

Function of H_2O_2 , Myeloperoxidase, and Hexose Monophosphate Shunt Enzymes in Phagocytizing Cells from Different Species¹

B. B. PAUL, R. R. STRAUSS, A. A. JACOBS, AND A. J. SBARRA

Department of Pathology and Medical Research, St. Margaret's Hospital, and Department of Obstetrics and Gynecology, Tufts University Medical School, Boston, Massachusetts 02125

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The effect of phagocytosis on the H_2O_2 production and myeloperoxidase (MPO) activities of leukocytes from various species was investigated. The intracellular distribution of MPO, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (6PGDH) of resting and phagocytizing guinea pig polymorphonuclear leukocytes has also been studied. Phagocytizing cells produce more H_2O_2 than the corresponding resting cells. This has been found to be true for human peripheral polymorphonuclear leukocytes, mouse peritoneal macrophage, and guinea pig and rat peritoneal polymorphonuclear leukocytes. All of these cells, except rabbit alveolar macrophages, have significant MPO activity. Generally an increased activity is noted with phagocytizing cells. Homogenization and differential centrifugation of guinea pig peritoneal polymorphonuclear leukocytes indicate that the whole homogenate and its fractions from phagocytizing cells have significantly higher MPO and NADPH oxidase activities, when compared to the corresponding fractions from the resting cells. The $27,000 \times g$ supernatant fluid from phagocytizing cells has 6-fold more MPO and 2.5-fold more NADPH oxidase activity than similar supernatant fractions from resting cells. The enzyme 6PGDH was unaffected by phagocytosis. The relationship of these stimulated activities to the intracellular bactericidal function of the phagocytes has been discussed.

It has been reported that H_2O_2 and myeloperoxidase (MPO)-containing granules from guinea pig polymorphonuclear leukocytes (PMN) act synergistically to form an effective bactericidal system against a number of bacteria (11, 12, 15). It has also been observed that the stimulated oxidative metabolic activities of phagocytizing PMN result in an increased production of H_2O_2 as measured by a direct fluorometric assay (21, 22). To determine whether the H_2O_2 -MPO bactericidal system is unique to guinea pig PMN or whether it is more of a general phenomenon, human, rabbit, and rat PMN, rabbit alveolar macrophages, mouse peritoneal macrophages, and mouse spleen lymphocytes were studied for their H_2O_2 and MPO activities at rest and after phagocytosis.

Furthermore, the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, glucose-6-phosphate dehydrogenase

(G6PDH), and 6-phosphogluconate dehydrogenase (6PGDH) are considered to be actively involved with the production of H_2O_2 and activation of MPO (10, 26, 31, 32, 35; R. R. Strauss et al., *Cancer Res.*, *in press*; R. R. Strauss et al., *Arch. Biochem. Biophys.* **135**:265-271, 1969). Therefore, it was thought that it would also be of interest to study the effect of phagocytosis on the activities and intracellular distribution of these three enzymes and MPO.

The results presented indicate that the phagocytic stimulation of H_2O_2 production and MPO activity occurs with all of the cell types studied except one. Both granule-bound enzymes, MPO and NADPH oxidase, are stimulated in the phagocytizing guinea pig PMN. Further, the extracellular reaction medium of phagocytizing cells has three- to fourfold more G6PDH activity than that of resting PMN. These phagocytosis-associated metabolic activities will be discussed in relation to a general bactericidal system that could function in all the cell types studied.

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MATERIALS AND METHODS

All chemicals used were of reagent grade. H₂O₂ (Superoxal, 30% H₂O₂) and guaiacol (C₇H₈O₂) were obtained from Merck & Co., Inc. (Rahway, N.J.). The 30% H₂O₂ was diluted 1,000 times with distilled water, and the concentration was determined from the 230 nm extinction coefficient. This solution was further diluted for any desired molarity. Guaiacol solutions were prepared by adding 1.1 ml of guaiacol to approximately 70 ml of distilled water, and then adding slowly 20 ml of 95% alcohol while shaking and finally adjusting the volume to 100 ml with H₂O. Horseradish peroxidase (grade II) was obtained from the Sigma Chemical Co., St. Louis, Mo. It contained 127.0 purpurogallin units per mg of protein. Potassium cyanide, certified A.C.S., was obtained from Fisher Scientific Co. (Fairlawn, N.J.). Sodium azide was purchased from the Eastman Kodak Company, Rochester, N.Y. Glucose-6-phosphate, 6-phosphogluconate, nicotinamide adenine dinucleotide phosphate (NADP⁺), and NADPH were all used as sodium salts and were obtained from Calbiochem (Los Angeles, Calif.). Reagent grade glycerol was purchased from Fisher Scientific Co.

