MICROTUBULE DYNAMICS AND GLUTATHIONE METABOLISM IN PHAGOCYTIZING HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

Glutathione oxidants such as tertiary butyl hydroperoxide were shown previously to prevent microtubule assembly and cause breakdown of preassembled cytoplasmic microtubules in human polymorphonuclear leukocytes. The objectives of the present study were to determine the temporal relationship between the attachment and ingestion of phagocytic particles and the assembly of microtubules, and simultaneously to quantify the levels of reduced glutathione and products of its oxidation as potential physiological regulators of assembly. Polymorphonuclear leukocytes from human peripheral blood were induced to phagocytize opsonized zymosan at 30°C. Microtubule assembly was assessed in the electron microscope by direct counts of microtubules in thin sections through centrioles. Acid extracts were assayed for reduced glutatione (GSH) and oxidized glutathione (GSSG), by the sensitive enzymatic procedure of Tietze. Washed protein pellets were assayed for free sulfhydryl groups and for mixed protein disulfides with glutathione (protein-SSG) after borohydride splitting of the disulfide bond.

Resting cells have few assembled microtubules. Phagocytosis induces a cycle of rapid assembly followed by disassembly. Assembly is initiated by particle contact and is maximal by 3 min of phagocytosis. Disassembly after 5–9 min of phagocytosis is preceded by a slow rise in GSSG and coincides with a rapid rise in protein-SSG. Protein-SSG also increases under conditions in which butyl hydroperoxide inhibits the assembly of microtubules that normally follows binding of concanavalin A to leukocyte cell surface receptors. No evidence for direct involvement of GSH in the induction of assembly was obtained. The formation of protein-SSG, however, emerges as a possible regulatory mechanism for the inhibition of microtubule assembly and induction of their disassembly.

KEY WORDS microtubule assembly (disassembly) · glutathione (disulfide) · mixed disulfides · polymorphonuclear leukocytes · phagocytosis

Despite recent intensive efforts at characterizing conditions for microtubule assembly in vitro, the physiological regulation of assembly and disassembly has remained obscure. Yet, it has been clear

for some time that the state of microtubule assembly is highly dynamic. This concept was first developed by Inoué in studies of microtubules in mitotic spindles (14) and was subsequently applied to cytoplasmic microtubules (26). Human polymorphonuclear leukocytes (PMN) have proved to be particularly favorable cells for the analysis of cytoplasmic microtubule assembly. Their relatively small size and symmetrical shape facilitate the identification of centrioles. The status of microtubule assembly can then be standardized by counting centriole-associated microtubules. Relatively few microtubules are found in unstimulated PMN, whereas a large and rapid increase in centriole-associated microtubules follows the binding of ligands such as concanavalin A (Con A) to the PMN cell surface (13, 23, 24). PMN are also phagocytes. Because phagocytosis results from the interaction of particulate ligands with the cell surface, it seemed possible that phagocytosis might induce microtubule assembly. On the other hand, phagocytosis is associated with the generation of oxidants such as hydrogen peroxide that can inhibit microtubule assembly in vitro (24, 25). We show here that microtubule assembly is initiated by contact and ingestion of phagocytic particles. Microtubules disassemble when phagocytosis is complete.

With this cycle established, we have studied the role of glutathione metabolism in the physiological regulation of microtubule assembly and disassembly. It was previously shown that the assembly of both cytoplasmic and mitotic spindle microtubules is prevented, and that existing microtubules are depolymerized by agents that oxidize glutathione (GSH) (22, 24, 27). The possibility was therefore raised that GSH and/or the products of its oxidation may control microtubule formation or breakdown. The sulfhydryl residues of tubulin (tubulin-SH) were suggested as likely targets of such regulatory interaction as the in vitro polymerization of tubulin is blocked by sulfhydryl reagents (17) as well as by relatively high concentrations of (oxidized) glutathione disulfide (GSSG) (20, 24).

