# Role of Oxygen in Phagocyte Microbicidal Action

### Robert C. Allen

Research and Development Division, ExOxEmis, Inc., Little Rock, Arkansas

Immune information in the form of inflammatory mediators directs phagocyte locomotion and increases expression of opsonin receptors such that contact with an opsonized microbe results in receptor ligation and activation of microbicidal metabolism. Carbohydrate dehydrogenation and  $O_2$  consumption feed reactions that effectively lower the spin quantum number (*S*) of  $O_2$  from 1 to 1/2 and finally to 0. Oxidase-catalyzed univalent reduction of  $O_2$  (*S* = 1; triplet multiplicity) yields hydrodioxylic acid (HO<sub>2</sub>) and its conjugate base superoxide,  $O_2^-$  (*S* = 1/2; doublet multiplicity). Acid or enzymatic disproportionation of superoxide yields  $H_2O_2$  (*S* = 0; singlet multiplicity). Haloperoxidase catalyzes  $H_2O_2$ -dependent oxidation of CГ yield-ing HOCl (*S* = 0), and reaction of HOCl with  $H_2O_2$  yields singlet molecular oxygen,  ${}^{1}O_2$  (*S* = 0; singlet multiplicity). The Wigner spin conservation rule restricts direct reaction of  $S = 1 O_2$  with S = 0 organic molecules. Lowering the *S* of  $O_2$  overcomes this spin restriction and allows microbicidal combustion. High exergonicity dioxygenation reactions yield electronically excited carbonyl products that relax by photon emission, i.e., phagocyte luminescence. Addition of high quantum yield substrates susceptible to spin allowed dioxygenation, i.e., chemiluminigenic substrates, greatly increases detection sensitivity and defines the nature of the oxygenating agent. Measurement of luminescence allows high sensitivity, real-time, and substrate-specific differential analysis of phagocyte dioxygenating activities. Under assay conditions where immune mediator and opsonin exposure are controlled, luminescence analysis of the initial phase of opsonin-stimulated oxygenation activity allows functional assessment of the opsonin receptor expression per circulating phagocyte and can be used to gauge the *in vivo* state of immune activation. — Environ Health Perspect 102(Suppl 10): 201–208 (1994)

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## Phagocyte Reduction and Oxygenation Activities

Phagocyte microbicidal action is a dynamic, metabolically linked process whereby reduction potential provides the driving force to convert molecular oxygen  $(O_2)$  into effective oxygenating agents. Increased phagocyte metabolism of glucose through the dehydrogenases of the hexose monophosphate shunt in combination with increased nonmitochrondrial O2 consumption is collectively referred to as phagocyte respiratory burst metabolism (1-3). Glucose dehydrogenation generates the equivalents for univalent reduction of O<sub>2</sub>, that in turn initiate the transducing reactions required for phagocyte generation of oxygenating agents. Microbe killing results from the reactions of these metabolically generated oxygenating agents with molecular components of the target microbe.

On initial consideration, the mobilization of reduction potential might seem an unlikely initial step in the direction of phagocyte generation of oxygenating agents. This apparent paradox is resolved by taking a quantum mechanical perspective. As predicted by the Pauli exclusion principle, the  $O_2$  we breathe is a diradical molecule with one electron occupying each of its two pi antibonding ( $\pi^*$ ) orbitals, and according to Hund's maximum multiplicity rule, both electrons have the same spin quantum number (s), i.e., both electrons will have an s value equal to 1/2 or -1/2(4). As such, the total or net spin quantum number (S) of  $O_2$  is either 1 or -1. Multiplicity is a spectroscopy expression related to S by the equation |2S|+1. Thus, in its lowest energy or ground state,  $O_2$  is a triplet multiplicity diradical molecule, i.e., |2(1)|+1=3, and is highly paramagnetic (5).

### Spin Conservation

Symmetry must be conserved in all chemical reactions. The Wigner spin conservation rules define the tendency of a reacting system to conserve spin angular momentum; i.e., the total orbital angular momentum of all the electrons of a reacting system is conserved (5-7). This conservation principle is illustrated by the Wigner–Witmer correlation rules presented in Table 1 (8). The more familiar Woodward–Hoffman rules define the conservation of orbital angular momentum of each electron separately. Reaction allowedness and feasibility require conservation of both total and individual symmetries (9).

Reactions of organic and biologic molecules with  $O_2$  are typically of very high

exergonicity, but such reactions do not occur spontaneously. Consider the heat of reaction calculations for the reaction of  $O_2$  with the double bond of ethylene (10):

- $\{C_2H_4 (+ 146 \text{ kcal/mole}) \rightarrow 2 \text{ CH}_2\}$  [1]
- $\{O_2 (+ 119 \text{ kcal/mole}) \rightarrow 2 \text{ O}\}$  [2]

{2

Net  $\Delta G = -93$  kcal/mole (equivalent to an einstein of 308 nm photons) [4]

This oxygenation reaction yields free energy magnitudinally greater than that typically considered in bioenergetics. The concept of spin conservation is at the crux of understanding why such high exergonicity reactions do not spontaneously occur. Ethylene, like other typical organic and biologic molecules, is a singlet multiplicity S=0 molecule, and as described in Table 1, the direct reaction of an S=0molecule with S=1 O<sub>2</sub> would proceed via a S=1 reaction surface to yield an S=1oxygenation product. Absolute reaction rate theory predicts a low probability for any reaction involving change in multiplicity (11,12). The transmission coefficient of the absolute reaction rate

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Address correspondence to Robert C. Allen, 3215 Woodcrest, San Antonio, TX 78209-3138. Telephone (210) 822-5091. Fax (210) 822-0948.