Isolation of different phagocytes. Human PMN were prepared by a previously described method (4, 16). They were collected from apparently healthy 17- to 22-year-old females.

Rabbit (20) and guinea pig (16) PMN were isolated as described previously. Rat PMN were collected 16 to 18 hr after intraperitoneal injection of 20 ml of a 12% solution of sodium caseinate. The cells were washed three times with Krebs-Ringer phosphate medium (KRPM) and resuspended in KRPM. The technique was similar to that used in harvesting guinea pig PMN (16). Mice were challenged intraperitoneally with 2 ml of sterile 0.05% glycogen. At 24 hr later, the mice were killed by cervical dislocation and immediately injected intraperitoneally with 4 ml of Hanks Buffered Glucose (HBG). After abdominal massage, the peritoneal cavity was opened and the cells were harvested. Before use, the cells were washed twice with HBG. Centrifugations were carried out at refrigerator temperatures and at 120 × g for 5 min.

For mouse spleen lymphocytes, the animals were killed as above. Immediately after death, the abdominal cavities were opened and the spleens were surgically removed and placed in cold HBG. After removing extraneous tissues, the spleens were minced gently. They were then placed in a syringe equipped with a Swinny filter adaptor containing a stainless-steel sieve. Digital pressure was applied so that the cells would pass through; the tissue and debris remained behind. The resulting cell suspension was centrifuged at 120 × g for 6 min. The pellet, containing spleen lymphocytes and red blood cells, was freed of red blood cells by the addition of cold distilled water and mixing for exactly 30 sec; 3.5% NaCl was now added to a final NaCl concentration of 0.9%. This suspension containing over 95% lymphocytes was centrifuged at 120 × g for 6 min and resuspended in cold KRPM, counted, and used. Rabbit alveolar macrophages were isolated by the procedure of Myrvik et al. (19).

Experimental design for H₂O₂ estimation. The technique used to estimate the metabolic H₂O₂ of guinea pig PMN, at rest and during phagocytosis, has been previously described (21, 22). Generally, 20 × 10⁶ to 25 × 10⁶ cells in 1 ml of KRPM were placed in each of three dialysis bags. Each bag was dialyzed against 19 to 24 ml of KRPM. One bag containing PMN only was held at 0 C and served to establish the dialyzable H₂O₂ released under conditions under which metabolism would be minimal. To one of the two remaining bags containing cells, heat-killed *Escherichia coli* (phagocyte-to-particle ratio, 1:100) were added, buffer was added to the other, and both were incubated at 37 C in a Dubnoff metabolic shaker for 30 min, while agitating at 90 oscillations/min. Rabbit cells were incubated for 45 min. Agitation of the 0 C control was accomplished by placing the reaction medium in an ice bath on a rotary shaker (90 oscillations/min). After the desired incubation time, the bags were removed and the dialysates were assayed for H₂O₂ fluorometrically (21, 22). Phagocytosis was monitored by making smears of the reaction mixture. Over 70% of PMN and macrophages was found to contain one or more particles per cell. Polystyrene latex spherules have also been used as particles with similar results.

Preparation of samples for enzyme assay. PMN were placed in each of two 10-ml glass homogenizing tubes (when larger quantities of PMN were required, siliconized Erlenmeyer flasks were used). To one of them heat-killed *E. coli* were added (particle-to-cell ratio, 100:1). The total volume in each was made to 4.0 ml with KRPM. Both tubes were incubated at 37 C in a Dubnoff metabolic shaker rotating at 90 oscillations/min. After 15 or 30 min, both tubes were removed from the water bath and 4.0 ml of cold 0.5 M sucrose was added. The contents of each tube were then centrifuged at 120 × g for 5 min in the cold. The supernatant fractions were assayed for extracellular enzyme. The pellets were suspended in 4.0 ml of 0.25 M sucrose, thoroughly mixed in a Vortex rotator, and assayed for cell-associated enzymes. For further fractionation, a sample of the pellet suspension was homogenized for 5 min with a Teflon-tipped pestle. A sample of the homogenate was diluted (1:1) with 0.25 M sucrose and centrifuged at 27,000 × g for 15 min in Servall refrigerated centrifuge. The 27,000 × g pellets (granules) were resuspended in 0.25 M sucrose. The homogenates, 27,000 × g granular fractions and supernatant fractions, obtained from both resting and phagocytizing cells, were assayed for enzyme activity.

