

Brief Definitive Reports

SUPEROXIDE PRODUCTION BY PHAGOCYtic LEUKOCYTES*

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It has recently been shown (1) that the release of superoxide (O_2^-) produced by the one electron reduction of oxygen (2) is increased in human polymorphonuclear leukocytes during phagocytosis. Information on mononuclear phagocytes is lacking in this context. Since superoxide is potentially a microbicidal agent, and since the modes of killing employed by macrophages are still largely unknown, we have extended to those cells observations on superoxide release. Our studies therefore compare O_2^- production by mononuclear phagocytes from lung and peritoneum with polymorphonuclear leukocytes (PMN), in two species.

Furthermore, the rate and extent of ingestion of particles, the metabolic responses of mononuclear phagocytes during ingestion, and the efficiency of bactericidal action have all been shown to be functions of the state of the cells. This is in turn a function of the prior treatment of the animal, e.g. administration of an eliciting agent, or infection with an organism such as *Listeria monocytogenes* to produce a macrophage "activated" by a lymphocyte-mediated phenomenon (3-7). To assess the possible role of superoxide in "activated" macrophages, normal, elicited and activated peritoneal macrophages were examined.

Materials and Methods

Superoxide Determination. Superoxide release was measured by modification of the method used by Babior et al. (1) in which superoxide-dependent cytochrome *c* reduction is determined spectrophotometrically at 550 nm, in the presence or absence of superoxide dismutase, an enzyme that catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (2). It should be stressed that this method determines only the extracellularly released O_2^- , since the detecting agent (cytochrome *c*) and the agent that insures specificity (superoxide dismutase) would not be expected to enter the cells.

Collection of Peritoneal Exudates. Polymorphonuclear leukocytes were harvested from the peritonea of 28-day old mice (CD-1 strain) 6 h after intraperitoneal injection of 3 ml of 12% sodium caseinate. Normal peritoneal macrophages were collected by washing the peritoneal cavities of 25 mice without prior treatment. Elicited macrophages were harvested from 25 mice 3 days after intraperitoneal injection of 1.5 ml of 1.2% sodium caseinate. These methods, and the cellular populations obtained, have been described previously (8). "Activated" macrophages (4) were collected by lavage from the peritoneal cavities of 25 mice 7 days after intravenous injection of 1×10^8 *Listeria monocytogenes* (cf. 4). Alveolar macrophages were released from the lung tissue of 25 mice by a modification of the method of Oren et al. (9) and from guinea pig lung by the method of Myrvik et al. (10). Polymorphonuclear leukocytes were harvested from the peritoneal cavities of guinea pigs 18 h after intraperitoneal injection of 30 ml of 12% sodium caseinate (9). Elicited macrophages were collected from the peritoneal cavities of guinea pigs 3-4 days after injection of 40 ml of 1.2% sodium caseinate (9).

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Measurements on Monolayers. All cells were collected in Hank's balanced salt solution (HBSS). They were washed, diluted with HBSS to approximately 3×10^6 cells/ml, and then allowed to adhere as monolayers on plastic petri dishes for 45 min at 37°C, as described by Michell et al. (11). The monolayers thus formed contained over 90% of the particular cell type in question. After washing the monolayers with HBSS several reaction mixtures containing cytochrome *c* (90 nmol), zymosan particles where appropriate, (approximately 15 particles/cell), superoxide dismutase (130 U, Truett Labs., Dallas, Texas) where appropriate, and serum to a final concentration of 10%, all in a final vol of 1.5 ml were added to duplicate cultures. If serum was omitted from the reaction mixture superoxide values dropped by almost an order of magnitude. One plate of each pair was kept at 0°C while the other was incubated for 30 min at 37°C after which time all supernatant fluids were centrifuged to remove zymosan particles and any cells that might have detached from the petri dishes. Cytochrome *c* reduction was then monitored spectrophotometrically (1). Protein assays were performed on the cells in the culture dishes according to Lowry et al. (12). Oxygen uptake was determined by Warburg respirometry and with the oxygen electrode; the data, except for those on PMN and alveolar macrophages have been reported previously (3, 6).