We find that microtubule assembly induced by phagocytosis is accompanied by only small changes in cellular levels of GSH and its oxidation products. However, the microtubule disassembly phase is preceded by a gradual increase in GSSG and is coincident with the generation of mixed disulfides of glutathione with protein (protein-SSG). A similar increase in protein-SSG is observed coincident with the prevention by the GSH oxidizing agent, tertiary butyl hydroperoxide, of Con A-induced microtubule assembly in human leukocytes. We suggest that mechanisms controlling the formation and reduction of protein-SSG may serve a regulatory role in microtubule disassembly in vivo.

MATERIALS AND METHODS

Definitions

Throughout the text, reduced glutathione is abbreviated as GSH and oxidized glutathione (glutathione disulfide) as GSSG. Total glutathione refers to the sum of GSH and GSSG.

Cells

Leukocyte suspensions containing 75–80% PMN and 20–25% mononuclear cells were isolated by dextran sedimentation of the majority of erythrocytes in freshly drawn heparinized human peripheral blood and hypotonic lysis of contaminating erythrocytes in the resulting leukocyte-rich plasma. The cells were collected by centrifugation at 4°C, washed, and diluted to 10^7 cells/ml in phagocytosis medium (*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid [HEPES]-buffered saline containing calcium, magnesium, and glucose [4]). Immediately before use the cell suspensions were warmed for 10 min without shaking in a 30° or 37°C bath. Portions (1.0 ml) of the suspensions were then transferred into capped 1.5-ml plastic Eppendorf centrifuge tubes held in a shaking bath. These tubes already contained 0.1 ml of buffer, drug solution, or phagocytic particles as indicated.

Phagocytosis

Zymosan A (Sigma Chemical Co., St. Louis, Mo.) was opsonized by incubation for 30 min at 37°C in fresh human serum. The zymosan was washed in phagocytosis medium and resuspended in medium to a concentration of 30 mg/ml. To initiate phagocytosis, 1.0 ml of cells prewarmed to 30°C was added to tubes containing 0.1 ml of zymosan (~11 zymosan particles/cell), and the suspensions were incubated with shaking at 30°C. Incubation at 30°C instead of 37°C reduced the rate of particle uptake and accompanying metabolic changes sufficiently to permit accurate sequential sampling and measurements.

Phagocytosis was terminated by brief centrifugation of the cell suspension in an Eppendorf microcentrifuge, followed by rapid withdrawal of the supernatant medium into separate tubes on ice. The cell pellet was either fixed with glutaraldehyde at room temperature for microscopy or extracted with acid at 4°C for measurement of GSH and its metabolites. The entire procedure of centrifugation and addition of fixative or acid required 30 s.

Electron Microscopy

Cells were fixed for 30 min with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were washed three times in buffer alone, postfixed in 2% aqueous OsO_4 for 30 min, washed in distilled water, and stained en bloc for 20 min at 37°C in 2% aqueous uranyl acetate as before (24). Cell pellets were dehydrated through a graded series of ethanols and embedded in Spurr resin. Thin sections (silver) cut with a diamond knife were picked up on Formvar-coated 100-mesh copper grids, stained with uranyl acetate and lead, and examined in a Philips 300 electron microscope.

Sections of PMN containing a centriole were photographed at a stage magnification of × 14,200 and printed at a final magnification of × 53,000. Microtubules were then counted within a 2-μm square centered on a centriole.

Acid Extraction of Leukocytes

0.25 ml of 10% trichloroacetic acid (TCA) in 0.02% hydrochloric acid (HCl) was mixed with cell pellets followed by 0.25 ml of 0.25% Triton X-100 containing 10 mg/ml bovine serum albumin (Sigma Fraction V, Sigma Chemical Co., defatted as in reference 6). The albumin had been treated previously with excess *N*-ethylmaleimide to destroy all free protein-SH groups and dialyzed exhaustively to remove unreacted *N*-ethylmaleimide. The suspensions of cells in acid were vortexed for 20 s and then held on ice for 15 min.