Table 1. The Wigner-Witmer spin correlation rules.

Reactant A	Reactant B	Potential energy surface	
Singlet $(S = 0)$ Doublet $(S = 1/2)$ Triplet $(S = 1)$ Singlet $(S = 0)$ Triplet $(S = 1)$ Singlet $(S = 0)$ Doublet $(S = -1/2)$	Singlet $(S = 0)$ Doublet $(S = -1/2)$ Triplet $(S = -1)$ Doublet $(S = 1/2)$ Doublet $(S = -1/2)$ Triplet $(S = 1)$ Quartet $(S = 11/2)$	Singlet $(S = 0)$ Singlet $(S = 1/2 - 1/2 = 0)$ Singlet $(S = 1 - 1 = 0)$ Doublet $(S = 1/2)$ Doublet $(S = 1/2)$ Triplet $(S = 1)$ Triplet $(S = -1/2 + 11/2 = 1)$	
Doublet $(S = -1/2)$	Quintet $(S = 2)$	Quartet $(S = -\frac{1}{2} + 2 = \frac{1}{2})$	

equation gauges the spin allowedness of a reaction (7).

### **Chemical Combustion**

We all have a first-hand experience with the phenomenon of burning. Hold a lit match to a piece of paper and the paper will burn. In this case, the match provides the activation energy required to spread the fire to the paper, i.e., to initiate combustion. Although ethylene does not spontaneously react with  $O_2$ , combustion is readily initiated with a spark. Even highly exergonic chemical combustion can require relatively large activation energies. Once initiated, the energy liberated by reaction maintains propagation.

Chemical combustion can be best understood as a radical (S > 0) propagation reaction. Initiation of burning requires the application of energy sufficient to produce homolytic cleavage of the organic molecular bonds, i.e., to convert the S=0 substrate molecule (<sup>1</sup>substrate) into S=1/2 reactants (<sup>2</sup>reactants):

<sup>1</sup>substrate + heat 
$$\rightarrow 2^{-2}$$
 reactants. [5]

The superscript preceding the symbol indicates the multiplicity of the symbolized reactant. The doublet multiplicity (S = 1/2) radicals produced by homolytic cleavage can react with triplet multiplicity (S = 1)  ${}^{3}O_{2}$  by radical-radical orbital overlap to yield covalent bonding,

<sup>2</sup>reactant + <sup>3</sup>O<sub>2</sub> 
$$\rightarrow$$
 <sup>2</sup>product +  $\Delta$ G [6]

as described in Table 1. The S=1/2 radical product of this oxygenation plus the free energy liberated ensure additional reaction with S=1 triplet multiplicity  ${}^{3}O_{2}$ ,

<sup>2</sup>product + <sup>3</sup>O<sub>2</sub> 
$$\rightarrow$$
 <sup>2</sup>product +  $\Delta$ G [7]

to produce S=1/2 radical products as required for radical propagation, i.e., continued burning, until the substrate or  ${}^{3}O_{2}$  is depleted. Reaction with another S=1/2 radical,

<sup>2</sup>product + <sup>2</sup>reactant 
$$\rightarrow$$
 <sup>1</sup>product +  $\Delta G$  [8]

i.e., radical-radical annihilation (S = 1/2-1/2 = 0), terminates the reaction and yields a singlet multiplicity product as shown in Table 1.

### Microbicidal Combustion: Changing the Spin Quantum Number of O<sub>2</sub>

Phagocytes exert a broad and lethal microbicidal action using the simple ingredients of metabolically generated reduction potential and O2. The character of respiratory burst metabolism suggests the possibility that phagocyte microbicidal action might involve combustionlike oxygenation reactions, but are phagocytes energetically and mechanistically capable of changing the spin quantum number of  $O_2$  from S=1to S=1/2, and finally to S=0? Doing so would overcome the symmetry restrictions to O<sub>2</sub> reactivity and, as such, the electrophilic reactive potential for direct O<sub>2</sub> reactivity would be realized. Chemical combustion (burning) requires radicalization of substrate, i.e., increasing the value of S, to guarantee reaction with triplet multiplicity O2. Phagocyte microbicidal combustion requires deradicalization of O2, i.e., decreasing the value of S, to guarantee its reaction with singlet multiplicity organic molecules (13). Note that reaction by either pathway satisfies the spin conservation requirements.

Activation of phagocyte respiratory burst metabolism is linked to lowering the Michaelis constant of membrane-associated NADPH:O<sub>2</sub> oxidoreductase for its reduced substrate NADPH (2). The univalent reduction of O<sub>2</sub> catalyzed by this flavoprotein oxidase (14-17):

$$2^{2}H (NADPH + H^{\dagger}) + 2^{3}O_{2} \rightarrow$$
$$2^{2}HO_{2} \leftrightarrow 2^{2}O_{2}^{-} + 2H^{\dagger}$$
[9]

is the first step in lowering the S value or multiplicity of O<sub>2</sub>. Hydrodioxylic acid (<sup>2</sup>HO<sub>2</sub>; perhydroxylic acid) and its conjugate base superoxide (<sup>2</sup>O<sub>2</sub><sup>-</sup>), the products of univalent reduction, are doublet multiplicity S = 1/2 molecules. Hydrodioxylic acid has a  $pK_a$  of 4.8, and its generation by the activated oxidase may be a major factor in the dynamic acidification of the relatively small phagolysosomal space (18). When the pH of the space is 4.8, the ratio of <sup>2</sup>HO<sub>2</sub> to <sup>2</sup>O<sub>2</sub> is unity. As the pH approaches the  $pK_a$ , the anionic repulsion that prevents direct disproportionation of <sup>2</sup>O<sub>2</sub> is no longer a barrier to reaction, and as such, the doublet-doublet annihilation reaction,

