. Thus, deformation of cells around beads would appear to depend on some form of cellular activity, possibly contractile events as suggested by Stossel and Hartwig (35), whereas deformation of cells by fluid shear stress would appear to be a passive phenomenon.

These effects of metabolic inhibitors and pharmacologic agents on phagocytosis and on deformability characteristics of macrophages, together with previous studies of effects of these agents on isolated actin and myosin, indicate that (a) contractile proteins are major determinants of cell deformability characteristics, (b) normal function of contractile proteins is requisite to phagocytosis, and (c) microtubules are not significant determinants of cell deformability. These observations and conclusions are consistent with those of Polard who suggests that "*Acanthamoeba* contractile proteins have a dual role in the cell; they may generate the forces for cellular movements and also act as cytoskeletal elements by controlling the consistency of the cytoplasm" (27). Clearly, further studies are needed to elucidate the interrelationships between energy metabolism and the function of contractile proteins and microtubules in macrophage deformability and phagocytosis.

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