Results and Discussion

All the mouse cells studied were shown to release superoxide into the medium at rest; PMN were the most active (Table I). During phagocytosis, normal mouse

TABLE I
Superoxide Release by Mouse and Guinea Pig Phagocytic Leukocytes, and its Relation to the Respiratory Burst of Phagocytosis

Cell type	Superoxide release			Respiration§		
	Rest*	Phago- cytosis*	Δ ‡	ΔO_2	% ¶	
Polymorphonuclear leukocyte	Mouse	12.1 14.6	25.3 29.3	13.7	181	8
	Guinea pig	4.8 2.0	37.1 30.3	29.2	81	36
Casein-elicited macrophage	Mouse	4.9 1.6	15.2 18.3	13.5	270	5
	Guinea pig	11.9 16.7	31.1 33.4	18.0	547	3
Normal peritoneal macrophage	Mouse	7.7 6.8	33.6 30.3	24.6	30	82
Listeria-activated macrophage	Mouse	5.0 3.5	14.9 12.3	9.4	168	6
Alveolar macrophage	Mouse	7.2 1.3	64.1 54.3	54.9	—§	—§
	Guinea pig	14.2 11.8	9.7 10.8	0	152	0

* Results of duplicate experiments (two separate batches of cells) are expressed here as nmol cytochrome *c* reduced/mg cell protein/30 min. Values for phagocytosis and rest have been obtained by subtracting cytochrome *c* reduction values in the presence of superoxide dismutase from those in which the enzyme was not included in the incubation mixture.

‡ Represents the average difference in O_2^- release due to phagocytosis.

|| Represents the difference in O_2 consumption due to phagocytosis, expressed as nmoles O_2 /mg cell protein/30 min. Mean values for multiple samples of various types of cell were taken from the literature or newly obtained (see Methods).

§ Respiratory data on mouse alveolar macrophages were not obtained (see text).

¶ Percentage of the respiratory burst which can be accounted for by O_2^- release.

peritoneal macrophages and alveolar macrophages exhibited an increment in O_2^- production that was at least twice that in any of the other cell types from mice. Elicited and "activated" macrophages produced quantities of superoxide that were comparable when at rest, and during phagocytosis.

As can also be seen in Table I, the resting values for guinea pig PMN were considerably lower than those for the mouse polymorphonuclear leukocyte. The increment during ingestion was however much greater than in the case of the mouse PMN. Guinea pig alveolar macrophages, on the other hand, while releasing high levels of superoxide at rest, were shown not to manifest an increment in the release of this agent during phagocytosis. This contrasts with the behavior of the same cell type in the mouse. Resting values for O_2^- release by elicited macrophages of guinea pig were found to be considerably higher than in the mouse, but the superoxide release due to phagocytosis was not strikingly different from that of mouse cells, being about double the resting value.

When one considers the proportion of the respiratory burst that is constituted by the net O_2^- release during phagocytosis, some interesting comparisons emerge. Superoxide release during the ingestion phase in the normal mouse peritoneal macrophage is responsible for over 80% of the relatively small respiratory increase that accompanies phagocytosis (Table I). This is remarkable, since, as mentioned, the methods available detect O_2^- only in the extracellular medium. The data thus indicate rather complete voiding of O_2^- from these cells. The amount of O_2^- actually found extracellularly is a function of the true production of O_2^- , destruction of O_2^- by a specific dismutase (cf. 13-15), and the rate of release of O_2^- from the cells to the external milieu.