$${}^{2}\text{HO}_{2} + {}^{2}\text{O}_{2}^{-} + \text{H}^{+} \rightarrow {}^{1}\text{H}_{2}\text{O}_{2} + {}^{1}\text{O}_{2}$$
 [10]

approaches maximum rate, i.e.,  $8 \times 10^7$  M<sup>-1</sup>sec<sup>-1</sup> (19). Note that Equation 10 is the same type of radical termination reaction described by Equation 8. If the reaction is a direct annihilation through an S=0surface, both H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> will be in the S=0, singlet multiplicity state as described in Table 1 (20). On the other hand, an indirect stepwise disproportionation involving high multiplicity catalysts, e.g., superoxide dismutase (21), can yield the mixed spin products <sup>1</sup>H<sub>2</sub>O<sub>2</sub> and ground state <sup>3</sup>O<sub>2</sub> (22).

Superoxide  $({}^{2}O_{2}^{-})$  can also participate in doublet-doublet annihilation reaction with nitric oxide ( ${}^{2}NO$ ), another S = 1/2reactant,

$$^{2}NO + ^{2}O_{2}^{-} \rightarrow ^{1}OONO^{-}$$
 [11]

to yield the peroxynitrite anion. As predicated by the spin symmetry conservation rules, neither  ${}^{2}O_{2}^{-}$  nor  ${}^{2}NO$  reacts readily with S=0 organic or biologic molecules, but since both have a common radical S=1/2 symmetric character, they readily react with each other. The reported rate constant for Reaction 11 is  $4 \times 10^{7}$  $M^{-1}sec^{-1}$  (23,24). Radical annihilation via a S=0 surface generates a product, i.e., peroxynitrite, with S=0 symmetry in common with most organic and biologic molecules, and as such, symmetry is not a barrier to direct reaction of peroxynitrite with these S=0 molecules (25).

### Haloperoxidase Activity

Although metabolically costly, the reduction of  $O_2$  to  $H_2O_2$  is necessary to eliminate the S=1 character that limits its direct reactivity.  $H_2O_2$ , a S=0 molecule, can participate in spin allowed reactions with S=0organic and biologic molecules. However,  $H_2O_2$  is a weak acid with a  $pK_a$  of 11.65, and in its protonated form,  $H_2O_2$  is relatively unreactive. The direct reactive capacity of  $H_2O_2$  increases with alkalinity, e.g., the Dakin reaction. At a physiologic pH of 7, the  $HO_2^-/H_2O_2$  ratio is approximately 1/100,000 and, as such, the actual concentration of  $HO_2^-$  available as a microbicidal oxygenating agent is modest.

Granulocytic leukocytes and blood monocytes contain haloperoxidases, i.e., myeloperoxidase and eosinophil peroxidase, that exert a lethal microbicidal action (26). These  $H_2O_2$ :halide oxidoreductases catalyze the oxidation of halide (X) to hypohalous acid (HOX) and the reduction of  $H_2O_2$  to  $H_2O$  (27,28). This oxidation-reduction reactive coupling can be described in the Nernst equation:

$$\Delta E = \frac{RT}{nF} ln \left( \frac{[H_2O][HOX]}{[X^-][H_2O_2][H^+]} \right)$$
[12]

 $\Delta E$  is the change in potential (volts), R is the gas constant, T is the absolute temperature, n is the number of electrons per gram-equivalent transferred, F is a faraday, and *ln* is the natural log of the reactants and products as shown (29). The influence of pH and type of halide on the  $\Delta E$  and  $\Delta G$  of the reaction is shown in Table 2. The exergonicity of either chloride (Cl<sup>-</sup>) or bromide (Br) oxidation, the primary reaction catalyzed by haloperoxidase, increases directly with proton availability. In the secondary reaction of Table 2, HOX, the product of the primary reaction, reacts with a second molecule of  $H_2O_2$  to yield singlet molecular oxygen (30). Note that for this secondary reaction, exergonicity is directly related to halogen electronegativity and inversely related to proton availability.

When  $[H_2O_2]$  is relatively low, some HOX can accumulate and react with biologic molecules, e.g., chloramine formation. Since both HOX production and consumption are dependent on  $[H_2O_2]$ , and since the reaction of HOX with  $H_2O_2$  is essentially diffusion controlled, it is likely that most HOX will be consumed in the secondary reaction. It is also likely that chloramines can react with  $H_2O_2$  to yield  ${}^{1}O_2$ .