Extraction was followed by centrifugation for 2 min at 13,000 rpm in an Eppendorf microcentrifuge, removal of the supernate, and washing of the insoluble protein pellet with additional acid. The combined supernates were then extracted five times with equal volumes of ether (to remove TCA); residual ether was removed under nitrogen, and portions of the extracts were used for measurement of GSH and GSSG. The protein pellet was sonicated in 0.4 ml of water to release any noncovalently bound sulfhydryl residues, reprecipitated with 0.4 ml of 10% TCA, and resonicated in 1.2 ml of water. Protein-SH and protein-SSG levels were measured in the sonicate.

Measurement of GSH and GSSG

Both total glutathione (GSH + GSSG) and GSSG alone were measured by modifications of the sensitive assay developed by Tietze (29). For measurement of total glutathione, 0.02 ml of the ether extract (above) was diluted in 0.38 ml of 5 mM EDTA in 0.1 M phosphate buffer, pH 7.5 (phosphate buffer). The following were then combined in a cuvette: 0.025–0.05 ml of sample, 0.1 ml of 1.18 mg/ml dithiobis-2-nitrobenzoic acid (DTNB), 0.1 ml of glutathione reductase (Sigma type III, Sigma Chemical Co., [10 U/ml]), 0.1 ml of 1 mg/ml NADPH (Sigma Type III, Sigma Chemical Co.) and phosphate buffer to a final volume of 0.5 ml. Absorbance of the solution at 412 nm was recorded for 6 min at room temperature. All samples were run in duplicate. Total glutathione was determined by use of standard curves generated by substituting known amounts of GSH for sample. Internal standards, in which known amounts of GSH were added to the sample, were included to ascertain that samples were free of substances that could interfere with GSH reductase activity.

For GSSG measurement, ether-extracted solutions were incubated for 60 min at room temperature with an equal volume of 0.04 M *N*-ethylmaleimide in phosphate buffer to complex reduced sulfhydryl groups (primarily GSH). The solutions were reextracted 10 times with equal volumes of ether; residual ether was removed by flushing with nitrogen, and 0.02 to 0.05-ml portions were assayed for GSSG by the method described above for total glutathione.

For both assays, blank tubes (no cells) were always run with a known quantity of GSSG added at the time of initial TCA precipitation. Recovery was consistently between 95 and 105%, even after 15 ether extractions.

Measurement of Protein-SH and Protein-SSG

Protein-SH was measured in duplicate from the absorbance at 412 nm of the supernate obtained by centrifugation from a mixture consisting of 0.1 ml of protein pellet sonicate, 0.4 ml of phosphate buffer, and 0.1 ml of 0.4 mg/ml DTNB (5, 25).

Protein-SSG was determined by releasing glutathione covalently bound to protein by borohydride ($NaBH_4$) reduction and then employing the modified Tietze assay previously described. Washed protein pellet sonicate (1 ml) was incubated with shaking at 50°C for 1 h with 1 ml of 1% $NaBH_4$ (Sigma Chemical Co.). The remaining $NaBH_4$ was hydrolyzed by addition of 0.4 ml of 30% TCA. The tubes were then heated for 15 min, cooled on ice for 5 min, and insoluble protein was removed by centrifugation. The supernatant volume was adjusted to 2.5 ml with phosphate buffer; 2 ml of acetone were added to ensure complete oxidation of the $NaBH_4$, and the samples were ether-extracted five times. Total glutathione was then determined in these extracted samples. Loss of glutathione was estimated from determinations in parallel tubes to which known amounts of GSH were added at the start of $NaBH_4$ reduction. In most experiments, the loss was ~50%. The loss of GSH occurred during the reduction step, was proportional to the time in $NaBH_4$, was the same over a range of added GSH concentrations, and was not altered by reduction under nitrogen. The tabular values cited have been corrected for this loss determined in each experiment.

Oxidative Metabolism

Several parameters of oxidative metabolism were measured in phagocytizing PMN: superoxide generation, determined by a modification of the colorimetric method of Babior et al. (1); H_2O_2 generation, measured spectro-

fluorimetrically as described by Root et al. (28); and hexose monophosphate shunt activity, determined from [1-^{14}C]glucose oxidation as before (24).