MPO assay. This enzyme was assayed by the guaiacol method of Chance and Maehly (3). The optimal reagent concentrations at 25 C were established with guinea pig PMN homogenates. The reaction mixtures contained in a total volume of 3 ml: phosphate buffer (pH 7.0), 18.0 μmoles; guaiacol, 50.0 μmoles; homogenates equivalent to 0.5 × 10⁶ to 1.0 × 10⁶ cells; and sucrose, 750.0 μmoles (0.25 M final concentration). The reaction was started by the addition of 0.1 μmole of H₂O₂. The results were calculated by using the equation for K_i values for peroxidase and are presented arbitrarily as guaiacol units for 100 × 10⁶ cells. Spectrophotometric readings were obtained in a model DB spectrophotometer at 470 nm (Beckman Instruments, Inc., Fullerton, Calif.).

NADPH-oxidase, G6PDH, and 6PGDH assay. These three enzymes were assayed in intact cells, homogenates, $27,000 \times g$ granules, and supernatant fractions. The techniques have been reported (B. B. Paul et al., unpublished data). Each preparation, usually 1 ml, was suspended in 2.3 ml of 95% glycerol. A sample of these glycerol-treated samples was used for the spectrophotometric assay of the enzymes at 340 nm in a Beckman model DB spectrophotometer. The assay medium for NADPH oxidase, G6PDH, and 6PGDH, in a total volume of 3 ml, was as follows. NADPH oxidase: phosphate buffer (pH 5.5), 300.0 μ moles; Mn^{2+} , 1.0 μ mole; ethanol, 200.0 μ moles; and PMN from a suspension in 70% glycerol containing 9.0×10^6 cells; total glycerol, 2,250.0 μ moles (7% final concentration); 0.5 μ mole of NADPH was added last to start the reaction. G6PDH: tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5), 200 μ moles; Mg^{2+} , 10.0 μ moles; glucose-6-phosphate, 5.0 μ moles; PMN from 70% glycerol suspension, 3.0×10^6 cells; total glycerol content, 750 μ moles

(2.33%); the reaction was initiated by the addition of 0.625 μ mole of $NADP^+$. 6PGDH: Tris, Mg^{2+} , and $NADP^+$ were used in the same concentration as for G6PDH. PMN were added from a suspension in 70% glycerol containing 9.0×10^6 cells (total glycerol content, 2,250.0 μ moles, 7% final concentration); 10.0 μ moles of 6-phosphogluconate was added last to start the reaction.

For G6PDH and 6PGDH, increases in optical density due to NADPH formation were recorded for at least 5 min. For NADPH oxidase, decrease in optical density due to oxidation of NADPH to $NADP^+$ was recorded for at least 15 min. Auto-oxidation of NADPH at pH 5.5 was measured in the absence of enzyme. This was always subtracted from the values obtained with cell fractions.

RESULTS

The metabolic H_2O_2 content of the different cell types is presented in Table 1. The H_2O_2 dialyzing

TABLE 1. Effect of phagocytosis on H_2O_2 production by leukocytes from various species^a

Species	$H_2O_2^b$		
	Resting	Phagocytizing	<i>P</i> ^c
Human peripheral PMN.....	122.3 \pm 6.3 ^d	197.3 \pm 8.1	<0.01
Mouse peritoneal macrophage.....	108.2 \pm 15.1	177.5 \pm 20.5	<0.05
Rabbit alveolar macrophage.....	59.2 \pm 17.6	69.0 \pm 21.8	>0.10
Rabbit peritoneal PMN.....	12.4 \pm 3.8	21.6 \pm 3.2	>0.10
Guinea pig peritoneal PMN.....	8.2 \pm 1.1	17.0 \pm 2.5	<0.01
Rat peritoneal PMN.....	6.9 \pm 1.5	11.6 \pm 0.6	<0.05

^a Incubations were carried out at 37 C for 30 min. Heat-killed *Escherichia coli* to phagocyte ratio was 100:1.