The first-order relaxation of  ${}^{1}\Delta g O_{2}$  to ground state  ${}^{3}O_{2}$  yields a 1268-nm photon (30), and measurement of this infrared emission has been taken as direct spectroscopic proof of  ${}^{1}O_{2}$  generation by haloperoxidase (31). A 1268-nm emission has also been reported from phorbol myristate acetate (PMA) stimulated eosinophil leukocytes but not from neutrophil leukocytes (32). The problem of documenting  ${}^{1}O_{2}$ generation in biologic systems is illustrated by the following riddle. What is definitive evidence for  ${}^{1}O_{2}$  generation and what is

definitive evidence that the <sup>1</sup>O<sub>2</sub> generated has not participated in reaction chemistry? The answer to both questions is the same: 1268-nm emission. Photon emission from chemically unreacted <sup>1</sup>O<sub>2</sub> represents wasted energy. On the other hand, the reaction of <sup>1</sup>O<sub>2</sub> with appropriate substrates can yield high energy endoperoxide and dioxetane products that can relax by photon emission in the visible range of the spectrum yielding chemiluminescence (33). Conditions that maximize <sup>1</sup>O<sub>2</sub> reactivity, and thus killing efficiency, are the same conditions where little or no 1268-nm emission is likely to be observed. Introducing microbes to a 1268-nm light-generating system will inhibit infrared emission in proportion to the <sup>1</sup>O<sub>2</sub> consumed in the microbicidal reaction.

The problem of  ${}^{1}O_{2}$ -specific detection has recently been overcome by the development of an 9,10-diphenylanthracene (DBA)-based assay for the measurement of  ${}^{1}O_{2}$  in complex biologic systems. Using DBA-coated beads and phorbol 12-myristate 13-acetate (PMA; 1.6 pmole/ml) as stimuli, Steinbeck et al. reported the generation of 14 nmole  ${}^{1}O_{2}/1.25 \times 10^{6}$  neutrophils. This quantity was equal to approximately 20% of  ${}^{3}O_{2}$  consumption by the activated neutrophils (34). Using Equations 9 and 10, there is the possibility that one  ${}^{1}O_{2}$  and

 
 Table 2. Haloperoxidase generation of hypohalous acid and singlet molecular oxygen: the effect of halide electronegativity and pH.

	Primary reaction								
	${}^{1}\text{H}_{2}\text{O}_{2}$ + ${}^{1}\text{X}^{-}$ + H <sup>+</sup> —XPO $\rightarrow$ ${}^{1}\text{HOX}$ + ${}^{1}\text{H}_{2}\text{O}$								
pН	$EH_2O_2$	-	ECI <sup></sup>	=	$\Delta E_1$	$\Delta G_1$			
4	1.5396	-	1.3760	=	0.1636	-7.53			
5	1.4805	-	1.3465	=	0.1340	-6.16			
6	1.4214	-	1.3170	=	0.1044	-4.80			
7	1.3623	-	1.2875	=	0.0748	-3.44			
pН	EH <sub>2</sub> O <sub>2</sub>	-	EBr <sup>-</sup>	=	ΔE <sub>1</sub>	$\Delta G_1$			
4	1.5396	-	1.2130	=	0.3266	-15.02			
5	1.4805	-	1.1835	=	0.2970	-13.66			
6	1.4214	-	1.1540	=	0.2674	-12.30			
7	1.3623	-	1.1245	=	0.2378	-10.94			
	Secondary reaction								
	$^{1}\text{HOX} + {}^{1}\text{H}_{2}\text{O}_{2} \rightarrow \text{H}^{+} + {}^{1}\text{X}^{-} + {}^{1}\text{O}_{2} + {}^{1}\text{H}_{2}\text{O}$								
pН	EHOCI		$\rm EH_2O_2$	=	$\Delta E_2$	$\Delta G_2$			
4	1.3760	-	0.4456	=	0.9304	-20.27			
5	1.3465	-	0.3865	=	0.9600	-21.63			
6	1.3170	-	0.3274	=	0.9896	-22.99			
7	1.2875	-	0.2683	=	1.0192	-24.35			
pН	EHOBr	-	EH <sub>2</sub> O <sub>2</sub>	=	$\Delta E_2$	$\Delta G_2$			
4	1.2130	_	0.4456	=	0.7674	-12.77			
5	1.1835	-	0.3865	=	0.7970	-14.13			
6			0.0074		0 0000	15 40			
0	1.1540	-	0.3274	=	0.8200	-15.49			

one  $H_2O_2$  are produced per two  ${}^3O_2$  consumed. Based on the primary and secondary reactions of Table 2, one  $^{1}O_{2}$  is expected to be generated per two H<sub>2</sub>O<sub>2</sub> consumed. If  $H_2O_2$  production is proportional to the  $^{3}O_2$ consumed, a maximum of three <sup>1</sup>O<sub>2</sub> could be generated for each four <sup>3</sup>O<sub>2</sub> consumed. Thus consumption of 72 nmole of  ${}^{3}O_{2}$ could yield a maximum of 54 nmole <sup>1</sup>O<sub>2</sub>. The actual measured yield was 14 nmole O<sub>2</sub> for an estimated efficiency of 26%. If the haloperoxidase activity described in Table 2 is solely responsible for generating <sup>1</sup>O<sub>2</sub>, one <sup>1</sup>O<sub>2</sub> could be generated for each two H<sub>2</sub>O<sub>2</sub> reacted and by extension for each two <sup>3</sup>O<sub>2</sub> consumed. Therefore, consumption of 72 nmole of  $^{3}O_{2}$  could yield a maximum of 36 nmole <sup>1</sup>O<sub>2</sub>, and thus the estimated efficiency is 40%.