Enzyme Assays

The activity of the cytoplasmic marker enzyme lactate dehydrogenase (LDH) was measured in both whole cell sonicates and incubation media according to Bergmeyer (3). The lysosomal enzyme, β-glucosaminidase, was measured in cell sonicates in incubation media by hydrolysis of the colorigenic substrate *p*-nitrophenyl-β-D-glucosaminide (Sigma Chemical Co.).

RESULTS

Uptake of Zymosan and its Metabolic Accompaniments

Opsonized zymosan has been used extensively to study phagocytosis in human PMN (e.g. reference 2). Uptake occurs rapidly under the conditions employed here, being almost maximal by 5 min (when each cell contains an average of 4.4 particles as determined by counting in the light microscope with Nomarski optics), and increasing very little (to 4.9 particles per cell) with a 10-min incubation period. We confirmed that no loss of the cytoplasmic marker enzyme lactate dehydrogenase from the cells into the medium occurs over 10 min of phagocytosis. Some loss of lysosomal enzymes was inferred from the presence extracellularly of 31% of the original cellular content of β-glucosaminidase after 5 and 10 min of exposure of cells to zymosan at 30°C. Consistent with other extensive studies (2, 7), we found that the first 10 min of phagocytosis was accompanied by the release of 9.4 nmol of superoxide/10^6 cells compared to no measurable release by control cells; by a 24-fold increase in H_2O_2 production; and by an eightfold increase in [1-^{14}C]glucose oxidation (presumably via the hexose monophosphate shunt). Importantly, this greatly accelerated oxidative metabolism continued linearly for at least 20 min of incubation of cells with zymosan, i.e., long after phagocytosis was essentially complete.

The Effect of Zymosan Ingestion on Microtubule Assembly

The results of microtubule counts in cells exposed for various lengths of time to zymosan are summarized in Table I. As previously described (23, 24), resting cells contained relatively few centriole-associated microtubules (line 1 in Table I). Cells incubated with 10% zymosan-treated serum also showed very few microtubules (line 7). By contrast, cells mixed with zymosan and immediately centrifuged and fixed contained approximately 12 microtubules per centriole (line 2). In addition, these cells showed prominent pseudopods commencing to encircle attached particles; very few zymosan particles were completely inside the cells.

Cells incubated with zymosan for 1 and 3 min before centrifugation and fixation showed particles both completely enclosed in the cytoplasm and in the process of being engulfed. The number of centriole-associated microtubules in such active cells was further increased to approximately 16 per centriole (lines 3, 4). (The difference in microtubule counts between 0.5 and 3.5 min is significant, $P < 0.01$.)

By 5 min most cell-associated particles were completely intracellular and the number of centriole-associated microtubules was reduced to about nine microtubules (line 5). Further incubation was now accompanied by a progressive decrease in centriole-associated microtubules towards the unstimulated levels (line 6). Thus,

TABLE I

The Effects of Phagocytosis on Microtubule Assembly in Human Leukocytes

Line	Incubation at 30°C	Microtubules ± SEM
1	Buffer, 0.5 and 10 min*	4.06 ± 0.83 (34)‡
2	Zymosan, 0.5 min§	12.25 ± 0.97 (28)
3	Zymosan, 1.5 min	14.71 ± 1.71 (14)
4	Zymosan, 3.5 min	16.50 ± 2.05 (18)
5	Zymosan, 5.5 min	8.81 ± 1.09 (36)
6	Zymosan, 9.5 min	1.86 ± 0.63 (14)
7	Zymosan-treated serum (10%), 4 min	2.00 ± 0.60 (13)

Two blocks from each of two or three separate experiments were sectioned and centrioles were photographed at × 14,000. The number of microtubules associated with each centriole could be counted after printing × 56,000.

* Microtubule counts were pooled after demonstration that no significant differences existed between cells prewarmed to 30°C for 10 min and further incubated at 30°C with shaking for either 0.5 or 10 min.