^b Expressed as nanomoles per 10^8 cells.

^c Probability of significance between resting and phagocytizing cells.

^d Mean \pm standard error of the mean for three or more experiments.

TABLE 2. Myeloperoxidase activities of resting and phagocytizing cells collected from different species^a

Homogenates from	Guaicol units ^b		
	Resting	Phagocytizing	<i>P</i> ^c
Human peripheral PMN	4.10 \pm 0.34 ^d	5.15 \pm 0.40	<0.07
Rat peritoneal PMN.....	0.63 \pm 0.13	1.48 \pm 0.08	<0.01
Rabbit peritoneal PMN ^e	0.51	0.60	
Guinea pig peritoneal PMN.....	0.48 \pm 0.05	0.78 \pm 0.12	<0.05
Mouse spleen lymphocytes ^f	0.062 \pm 0.004	0.074 \pm 0.005	<0.08
Mouse peritoneal macrophages.....	0.028 \pm 0.009	0.024 \pm 0.004	>0.7
Rabbit alveolar macrophages.....	<0.01	<0.01	

^a Incubations were carried out at 37 C for 30 min. Heat-killed *E. coli* to phagocyte ratio was 100:1. The results are average of three or more experiments.

^b Expressed per 10^8 cells.

^c Mean \pm standard error of the mean.

^d Probability of significance between resting and phagocytizing cells.

^e Two experiments only.

^f Fifty weeks old.

out of the cell at 0 C has been subtracted from the values reported. Phagocytosis causes an increase in the metabolic H₂O₂ over that noted in resting cells in all of the cell types except those of the rabbit. Under the conditions tested, significant differences in the H₂O₂ production by the various cell types studied have been noted. Human peripheral PMN produce the greatest amount of H₂O₂ and rat peritoneal PMN the least.

MPO activity of the various leukocytes after incubation in the absence and presence of particles is presented in Table 2. The enzyme was assayed in the total homogenate. Human peripheral PMN have the highest MPO activity, whereas rabbit alveolar macrophages have insignificant activity. In general, phagocytizing cells appear to have higher activity when compared to the corresponding resting cells.

The effect of phagocytosis on the MPO activity of various homogenate fractions of guinea pig PMN is presented in Table 3. The extracellular reaction medium collected from phagocytizing

cells has three to four times more activity than that of resting cells. The homogenate and the 27,000 × g pellet from the phagocytizing cells show a 62 and 73% increase, respectively, in MPO activity, when compared to the corresponding resting cells. Furthermore, the 27,000 × g supernatant fraction from phagocytizing cells shows a sixfold increase in MPO activity, when compared to that of the corresponding resting cells.

The intracellular distribution of NADPH oxidase of guinea pig PMN after resting or phagocytosis can be seen in Table 4. Phagocytosis causes a twofold increase in the cell-associated NADPH oxidase activity. The 27,000 × g supernatant from the phagocytizing cells has 2.5-fold increased activity, when compared with the supernatant from the corresponding resting cells. The granule-bound enzyme activity of phagocytizing cells is 72% higher than the corresponding granular fraction from resting cells. The extra-

TABLE 3. Effect of phagocytosis on the myeloperoxidase activities of guinea pig polymorphonuclear leukocyte homogenate fractions^a

Fractions	Guaiacol units		
	Resting	Phagocytizing	P ^b
Extracellular.....	0.01 ± 0.001 ^c	0.03 ± 0.007	<0.05
Whole homogenate.....	0.47 ± 0.050	0.75 ± 0.120	<0.05
27,000 × g Pellet.....	0.36 ± 0.024	0.45 ± 0.060	<0.02
27,000 × g Supernatant.....	0.03 ± 0.002	0.17 ± 0.026	<0.001

^a In the presence of 1 mM KCN or NaN₃ [inhibitors for myeloperoxidase (MPO)], no increase in optical density or formation of tetraguaiacol color occurred, indicating the extracellular fraction contained MPO. Incubations were carried out at 37 C for 30 min. Heat-killed *E. coli* to phagocyte ratio was 100:1. The results are averages of three or more experiments.