## Luminescence as an Energy Product of Oxygenations

Generation of photons in the visible range of the spectrum requires highly exergonic reactions. For example, 650-nm (red) photons and 400-nm (blue) photons have  $\Delta G$ 's equivalents to -44 and -72 kcal/mole photon, respectively. With few exceptions, biochemical reactions do not yield  $\Delta G$ 's sufficient to generate such photons. Oxygenations, O<sub>2</sub> reactions yielding O<sub>2</sub>containing products, are such exceptions. In the previous example (Equations 1-4), dioxygenation of ethylene was shown to yield a net  $\Delta G$  of -93 kcal/mole. Biologic dioxygenations of carbon-carbon double bonds yielding endoperoxide, dioxetane, or dioxetanone intermediate, and ultimately vielding aldehyde or ketone products, can generate exergonicity sufficient for electronic excitation and ultimately photon emission in the visible (700-340 nm photons) range of the spectrum. The transformation of  $O_2$  from the S=1 to the S=1/2and ultimately to the S=0 state effectively removes the mechanistic barrier to dioxygenation. Thus, the electrophilic potential for direct oxygenation is realized.

Luminescence is an energy product of phagocyte microbicidal activity (13). In fact, phagocyte luminescence was first observed during experiments to test the hypothesis that changing the S of  $O_2$  from 1 to 1/2 to 0 was required for effective  $O_2$ dependent microbicidal action. Lowering the S of  $O_2$  from 1 to 0 would involve transductions driven by respiratory burst metabolism. If the resulting low multiplicity reactants participate in microbicidal oxygenation reactions, then it is likely that a portion of the reactions proceed via endoperoxide or dioxetane intermediates to yield excited carbonyl products that can relax by photon emission. The observation of luminescence from activated phagocytes provides an energy-based confirmation of the important role of oxygenations in phagocyte microbicidal action.

### Chemiluminigenic Probing: Differential Measurement of Oxygenation Activities

Although of theoretical importance, there are several limitations to using native luminescence to quantify phagocyte oxygenation activity. Typically, native substrates are of relatively low luminescence quantum yield, and the nature of the substrate varies with the microbe and the test condition. A chemiluminigenic substrate (C) is an organic molecule susceptible to dioxygenation with high quantum (luminescence) yield. Introducing a C to the phagocyte test systems increases the luminescence yield magnitudinally (35,36). Using a C with relatively well-established reactive characteristics also defines the nature of the oxygenating agent (X) measured (37). As such, the luminescence intensity (dL/dt) is functionally and quantitatively linked to both the concentration of oxygenating agent ([X]) generated by the phagocyte and the concentration of C ([C]) available for reaction:

$$dL/dt = k[X][C]$$
 [13]

where k is the proportionality constant. When the [C] is much greater than the [X], the reaction order approximates zero with respect to C, i.e., C is not rate limiting, and the relationship simplifies to:

$$dL/dt = k[X]$$
 [14]

Phagocyte oxidase function can be measured as the luminescence product of dimethylbiacridinium (DBA<sup>++</sup>; lucigenin) dioxygenation (38). Under alkaline conditions, i.e., as the pH approaches the pKa of  $H_2O_2$ , lucigenin reacts directly with peroxide,

$${}^{1}\text{DBA}^{2*} + {}^{1}\text{HO}_{2}^{-} ({}^{1}\text{O}_{2}^{2-}) \rightarrow$$

$$2 {}^{1}N\text{-methylacridone} + \text{photon}$$
[15]

Under neutral to mildly acid conditions, DBA<sup>++</sup> can be univalently reduced resulting in change from the S=0 to the S=1/2 state,

$${}^{1}\text{DBA}^{2+} + e^{-} ({}^{2}\text{O}_{2}^{-}) \rightarrow {}^{2}\text{DBA}^{+} + ({}^{3}\text{O}_{2})$$
[16]

In its radicalized form, <sup>2</sup>DBA<sup>+</sup> can directly react with <sup>2</sup>O<sub>2</sub> in a doublet-doublet annihilation via a S = 0 surface,

$$^{2}\text{DBA}^{+} + ^{2}\text{O}_{2}^{-} \rightarrow$$

2<sup>1</sup>*N*-methylacridone + photon [17]

One of the two *N*-methylacridone produced will have an excited ketone carbonyl function that can relax to ground state by photon emission. Note that both the onestep peroxidation reaction and the two-step radical intermediate reaction with  ${}^{2}O_{2}^{-}$ yield the same product. Recall that the disproportionation of  ${}^{2}O_{2}^{-}$  to yield  ${}^{1}H_{2}O_{2}$  is second order with respect to superoxide, i.e.,  $[{}^{2}O_{2}^{-}]^{2}$ . At physiologic pH, the DBA<sup>++</sup> reaction is also either second order with respect to  ${}^{2}O_{2}^{-}$  or a mixed second order reaction, i.e., first order with respect to  $[{}^{2}O_{2}^{-}]$ .

DBA<sup>++</sup> does not react with HOCl or  $^{1}\text{O}_{2}$  to yield luminescence and, as such, DBA<sup>++</sup> is not a substrate for the measurement of haloperoxidase activity. In fact, these oxidizing and dioxygenating agents will tend to competitively inhibit oxidase-dependent DBA<sup>++</sup> luminescence. The net reaction by any of the above considered pathways is:

$$DBA^{2*} + O_2 + 2 e^- \rightarrow$$
  
2<sup>1</sup>N-methylacridone + photon  
[18]

and, as such, can be considered as a reductive dioxygenation (RDOX) reaction.