‡ Numbers in parentheses represent the numbers of centrioles examined for each condition.

§ In this and subsequent tables, 0.5 min represents the time between placing cell suspensions in the centrifuge and addition of fixative to the packed cell plus zymosan pellet.

microtubule assembly was essentially coterminous with particle attachment and ingestion.

The Effect of Phagocytosis on Glutathione Metabolism

Resting human PMN from a number of different normal adult donors maintained a GSH:GSSG ratio very close to 100. However, as noted before (24), different cell preparations showed a relatively broad range of glutathione concentrations. Thus, over approximately 20 experiments with cells from different adult donors, total glutathione levels varied from 0.9–1.9 nmol/10^6 cells.

Assays of GSH and GSSG in phagocytizing cells also showed a substantial variability in the extent, although not in the direction, of changes in concentration. We have therefore chosen to illustrate in Fig. 1 the results of three separate experiments which indicate the range of changes in glutathione seen in phagocytic cells. The averages of these same data are presented in Table II.

A steady decrease in GSH beginning early in phagocytosis (by at least 1.5 min) and continuing to an average of 75% of resting GSH levels after 10 min of phagocytosis is immediately apparent. This loss of GSH is paralleled by a rapid rise in GSSG levels to approximately twice the resting levels by 5–10 min of phagocytosis. When experiments were continued to 15 and 20 min of phagocytosis, no consistent further changes in GSH or GSSG were noted. Glutathione levels were not changed by incubation of cells at 30°C in buffer alone, in 10% zymosan-treated serum, or by their incubation with nonopsonized zymosan (which fails to either adhere to or be ingested by the cells).

It had been apparent in preliminary experiments that GSSG levels did not rise sufficiently to account in a quantitative manner for the decrease in GSH levels. We reasoned that glutathione no longer detectable in acid extracts of leukocytes might be incorporated into the protein pellet of cells via formation of mixed disulfides with proteins. Therefore, assays of protein-SSG, as well as parallel protein-SH assays, were performed in the same experiments.

The protein-SH content of leukocyte extracts is high and shows no statistically significant change during phagocytosis. Thus, the experiments of Table II and Fig. 1 included 21 duplicate assays of protein-SH in acid precipitates of cells that phagocytized between 0 and 10 min. The resulting data showed a constant protein-SH level of 5.52 ± 0.01 (SEM) nmol/10^6 cells. The high value of this measurement very likely made it insensitive to more subtle reactions of protein sulfhydryls.