^b Mean ± standard error of the mean.

^c Probability of significance between resting and phagocytizing cells.

TABLE 4. Effect of phagocytosis on the intracellular distribution of NADPH oxidase in different guinea pig polymorphonuclear leukocytes (PMN) and fractions^a

Sample	NADPH oxidized ^b		
	Resting	Phagocytizing	P ^c
Extracellular.....	0.73 ± 0.23 (4) ^d	0.87 ± 0.17 (4)	>0.7
Cell associated.....	6.92 ± 1.23 (6)	13.29 ± 2.43 (6)	<0.05
Homogenate.....	4.00 ± 0.67 (14)	6.60 ± 1.40 (8)	>0.1
Granules ^e	1.39 ± 0.22 (4)	2.39 ± 0.34 (4)	<0.05
Supernatant ^e	1.38 ± 0.41 (5)	3.49 ± 0.71 (4)	<0.05

^a Incubation for 15 min with or without heat-killed *E. coli*; PMN to *E. coli*, 1:100.

^b Expressed as micromoles of NADPH oxidized per minute per 10¹⁰ PMN or fractions.

^c Probability of significance between resting and phagocytizing cells.

^d Mean ± standard error of the mean. Figures in parentheses represent number of experiments.

^e Granules and supernatant fraction resulting from centrifugation at 27,000 × g for 15 min.

TABLE 5. Effect of phagocytosis on the intracellular distribution of G6PDH in guinea pig polymorphonuclear leukocytes (PMN) and fractions^a

Sample	NADPH formed ^b		
	Resting	Phagocytizing	P ^c
Extracellular	4.41 ± 0.65 (7) ^d	15.3 ± 3.40 (6)	<0.01
Cell associated	139.1 ± 8.70 (13)	136.2 ± 8.70 (13)	>0.8
Homogenate	80.8 ± 5.42 (7)	106.7 ± 10.06 (3)	>0.060
Granules ^e	2.99 ± 1.45 (3)	2.31 ± 1.24 (3)	>0.7
Supernatant ^e	67.0 ± 14.40 (5)	82.7 ± 15.90 (4)	>0.4

^a Incubation for 15 min at 37 C with or without heat-killed *E. coli*; PMN to *E. coli*, 1:100.

^b Expressed in micromoles of NADPH formed per minute per 10¹⁰ PMN or fractions.

^c Probability of significance between resting and phagocytizing cells.

^d Mean ± standard error of the mean. Number of experiments are indicated in the parentheses.

^e Granules and supernatant fractions resulting from centrifugation at 27,000 × g for 15 min.

TABLE 6. Effect of phagocytosis on the intracellular distribution of 6PGDH in guinea pig polymorphonuclear leukocytes (PMN) and fractions^a

Sample	NADPH oxidized ^b		
	Resting	Phagocytizing	P ^c
Extracellular	0.45 ± 0.24 (7) ^d	2.09 ± 1.17 (7)	>0.2
Cell associated	30.0 ± 6.30 (3)	23.7 ± 6.30 (3)	>0.5
Homogenate	27.5 ± 2.5 (7)	26.2 ± 4.6 (3)	>0.7
Granules ^e	1.40 (2)	0.39 (2)	
Supernatant ^e	17.0 ± 2.60 (5)	18.3 ± 2.80 (4)	>0.8

^a Incubation for 15 min at 37 C for resting or phagocytizing cells with or without heat-killed *E. coli*. PMN to *E. coli*, 1:100.

^b Expressed as micromoles of NADPH formed per minute per 10¹⁰ PMN or fractions.

^c Probability of significance between resting and phagocytizing cells.

^d Mean ± standard error of the mean. The number of experiments is indicated in the parentheses.

^e Granules and supernatant fractions resulting from 15 min of centrifugation at 27,000 × g.

cellular fractions from resting and phagocytizing cells show little activity.

The effect of phagocytosis on the intracellular distribution of G6PDH is presented in Table 5. The extracellular fraction from the phagocytizing cells has three- to fourfold more activity than the corresponding resting cell fraction. The activity of the other fractions from phagocytizing cells is not significantly different from the corresponding fractions of the resting cells.