The O_2^- release by phagocytizing guinea pig PMN accounts for 35% of the respiratory increment. Phagocytizing mouse polymorphonuclear leukocytes, elicited macrophages (mouse and guinea pig) and "activated" macrophages, on the other hand, show a superoxide release that comprises no more than 8% of the respiratory burst that accompanies ingestion of particles by these cells. Unfortunately, we were unable to gather accurate respiratory data on mouse alveolar macrophages due to difficulties in obtaining enough viable cells for these measurements. The results we did obtain, however, indicate that the respiratory increment due to phagocytosis is not large, in a relative sense, a finding consistent with the situation in guinea pig alveolar macrophages. It appears, in the mouse alveolar macrophages, that a large part of the respiratory burst may be accounted for by superoxide formation. In the guinea pig alveolar macrophage, where there is also a minimal respiratory increment during phagocytosis, there is no release of superoxide (Table I).

All the O_2^- measurements were made using zymosan as the phagocytizable particle, while the respiratory data cited were obtained using particles other than zymosan (3, 6). In our studies on guinea pig alveolar macrophages and polymorphonuclear leukocytes, however, oxygen-consumption did not vary appreciably when zymosan was used in place of polystyrene particles under conditions where uptake was maximal (9).

Treatment of leukocytes with small amounts of surface-active agents such as digitonin (20 $\mu\text{g}/\text{ml}$) has been shown to mimic the respiratory increment of phagocytosis (16). Digitonin also caused a substantial increase in superoxide

release by all cells of the monocytic series; values for superoxide release were found to increase by at least a factor of three. It was not necessary to include serum in the reaction mixture during these experiments.

The possibility exists, as pointed out by Babior et al. (1), and more recently by Yost and Fridovich (17), that an agent as reactive as superoxide might well have importance in leukocytic killing of ingested microorganisms. The theory is not at present well substantiated, and, in fact, Klebanoff (18) has advanced evidence to point out that superoxide is active only after conversion to H_2O_2 , which with myeloperoxidase and halide forms the most potent system, at least in polymorphonuclear leukocytes (19).

As mentioned previously, the precise mechanisms by which cells of the monocytic series kill invading microbes are not known. These cells do not contain appreciable amounts of peroxidase (8) and only recently has lysozyme activity been demonstrated (20). It is conceivable that superoxide, by (enzymatic?) mechanisms as yet undetermined, may play an important role in their microbicidal action. The fact that so large a fraction of the respiratory response of normal macrophages is accounted for by superoxide release sharpens this possibility. On the other hand, on the basis of the data currently available, increased superoxide production can not be invoked to explain the enhanced microbicidal ability of "activated" macrophages. The limitations of the currently available methods, that detect only extracellular O_2^- , however, do not permit one to preclude that possibility.

Apart from questions concerning the intracellular role of O_2^- , especially the question of intracellular localization as it relates to putative antibacterial action, one might also point to the increasing interest in the "secretion" of such potent agents as enzymes (e.g. 21), H_2O_2 (22), and O_2^- (22), and O_2^- (1) by phagocytizing cells. These might attack extracellular substances, or cells, with important consequences including damage to extracellular bacteria.

Summary

Mononuclear phagocytic leukocytes, as well as polymorphonuclear leukocytes, produce and release superoxide at rest, and this is stimulated by phagocytosis. Of the mouse monocytic cells studied, alveolar macrophages released the largest amounts of superoxide during phagocytosis, followed by normal peritoneal macrophages. Casein-elicited and "activated" macrophages released smaller quantities. In the guinea pig, polymorphonuclear leukocytes and casein-elicited macrophages were shown to release superoxide during phagocytosis whereas alveolar macrophages did not. Superoxide release accounted for only a small fraction of the respiratory burst of phagocytosis in all but the normal mouse peritoneal macrophage, the guinea pig polymorphonuclear leukocyte, and probably the mouse alveolar macrophage. There are obviously considerable species differences in O_2^- release by various leukocytes that might reflect both the production and/or destruction (e.g. by dismutase) of that substance.