The net dioxygenation of a cyclic hydrazide, e.g., luminol, to yield luminescence does not require electrons or reducing equivalents. Haloperoxidase-containing phagocytes can catalyze dioxygenation of S=0 luminol via the sequential two equivalent oxidation:

<sup>1</sup>luminol + <sup>1</sup>HOCl 
$$\rightarrow$$
  
<sup>1</sup>diazaquinone + <sup>1</sup>Cl<sup>-</sup> + <sup>1</sup>H<sub>2</sub>C  
[19]

followed by a two equivalent reductive dioxygenation:

<sup>1</sup>diazaquinone + <sup>1</sup>H<sub>2</sub>O<sub>2</sub>  $\rightarrow$ <sup>1</sup>aminophthalate + <sup>1</sup>N<sub>2</sub> + photon [20]

The possibility also exists that  ${}^{1}O_{2}$  or a related singlet multiplicity dioxygenating agent, e.g., O-O-Cl<sup>-</sup>, can directly dioxygenate luminol in a nonradical, spinallowed manner to yield electronically

excited aminophthalate. This pathway is suggested by the electrophilic reactivity of  ${}^{1}O_{2}$  with  $\pi$ -systems and its tendency to form dioxetanes and endoperoxides (36). Although presently unproved, the haloperoxidase catalyzed dioxygenation of luminol might also proceed by the reaction:

<sup>1</sup>luminol + <sup>1</sup>O<sub>2</sub> 
$$\rightarrow$$
  
<sup>1</sup>aminophthalate + <sup>1</sup>N<sub>2</sub> + photon  
[21]

The essential relatedness of the luminol luminescence reactions catalyzed by either mechanistic pathway can be appreciated by recalling that the reaction of HOCl with  $H_2O_2$ , the sequential reactants of Equations 19 and 20, yields  ${}^1O_2$ , the reactant of Equation 21. Note the secondary reaction of Table 2.

Haloperoxidase-deficient macrophages also catalyze the luminol luminescence reaction, but the specific activity per phagocyte is greatly decreased (35). Luminol dioxygenation by macrophages (39), and possibly a portion of the activity of some haloperoxidase-containing phagocytes, may involve NO synthase-generated reactants such as peroxynitrite ( $^{1}OONO^{-}$ ), the product of Equation 11; e.g.,

luminol + <sup>1</sup>OONO<sup>-</sup> 
$$\rightarrow$$
 <sup>1</sup>aminophthalate  
+ <sup>1</sup>NO<sup>-</sup> + <sup>1</sup>N<sub>2</sub> + photon [22]

As an additional possibility, luminol luminescence may also be catalyzed by a sequential radical mechanism involving  ${}^{2}O_{2}^{-}$  as a reductive oxygenating agent (36). Such reaction requires the initial radical dehydrogenation of luminol by a radical oxidant such as hydroxyl radical:

<sup>1</sup>luminol + (univalent oxidant, e.g., <sup>2</sup>OH)  

$$\rightarrow$$
  
<sup>2</sup>luminol<sup>+</sup> + (reduced oxidant, e.g., <sup>1</sup>H<sub>2</sub>O)  
[23]

Once generated, the univalently oxidized luminol radical can now participate in a doublet-doublet annihilation reaction with superoxide:

<sup>2</sup>luminol<sup>+</sup> + <sup>2</sup>O<sub>2</sub><sup>-</sup> 
$$\rightarrow$$
 <sup>1</sup>aminophthalate  
+ <sup>1</sup>N<sub>2</sub> + photon [24]

Evidence has been presented that classic (halide-independent) peroxidases can also catalyze the radical dehydrogenation of luminol, ultimately resulting in luminol dioxygenation and luminescence (40,41). By whatever mechanism, and in contrast with the net DBA<sup>++</sup> reaction described above, the net luminol reaction responsible for luminescence is a simple dioxygenation (DOX) reaction,

luminol + 
$$O_2 \rightarrow$$
  
aminophthalate +  $N_2$  + photon [25]

and as such, provides a measure of the phagocyte DOX capacity.

### Estimating Phagocyte Opsonin Receptor Reserve: Theory

The humoral-phagocyte axis of the inflammation response is essentially an information-effector system directed to the task of killing pathogenic microbes. Tissue injury or infection elicits the synthesis and release of immunologic information in the form of cytokines and other inflammatory mediators. These mediators affect the expression of membrane receptors as required for effective phagocyte–endothelial contact, diapedesis and locomotion through the interstitial space to the site of injury or infection.

Expression of phagocyte opsonin, i.e., complement and immunoglobulin, receptors increases with the level of exposure to a diverse range of inflammatory mediators, including complement-derived C5a (complement anaphylatoxin), cell-derived platelet activator factors (PAF), leukotrienes, tumor necrosis factors (TNF), colony-stimulating factors (CSF), and certain interleukins such as IL-8, as well as microbe-derived factors such as N-formylmethionyl peptides. It should be emphasized that opsonin receptors are rapidly expressed on exposure to mediators and that this increased expression does not require neosynthesis or prolonged incubation. Opsonin receptor expression can be increased with little or no activation of respiratory burst metabolism. However, opsonin-opsonin receptor ligation triggers respiratory burst metabolism. Under properly controlled conditions of testing, phagocyte opsonin receptor expression can be functionally linked to the acceleration phase of the respiratory burst and measured as luminescence (42-44).

Phagocyte opsonin receptor expression is dependent on exposure to inflammatory mediators,

$$R/P = k[P][I]$$
 [26]

where R/P is the opsonin receptor (R) expressed per phagocyte (P), k is the proportionality constant, [P] is the concentration of phagocytes and [I] is the concentration of inflammatory mediators capable of increasing opsonin receptor expression. [Imax] is a concentration of inflammatory mediator sufficient to produce maximum opsonin receptor expression Rmax/P.