Similarly, the intracellular distribution of the enzyme 6PGDH of guinea pig PMN after resting or phagocytosis may be seen in Table 6. The extracellular fraction has some activity. The majority of the activity is in the cell-associated fractions in both resting and phagocytizing cells. On further fractionation, most of the activity is in the supernatant fraction. The granules show minimal activity. Phagocytosis does not seem to effect this enzyme significantly.

DISCUSSION

It has been established that the phagocytosis-associated stimulation in metabolic events is intimately related to the intracellular bactericidal activities of the phagocytes (6, 9, 25, 26, 28, 29; R. R. Strauss et al., *Cancer Res.*, *in press*). The results of this investigation show that H₂O₂, an end product of the stimulated HMS (8, 10, 16, 21, 22, 23), is not only present in all of the different cell types examined, but it is also produced in increased quantity due to particle uptake in most of the cell types studied. It should also be noted that there is considerable variability in H₂O₂ concentrations in the different cell types. This may be related to cellular MPO and catalase.

It has been reported that in addition to H₂O₂, MPO is also involved in the bactericidal activity of the cell (11-15, 17, 18). The MPO levels of resting cells, but not of phagocytizing cells, have

been reported (27). Systematic studies on the effect of phagocytosis on MPO activity of cells from various species, to the best of our knowledge, have not been reported. In this study, we have found that generally an increased activity is associated with phagocytizing cells. Further, the MPO activity of mouse spleen lymphocytes is related to the age of the animal. Spleen lymphocytes from young adult mice (8 to 12 weeks) have threefold higher MPO than those of the one-year-old mice reported here. The bactericidal activity of these lymphocytes is being investigated.

In studies with fractions of guinea pig PMN homogenates, the enzyme appeared to be redistributed in the cell. The supernatant (27,000 × g) fraction of phagocytizing cells was found to have significantly more enzyme than the corresponding fraction obtained from resting cells. Surprisingly, the extracellular MPO activity was noted with both resting and phagocytizing cells. In all cases, however, the activity of preparations from phagocytizing cells was significantly higher than that obtained from resting cells. This activation and release of MPO would make it more available for interaction with H₂O₂ and bacteria. Using human PMN, Baehner et al. (1) showed a 25% increased release of MPO to the extracellular medium. Evans and Rechigl (5) by using 3-amino-1,2,4-triazole noted a 50% inhibition in MPO activity of phagocytizing granules and no inhibition of MPO in resting granules. They interpreted this as stimulation of MPO due to phagocytosis.

In most cell types, H₂O₂ and MPO are present, and one or both are activated during phagocytosis. This suggests that this synergistic bactericidal mechanism of H₂O₂-MPO reported in guinea pig PMN (11, 15) may be of a more general nature. Recently, many investigators have reported the effectiveness of H₂O₂ and MPO as an efficient bactericidal system of phagocytes against a variety of bacteria (2, 14, 17, 18) and fungi (13). This does not preclude the fact that other factors, such as leukin (30), phagocytin (7), or cationic proteins (34), may act in conjunction with, or in addition to, this bactericidal system. It has been proposed that halide ions, in vitro, enhance the bactericidal activity of the H₂O₂-MPO complex (11, 12). The precise mechanism of this halide enhancement of bactericidal activity is under investigation.

Studies on the three enzymes, NADPH oxidase, G6PDH, and 6PGDH (Tables 4-6), help in further understanding of the phagocytosis-associated HMS activity and its relation to the MPO-H₂O₂ bactericidal system. The stimulated

NADPH oxidase may serve to increase the oxidation of NADPH to NADP⁺, resulting in H₂O₂ production (10, 16, 21, 22, 23, 26, 31). The H₂O₂ could activate and combine with MPO (11, 12, 15, 32). The overall activities of the two dehydrogenases, G6PDH and 6PGDH, appear not to be significantly affected by phagocytosis. The only significant effect noted is the 10% release of G6PDH to the extracellular medium of phagocytizing cells, compared to 3% in resting cells. Increase in permeability of the cells due to particle uptake and degranulation might be contributory to this effect. 6PGDH appears to be greater in homogenates prepared from phagocytizing cells; however, the significance is questionable. Further experiments should clarify this.

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