The amount of covalently bound glutathione

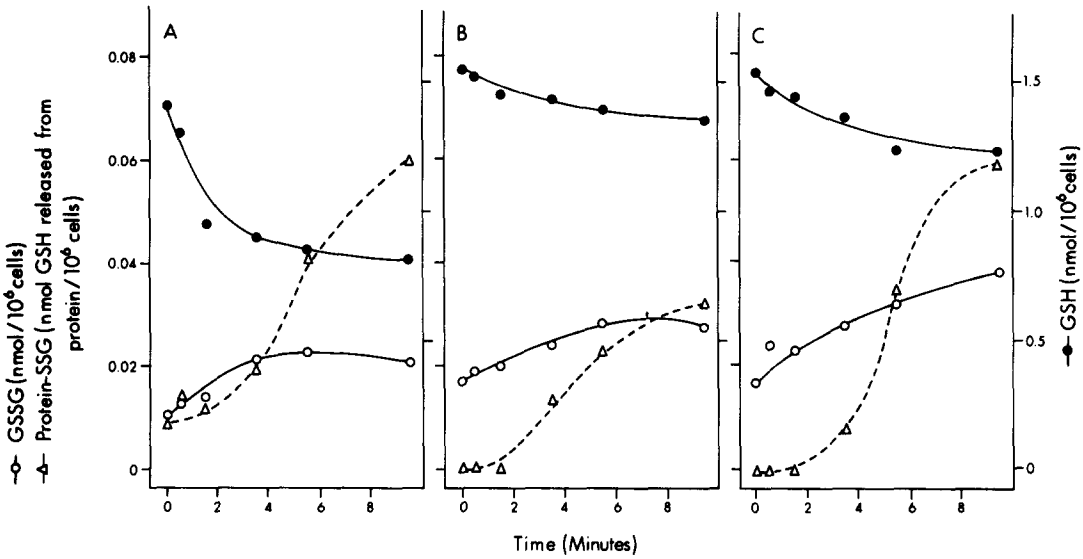

FIGURE 1 Changes in GSH, GSSG and protein-SSG during phagocytosis. The time-course of changes in glutathione concentrations is shown for three separate experiments in which leukocytes from different donors were used. Each point is the average of duplicate determinations that agreed within 5%. The results in Table II are averages taken from these separate experiments.

released from $NaBH_4$-treated acid precipitates (protein-SSG) showed very little increase over control during the first 3 min of phagocytosis. However, the amount of protein-SSG rose after 5 min to at least equal the total cellular content of GSSG and substantially exceeded the cellular GSSG content at 9.5 min (Fig. 1, Table II). Measurements at 10 and 20 min of phagocytosis showed little or no further increase in protein-SSG above the value of 0.04–0.05 nmol/10^6 cells achieved at 9.5 min.

Even with the identification of this protein-associated pool of glutathione, the total recovery of glutathione at 9.5 min is only 80%. One possible explanation is incomplete reduction of mixed disulfides, leading to an underestimation of protein-SSG concentration. This is currently under investigation. Alternatively, some glutathione may be released into the medium during phagocytosis or may be destroyed by specific or nonspecific peptidases that are activated during phagocytosis. These possibilities are difficult to assess. We could not determine the significance of a small (1–6% of the total) but consistent level of glutathione in the incubation medium of resting cells. We found, however, that GSH added to cell suspensions persisted in the medium of resting cells but was destroyed in the medium of phagocytizing cells.

The Effect of Tertiary Butyl Hydroperoxide on Glutathione Metabolism

The GSH oxidant tertiary butyl hydroperoxide inhibits Con A-induced microtubule assembly in leukocytes (24). If protein-SSG is a critical product related to microtubule inhibition, then protein-SSG should rise in butyl hydroperoxide-treated as well as phagocytizing normal cells.

The results in Table III confirm this prediction. Tertiary butyl hydroperoxide causes a marked drop in GSH and an increase in GSSG that is considerably larger than that associated with phag-

TABLE II

The Concentrations of GSH, GSSG, and Protein-SSG in Phagocytizing Neutrophils

Incubation at 30°C	GSH	GSSG	Protein-SSG	Recovery* of glutathione
	nmol/10^6 cells		*nmol GSH released from protein/ 10^6 cells*	%
Buffer, 0.5 min	1.49	0.015	0.003	100
Zymosan, 0.5 min	1.43	0.018	0.005	96.6
Zymosan, 1.5 min	1.28	0.019	0.004	86.8
Zymosan, 3.5 min	1.23	0.024	0.015	84.9
Zymosan, 5.5 min	1.15	0.028	0.033	81.4
Zymosan, 9.5 min	1.13	0.029	0.050	81.3
Buffer, 10 min	1.41	0.013	0.004	94.6

The cells were incubated at 30°C for 10 min in buffer alone before transfer into tubes containing zymosan. Data are the averages of results obtained in duplicate in three separate experiments. The duplicates agreed ± 5% within each experiment. However, significant variability was observed between experiments (see Fig. 1).

* Glutathione recovery = percent (GSH + 2GSSG + P-SSG) of resting cells taken at 0.5 min.