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References

1. Babior, B., R. Kipnes, and J. Curnutte. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**:741.
2. McCord, J., and I. Fridovich. 1969. Superoxide dismutase. An enzyme function for erythrocyte (hemocuprein). *J. Biol. Chem.* **244**:6049.
3. Karnovsky, M. L., S. Simmons, E. A. Glass, A. W. Shafer, and P. D'Arcy Hart. 1970. Metabolism of Macrophages. In *Mononuclear Phagocytes*. Ralph Van Furth, editor. Blackwell Scientific Publications, Ltd., Oxford. 103.
4. Stubbs, M., A. Kühner, E. A. Glass, J. R. David, and M. L. Karnovsky. 1973. Metabolic and functional studies on activated mouse macrophages. *J. Exp. Med.* **137**:537.
5. Ratzan, K., D. Musher, G. Keusch, and L. Weinstein. 1972. Correlation of increased metabolic activity, resistance to infection, enhanced phagocytosis and inhibition of bacterial growth by macrophages from Listeria- and BCG-infected mice. *Infec. Immun.* **5**:499.
6. Karnovsky, M. L., J. Lazdins, and S. Simmons. 1974. Metabolism of Activated Mononuclear Phagocytes at Rest and During Phagocytosis. Ralph Van Furth, editor. Blackwell Scientific Publications, Ltd., Oxford. In press.
7. Mackaness, G. 1964. The immunological basis of acquired cellular resistance. *J. Exp. Med.* **120**:105.
8. Simmons, S. R., and M. L. Karnovsky. 1973. Iodinating ability of various leukocytes and their bactericidal activity. *J. Exp. Med.* **138**:44.
9. Oren, R., A. E. Farnham, K. Saito, E. Milofsky, and M. L. Karnovsky. 1963. Metabolic patterns in three types of phagocytizing cells. *J. Cell Biol.* **17**:487.
10. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1961. Lysozyme content of alveolar and peritoneal macrophages from the rabbit. *J. Immunol.* **86**:128.
11. Michell, R. H., S. J. Pancake, J. Noseworthy, and M. L. Karnovsky. 1969. Measurements of rates of phagocytosis. The use of cellular monolayers. *J. Cell Biol.* **40**:216.
12. Lowry, O., N. Rosebrough, and A. Farr. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
13. DeChatelet, L. R., C. E. McCall, L. C. McPhail, and R. B. Johnston, Jr. 1974. Superoxide dismutase activity in leukocytes. *J. Clin. Invest.* **53**:1197.
14. Beckman, G., E. Lundgren, and A. Tarnvik. 1973. Superoxide dismutase isozymes in different human tissues, their genetic control and intracellular localization. *Hum. Hered.* **23**:338.
15. Salin, M. L., and J. M. McCord. 1974. Superoxide dismutase in polymorphonuclear leukocytes. *J. Clin. Invest.* **54**:1005.
16. Graham, R. C., M. J. Karnovsky, A. W. Shafer, E. A. Glass, and M. L. Karnovsky. 1967. Metabolic and morphological observations on the effect of surface-active agents on leukocytes. *J. Cell Biol.* **32**:629.
17. Yost, F. J., and I. Fridovich. 1974. Superoxide radicals and phagocytosis. *Arch. Biochem. Biophys.* **161**:395.
18. Klebanoff, S. J. 1974. Role of the superoxide anion in the myeloperoxidase-mediated antimicrobial system. *J. Biol. Chem.* **249**:3724.
19. Klebanoff, S. J. 1974. Antimicrobial systems of the polymorphonuclear leukocyte. Conference on The Phagocytic Cell in Host Resistance. Orlando, Florida. In press.
20. Gordon, S., and Z. Cohn. 1974. Mononuclear Phagocytes. Ralph Van Furth, editor. Blackwell Scientific Publications, Ltd., Oxford. In press.

21. Skosey, J. L., E. Damgaard, D. Chow, and L. Sorrensen. 1974. Modification of zymosan induced release of lysosomal enzymes from human polymorphonuclear leukocytes by cytochalasin B. *J. Cell Biol.* **62**:625.
22. Paul, B., and A. J. Sbarra. 1968. The role of the phagocyte in host-parasite interactions. XIII. The direct quantitative estimation of H_2O_2 in phagocytizing cells. *Biochim. Biophys. Acta.* **156**:168.