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# Identification and Quantitation of Superoxide Anion: Essential Steps in Elucidation of the Phagocyte "Respiratory Burst"

William M. Nauseef

As early as Metchnikoff's observations of cells wandering in starfish (1), investigators have recognized the prominent role of phagocytes in innate host defense against microbial invasion, although the mechanisms by which they kill ingested organisms eluded definition. Between 1930 and 1970, observations from the study of phagocytes in vitro and recognition of the clinical syndrome of chronic granulomatous disease (CGD) converged to link the abrupt increase in $O_2$ consumption by stimulated phagocytes, a phenomenon initially described as a "burst of extra respiration" (2), to phagocyte-mediated antimicrobial action (3, 4). Studies at that time demonstrated that the respiratory burst 1) required an agonist, as resting phagocytes did not generate oxidants, 2) occurred exclusively in phagocytes, and 3) resisted inhibition by cyanide or azide (5, 6), one of the early clues that it did not reflect mitochondrial activation. Studies of the biochemistry of the respiratory burst oxidase engendered substantial controversy, although investigators in the field circa 1970 agreed that the system was flavin- and NADPH-dependent, relied on a functional hexose monophosphate shunt, and released hydrogen peroxide extracellularly (7). The composition, subcellular location, biochemistry, and regulation of the respiratory burst oxidase, however, all remained undefined.

The seminal discovery of superoxide dismutase (SOD) by McCord and Fridovich (8) made it possible to examine and quantitate its substrate, superoxide anion. Superoxide anion arises from the single electron reduction of molecular oxygen ($O_2$), resulting in an unstable product with an unpaired electron ($O_2^{\cdot-}$). Perhaps because the name of the anion augured potent bactericidal action, Bernie Babior et al. (9) chose to determine whether stimulated human neutrophils generated $O_2^{\cdot-}$. In only four pages, two figures, and one table, they reported that neutrophils fed latex particles promoted agonist-dependent, SOD-inhibitable reduction of ferricytochrome *c*. Because each molecule of reduced ferricytochrome *c* reflects the action of a single electron, and the inclusion of SOD limits consideration to only $O_2^{\cdot-}$-mediated events, Babior et al. simultaneously developed a specific assay to quantitate phagocyte-generated extracellular $O_2^{\cdot-}$ and identified the proximal product of the phagocyte NADPH oxidase (NOX). Although the latter observation provided a critical advance in the field, assignment of substantial antimicrobial action to $O_2^{\cdot-}$ proved off the mark, as $O_2^{\cdot-}$ exhibits reactivity in aqueous solution that falls short of "super" (10) and exerts little, if any, bactericidal action (11). Rather, subsequent studies demonstrated that $O_2^{\cdot-}$ serves as progenitor of hydrogen peroxide (12), which the granule protein myeloperoxidase efficiently consumes to generate hypochlorous acid (13, 14), a highly reactive species that chlorinates and kills ingested microbes (15). The presence of multiple interacting reactive agents in phagosomes and the variability in vulnerable targets in microbes have made identification of the precise chemistry of antimicrobial action within phagocytes an unmet challenge (16, 17).

The ferricytochrome *c* assay system and the identification of the proximal product of the phagocyte oxidase catalyzed several advances in the field, three of which are highlighted here. First, elucidation of the nature and composition of the phagocyte oxidase followed from Babior et al.'s report. Extending earlier published observations that neutrophils from rabbits and horses possess an unusual *b*-type cytochrome that could operate in the phagocyte oxidase (18, 19), Segal et al. (20) demonstrated that membranes from normal human neutrophils contain cytochrome $b_{245}$, which is absent from patients with X-linked CGD. In a series of elegant studies between 1978 and 1987, Segal provided evidence that cytochrome $b_{245}$ serves as the electron transferase in the superoxide-generating system of phagocytes, a hypothesis that provoked significant controversy (e.g., see Refs. 21, 22). However, the Orkin laboratory proved Segal correct, identifying the membrane protein lacking in X-CGD neutrophils as flavocytochrome *b*, now known as gp91$^{phox}$, as essential for oxidase activity (23). Containing two nonidentical heme groups stacked in the membrane (24), gp91$^{phox}$ shuttles electrons from cytoplasmic NADPH via FAD across the membrane to molecular oxygen to generate $O_2^{\cdot-}$.

Second, the availability of a quantitative assay of activity provided a tool essential to gauge enzyme activity as investigators attempted to purify the $O_2^{\cdot-}$ generating system from membrane-enriched preparations of stimulated phagocytes. However, technical challenges that rendered the material

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Abbreviations used in this article: CGD, chronic granulomatous disease; NOX, NADPH oxidase; SOD, superoxide dismutase.



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unstable and rapidly inactivated crippled progress (25). While analyzing the role of unsaturated fatty acids in promoting $O_2^{\cdot-}$ production by guinea pig macrophages, Bromberg and Pick (26) developed a cell-free oxidase system whereby cytosol and membranes from resting phagocytes, each alone unable to support oxidase activity, are combined under defined conditions to support FAD-, NADPH-dependent $O_2^{\cdot-}$ generation (reviewed in detail in Ref. 27). Soon after that report, three other laboratories independently developed cell-free systems and confirmed that under very specific conditions, $O_2^{\cdot-}$ generation could be supported by combining cytosol and membranes from resting phagocytes (28–30). Coupled with the ferricytochrome *c* assay, use of the cell-free system made possible the discovery of cytoplasmic proteins required for normal phagocyte oxidase activity (reviewed in Ref. 31). With the identification of deficiencies in $p47^{phox}$ or $p67^{phox}$ in neutrophils of nearly all patients with autosomal CGD, the genotypic basis for all known forms of CGD was defined (32).

Third, recognition of the phagocyte oxidase as an $O_2^{\cdot-}$ generator led to the unanticipated realization that the system was not an exclusive property of phagocytes. With quantitation of $O_2^{\cdot-}$ production by transfectants expressing a homolog of $gp91^{phox}$, the family of NOX proteins was conceived (33). According to contemporary understanding, and in stark contrast to earlier views, most tissues possess at least one member of the NOX protein family, and many cells express more than one NOX protein, often segregated into distinct subcellular compartments (reviewed in Ref. 34). Because many of the novel NOX proteins generate oxidants intracellularly, precise quantitation poses a challenge to investigators, creating an opportunity for another seminal observation in oxidase biology, one on equal footing with Babior et al.'s (9) brief communication in 1973.

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