TABLE III

The Effect of Tertiary Butyl Hydroperoxide on GSH, GSSG, and Protein-SSG

Incubation at 37°C	GSH	GSSG	Protein-SSG	Recovery of glutathione
	nmol/10^6 cells		*nmol GSH released from protein/ 10^6 cells*	%
Buffer, 0.5 min	1.90	0.010	0.008	100
Tertiary butyl hydroperoxide, 50 nmol/10^6 cells				
0.5 min	1.50	0.134	0.026	93.1
5.5 min	1.54	0.096	0.044	92.1
Buffer, 5 min	1.90	0.008	0.001	99.4

For details see legend to Table II.

ocytosis. A rapid concurrent increase in protein-SSG to levels similar to those seen in phagocytizing PMN is measured in butyl hydroperoxide-treated cells.

DISCUSSION

It was previously demonstrated that binding of the plant lectin Con A to cell surface receptors induces microtubule assembly in human leukocytes (13, 23, 24). Exposure of leukocytes to pharmacological agents that can oxidize GSH inhibits this assembly response and causes the disassembly of existing centriole-associated microtubules (24). Weissmann and co-workers have observed that microtubule assembly is also increased after the interaction of opsonized zymosan and zymosan-treated serum with cytochalasin B-treated (nonphagocytic) PMN (11, 30).

We show here that microtubule assembly is induced by exposure of PMN to opsonized zymosan particles under conditions that permit phagocytosis to proceed normally. The shortest period of contact of particles with the cell (the 30-s centrifugation and fixation) is sufficient to induce a near-maximal assembly response. In contrast, 10% zymosan-treated serum fails to induce microtubule formation. This eliminates soluble factors generated during opsonization of zymosan as the stimulus to microtubule assembly in the present experiments.

The number of centriole-associated microtubules remains high through 3 min of phagocytosis. However, cells cease to phagocytize by ~5 min, and the number of centriole-associated microtubules is also reduced at 5 min. A further reduction in microtubules is apparent at 10 min. This disassembly appears not to be related to general cell damage. Thus, the increased cellular metabolism associated with phagocytosis continues over at least 20 min of incubation of cells with zymosan, and the cytoplasmic marker enzyme LDH is not further released from the cells.

Definition of this cycle of assembly of microtubules followed by their disassembly in the presence of a physiological stimulus has provided a unique system to explore the hypothesis (22, 24, 27) that GSH metabolism is involved in modulating the in vivo assembly and disassembly of microtubules.

No direct evidence that GSH is involved in regulating microtubule polymerization has been obtained. The present data show that microtubule assembly during the first 3 min of phagocytosis occurs in parallel with a small but reproducible decrease in GSH and a rise in GSSG. Some of this GSH may be consumed for reduction of tubulin-SH before its polymerization. However, we found previously that the assembly of microtubules induced by Con A occurred without any change in leukocyte GSH levels (24). We also found that microtubules can assemble in response to Con A in a patient whose leukocytes contain only 20% of normal glutathione due to a deficiency in GSH synthetase.[1] These data do not rule out a role for GSH in the maintenance of tubulin-SH or in some other process required for microtubule assembly. However, they clearly indicate that in PMN, GSH is not limiting for assembly, i.e., its level is not regulatory. In addition, because microtubule assembly can occur at 20% of the normal GSH level, which is lower than any depression of GSH during phagocytosis, it is clear that decreased GSH is not responsible for microtubule disassembly. Some mechanism that couples GSH to assembly could, of course, regulate the influence of GSH.

GSSG has been proposed before as a possible mediator of microtubule disassembly. In vitro GSSG inhibits microtubule assembly although unphysiologically high concentrations are required (20, 24). Previous evidence for a regulatory role of GSSG in vivo was based on inhibition of microtubule polymerization after treatment of both normal and chronic granulomatous disease leukocytes with the GSH oxidants, tertiary butyl hydroperoxide, and diamide (25). In the normal cells, inhibition could result from modification of tubulin by either GSSG or by H_2O_2 and other endogenous oxidants that might accumulate in GSH-depleted cells. Because chronic granulomatous disease cells fail to generate these oxidants in significant amounts, GSSG seemed the most likely substance leading to microtubule inhibition. Further support for this view is the demonstration that GSH synthetase-deficient cells were resistant to microtubule inhibition by butyl hydroperoxide.[1] Presumably, insufficient GSSG could be generated in these cells to affect microtubule integrity despite normal GSH peroxidase levels.