Phagocyte receptor ligation of opsonins, such as complement and/or immunoglobulin-coated microbes, activates respiratory burst metabolism yielding oxygenating agents (X),

$$dX/dt = k[R/P][P][O] = k[R][O]$$
 [27]

where dX/dt is the rate of oxygenating agent generation, [O] is the concentration of opsonin, and the other components are as previously described. If the conditions of testing are set such that [O] is much greater than [R], the equation simplifies to,

$$dX/dt = k[R].$$
 [28]

Since dL/dt is a function of [X], it follows that

$$dL/dt = k[R].$$
 [29]

A commercially available luminescence system is now available for measurement of basal and stimulated phagocyte oxidase and oxidase-driven haloperoxidase activities. The system also measures both the circulating opsonin receptor expression (CORE) and the maximum opsonin receptor expression (MORE) per phagocyte. The CORE value is obtained by testing a micrometer quantity of unmodified blood under conditions where [O] and [C] are nonlimiting; i.e.,

$$dL/dt = k[R] = k[P][Iin vivo]$$
 [30]

The MORE value is obtained by simultaneously testing an equivalent quantity of the same blood specimen with the same nonlimiting concentrations of O and C but with sufficient inflammatory mediator, i.e., *Imax*, to ensure maximum opsonin receptor expression but minimum azurophilic degranulation and minimum direct activation of respiratory burst metabolism. The resulting luminescence response to O represents maximum receptor activity,

$$dL_{max}/dt = k[R_{max}] = k[P][I_{max}]$$
[31]

The luminescence responses L and  $L_{max}$  reflect the CORE and MORE, respectively, and can be presented as a ratio, the CORE/MORE ratio or inflammatory index (42–44). Low ratio values reflect minimum *in vivo* exposure to inflamma-

tory mediators. The ratio increases to reflect the level of *in vivo* exposure to inflammatory mediators. The ratio can also be presented in reciprocal form as the percentage opsonin receptor reserve (%ORR) per phagocyte,

$$1 - (L/L_{max}) \times 100 = \% ORR$$
 [32]

The %ORR decreases in proportion to the degree of *in vivo* stimulation.

### Estimating Phagocyte Opsonin Receptor Reserve: The Application

### Analysis of Phagocyte Opsonin Receptor Expression

Materials and Methods. Basal and stimulated phagocyte oxidase and oxidase-driven haloperoxidase activities were measured with the AXIS luminescence system and reagents (ExOxEmis Inc., San Antonio, TX). Both the CORE and the MORE per phagocyte were also measured using this system. The K<sub>3</sub>EDTA-anticoagulated whole blood collected for complete blood count with differential was kept at ambient temperature (20-23°C) and tested within 2 hr of venipuncture. Just prior to measurement, 100 µl of whole blood was added to 9.9-ml blood-diluting medium (BDM, an AXIS reagent) for a 1:100 dilution. The diluted blood was loaded into the luminometer (Berthold LB953 AXIS modified, Wildbad, Germany) for automatic injection into prefabricated test tubes containing the indicated coating of inflammatory mediator or PMA. Tubes coated with PMA were used for opsonin receptorindependent measurement of oxidase and oxidase-driven haloperoxidase activities.

On initiation of measurement, the luminometer injected 600 µl of either luminol balanced salt solution (LBSS, an AXIS reagent) or dimethylbiacridinium (DBA<sup>++</sup>, i.e., lucigenin) balanced salt solution (DBSS, an AXIS reagent), and 100 µl of diluted blood, i.e., 1 µl equivalent of whole blood, into each tube. LBSS and DBSS contain non-rate-limiting quantities of luminol and DBA<sup>++</sup> as the respective chemiluminigenic substrate (C) to ensure that the luminescence response reflects the quantity of oxygenating agent generated by the activated leukocyte and not the availability of C. All of the luminescence measurements were in triplicate and were taken over a 20-min interval (approximately 1 measurement/1.5 min). The absolute polymorphonuclear neutrophil leukocyte (PMN) count was used to calculate the specific luminescence activity expressed as total counts/PMN/20 min. Alternatively, specific activity can be expressed as counts/phagocyte/20 min. The total phagocyte count is the total leukocyte count minus the lymphocyte count.

Opsonin receptor-dependent oxidasedriven MPO activity was measured with LBSS as the C medium. Human complement opsonized zymosan (hC-OpZ, an AXIS reagent) was used as the opsonin. Response to this opsonin was tested in the absence and presence of sufficient inflammatory agent to produce maximum neutrophil opsonin receptor expression. The data presented in Table 3 were obtained using uncoated tubes, recombinant human C5a-coated tubes (C5a, 20 pmole tubes, an AXIS reagent) and PAFcoated tubes (PAF, 10 pmole tubes, an AXIS reagent) with 100 µl complementopsonized zymosan (10<sup>8</sup> zymosan particles per test).

Figure 1 presents the plot of luminescence velocity, dL/dt, expressed as megacounts per minute (CPM×10°) (the ordinate) versus time in minutes (the abscissa) for both CORE (lower curve) and C5a-MORE (upper curve) responses to O, hC-OpZ. The leukocyte count was 5100/µl with an absolute differential count of 3200 segmented PMN, 205 monocytes, 51 eosinophils and 1630 lymphocytes/µl. Each microliter of blood tested contained 3200 PMN and 3456 phagocytes. The %ORR is calculated from the initial 10-min integral response. During this initial period, activation is causally linked to opsonin receptor expression. After this initial period, phagocyte metabolic capacity begins to exert a limiting effect on the luminescence response. The initial integral CORE (i.e., L) response is  $2.6 \times 10^6$  counts/10 min, and the C5a-MORE ( $L_{max}$ ) response is  $13.0 \times 10^6$  counts/10 min. Therefore, the inflammatory index is 0.20 and the %ORR is 80. This %ORR value is within the normal range of  $77 \pm 10$  (SD) for healthy subjects (n = 20). Table 3 presents oxidase-dependent and oxidase-haloperoxidase-dependent (both opsonin receptor dependent and independent activities) integral 20-min specific luminescence responses for the same blood specimen. Imax primed, hC-OpZ-stimulated phagocytes typically show peak dL/dt by 15 min. Once peak dL/dt is reached, phagocyte metabolic capacity exerts a limiting effect. As such, the 20-min MORE response reflects metabolic capacity. The MORE responses are essentially the same with either C5a or PAF as immunologic modulator.

Table 3. Whole blood luminescence analysis.

С	I	0 or PMA	Na azide	L,	
medium	immune primer	stimulus	inhibitor	Cts/PMN/20 min	Phagocyte function measured
LBSS	None	None	None	13	Basal DOX activity
LBSS	C5a, 20 pmole	None	None	22	C5a-primed DOX activity
LBSS	PAF, 10 pmole	None	None	35	PAF-primed DOX activity
LBSS	None	hC-OpZ	None	2,596	CORE activity
LBSS	C5a, 20 pmole	nC-OpZ	None	12,354	C5a-MORE activity
LBSS	PAF, 10 pmole	hC-OpZ	None	11,385	PAF-MORE activity
LBSS	None	PMA, 5 nmole	None	1,844	PMA-stimulated DOX activity
LBSS	None	None	None	19	Basal RDOX activity
LBSS	None	PMA, 10 pmole	None	387	PMA-stimulated RDOX activity
					Azide inhibition, %
LBSS	None	None	5 nmole	15	-15
LBSS	C5a, 20 pmole	None	5 nmole	20	9
LBSS	PAF, 10 pmole	None	5 nmole	22	37
LBSS	None	hC-OpZ	5 nmole	464	82
LBSS	C5a, 20 pmole	hC-OpZ	5 nmole	2,426	80
LBSS	PAF, 10 pmole	hC-OpZ	5 nmole	2,501	78
LBSS	None	PMA, 5 nmole	5 nmole	370	80
DBSS	None	None	5 nmole	21	-11
DBSS	None	PMA, 10 pmole	5 nmole	423	-9

Reaction initiated by addition of 1 µl blood suspended in 99 µl diluting medium to the prefabricated tubes as indicated. Each µl blood contained 3200 segmented PMS leukocytes, 1600 lymphocytes, 200 monocytes, and 50 eosinophils. The tubes contained the indicated quantity of primer or PMA, plus 600 µl LBSS or DBSS and, where indicated, 100 µl hC-0pZ. The blood was tested in triplicate measurements within 2 hr of venipuncture. AXIS reagents were used throughout and measurements were taken with a Berthold LB953 luminometer.



Figure 1. The CORE and C5a-MORE luminescence responses of 1 liter blood with LBSS as C and hC-OpZ (10<sup>8</sup> particles) as 0. Reaction was initiated by injection of diluted blood at time zero. All specimens were tested in triplicate.

Relatively low concentrations of azide  $(N_3^-)$  inhibit haloperoxidase (37,45). The selective inhibitory effects of sodium azide (5 nmole/test; 6  $\mu$ M final) on the LBSS and DBSS luminescence responses of PMN to O and PMA stimulation are presented in the lower portion of Table 3. The effects of azide on the CORE and C5a-MORE raw luminescence responses are presented in Figure 2. Although a final concentration of 6  $\mu$ M of azide inhibits luminescence by approximately 80%, the

%ORR is increased slightly from 80 to 86%. This small increase may reflect a greater susceptibility of CORE activity to azide inhibition. As shown in Table 3, azide also inhibits PMA-stimulated LBSS luminescence by approximately 80%.

Azide does not inhibit DBSS luminescence in response to PMA (10 pmole/test). As shown in Table 3, azide produces a mild augmentation that probably reflects inhibition of MPO-mediated oxidation activity which can interfere with DBA<sup>++</sup>



Figure 2. The conditions were the same as described for Figure 1, except that 5 nmole of sodium azide was included per test (6  $\mu$ M azide final concentration) as a myeloperoxidase inhibitor.



reductive dioxygenation. The effect of azide on the raw DBSS luminescence responses from PMA-stimulated blood is illustrated in Figure 3. The lower and upper curves depict the responses to PMA (10 pmole/test) in the absence and presence of azide, respectively.

All of the luminescence data presented were obtained from less than 0.5 ml blood and required less than 1 hr for setup, measurement, and analysis. Luminescence testing allows rapid, high sensitivity differential analysis of phagocyte oxygenation activities. If the conditions of analysis are properly controlled, opsonin receptor expression can also be measured, and since opsonin receptor expression is in proportion to the degree of inflammatory mediator exposure, analysis of the circulating phagocyte provides a gauge of in vivo inflammation. The AXIS system is ultimately designed for use in patient diagnosis and management (46,47). However, the system also has broad potential for assessing inflammation and antiinflammatory activity using in vivo and in vitro models (48).

Figure 3. The temporal luminescence responses of 1  $\mu$ l blood with DBSS as C and PMA (10 pmole/test) as chemical stimulus. The upper and lower curves depict the luminescence responses in the presence and absence of sodium azide (5 nmole/test; 6  $\mu$ M final), respectively.

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