We show here that phagocytosis is accompanied

[1] Oliver, J. M., S. P. Spielberg, C. B. Pearson, and J. D. Schulman. 1977. Microtubule assembly and function in normal and glutathione synthetase deficient polymorphonuclear leukocytes. Manuscript submitted for publication.

by a progressive but modest rise in GSSG to approximately double the resting levels. Tertiary butyl hydroperoxide causes an immediate large increase in GSSG, to greater than 10 times the resting amounts. Both microtubule disassembly following phagocytosis and the inhibition of Con A-induced assembly of microtubules due to butyl hydroperoxide are coincident with a greater than 10-fold elevation of protein-SSG to ~0.04–0.05 nmol/10^6 cells. These data suggest that microtubule disassembly and inhibition of microtubule assembly may be closely correlated with the formation of protein-SSG.

What concentration of mixed disulfide is associated with microtubule inhibition? The findings reported here and the previous results with GSH synthetase-deficient cells set at least a lower limit compatible with assembly. Thus, the GSH-deficient cells generate a maximum of only 0.014 nmol of protein-SSG when incubated between 0.5 and 5.5 min with 50 nmol of butyl hydroperoxide/10^6 leukocytes.[2] The same low level of protein-SSG is present in zymosan-treated normal cells after 3 min of phagocytosis. In both cases, high numbers of microtubules exist in the cells. A further increase to 0.03 nmol of protein-SSG/10^6 cells after 5 min of phagocytosis in the normal cells is correlated with partial microtubule depolymerization (Tables I, II).

The proposal that microtubule disassembly may be regulated in vivo through the formation and reduction of mixed disulfides between protein-SH and GSSG was first suggested on theoretical grounds by Rebhun and co-workers (22, 27). Our data suggest that an equilibrium between GSH, GSSG, and protein-SSG is shifted towards protein-SSG when GSSG is elevated by GSH oxidants and when cells have completed phagocytosis. This shift towards protein-SSG is correlated with inhibition of microtubule assembly. We emphasize that no causal relationship between protein-SSG formation and microtubule inhibition has been established. Further, should a causal relationship exist, we do not know whether tubulin itself or other proteins that influence the polymerization of tubulin are the immediate targets of protein-SSG formation. Studies of the fate of radioactive GSH in oxidant-treated and phagocytizing leukocytes now in progress will provide clarification.

Other workers have identified mixed disulfides in many different animal cells and tissues (e.g., references 8, 9, 12, 15, 16, 19, 21). In particular, their presence in tissues not subject to unusual oxidative stress and high GSSG levels indicates that protein-SSG formation may be subject to enzymatic regulation very likely involving thiol transferases. The abnormally high GSSG levels in butyl hydroperoxide-treated cells could overwhelm these regulatory enzymes and force protein-SSG formation. By contrast, the fact that GSSG is not increased very substantially in phagocytizing cells suggests that microtubule disassembly via protein-SSG formation may be a physiologically "programmed" event that follows ingestion of particles and their fusion with lysosomes, but may in fact precede the subsequent killing (in the case of bacterial ingestion) and digestion of internalized materials. Microtubule integrity is required for leukocyte chemotaxis (10, 18) and for the segregation of membrane components during phagocytosis (4). It is tempting to speculate that postphagocytosis disassembly of these structures would prevent directed migration of the engorged cell whose remaining function is digestion.

In summary, we have shown that phagocytosis in human PMN is accompanied by a rapid assembly followed by disassembly of centriole-associated microtubules. Microtubule disassembly is highly correlated with elevation of protein-SSG levels. Similarly, the GSH oxidizing agent, tertiary butyl hydroperoxide, inhibits Con A-induced microtubule assembly and concomitantly elevates protein-SSG levels in leukocytes. We propose that microtubule depolymerization may be influenced in vivo by the reversible formation and reduction of protein-SSG.

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