Review Article

ROLE OF OXYGEN-DERIVED FREE RADICALS AND METABOLITES IN LEUKOCYTE-DEPENDENT INFLAMMATORY REACTIONS

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REVIEW ARTICLE

Role of Oxygen-Derived Free Radicals and Metabolites in Leukocyte-Dependent Inflammatory Reactions

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SINCE the initial observations of Metchnikoff in the late 1800s, describing the presence of phagocytic cells in tissues, a large body of information has accumulated defining the roles of phagocytes in acute and chronic inflammatory reactions. When a host tissue is challenged by a pathologic insult of either an immunologic or nonimmunologic nature, an inflammatory reaction may occur, with subsequent clearance of the pathologic stimulus by phagocytic cells. Tissue injury may result from either the direct effects of the pathologic agent or as a consequence of an inflammatory cell influx. Three of the most extensively studied of the phagocytic cells are the polymorphonuclear leukocyte (PMN), the tissue macrophage, and the circulating monocyte. Each of these cells plays a key role in acute and/or chronic inflammatory responses.

When pathogenic agents are formed or deposited in host tissues, circulating neutrophils and/or monocytes may be recruited from the intravascular compartment to the site of insult by the local generation of soluble mediators. The process by which the cells migrate from the circulation to an inflammatory site is thought to involve the mechanism of directed cell migration, chemotaxis (reviewed,[1,2]). *In vitro* studies have shown that when neutrophils and monocytes are exposed to a chemotactic agent, the cell orients itself and migrates in the direction of increasing concentration of the chemical stimulus. In addition, many of the particulate and soluble phlogistic mediators generated at sites of inflammation have been shown to activate the metabolic processes of phagocytic cells, a consequence of which is an increase in the capacity of these cells to ingest pathogenic substances. This activation process may be initiated as a result of binding of mediators to specific receptors on the cell surface, or it may be initiated by the process of phagocytosis of particulate material.

The biochemical mechanisms involved in cellular activation and phagocytosis are complex and reviewed in detail elsewhere.[3,4] Briefly, when a foreign particle is deposited in host tissue, it may or may not be coated with specific host proteins such as immunoglobulins and/or complement, dependent on its organic makeup. These proteins, defined as opsonins, can enhance recognition of the particle by phagocytes through binding to specific receptors on the phagocyte cell surface (eg, Fc receptor, C3b receptor). After recognition, these foreign particles are engulfed by the phagocyte and internalized within cytoplasmic vacuoles, to which are fused lysosomal granules, forming the phagolysosome. These fused granules discharge into the phagocytic vacuole potent enzymes capable of degrading a wide variety of biologic substances, including bacterial cell membranes, collagen, elastin, and mucopolysaccharides. The process of fusion of the lysosomal granule with the phagosome is known as degranulation. It should be noted that degranulation to the exterior of the cell (fusion with the cell membrane) can be initiated in the absence of particulate stimuli, by interaction of surface receptors of neutrophils with chemotactic peptides, although cell pretreatment with cytochalasin B is usually necessary.

Upon recognition of a phagocytic or soluble stimulus, both neutrophils and macrophages experience a "respiratory burst," which is characterized by an in-

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crease in oxygen consumption, activation of the hexose monophosphate shunt, and generation of reactive oxygen-derived free radicals and their metabolic products. *In vitro* studies have shown that during the process of phagocytosis neutrophils, monocytes, and tissue macrophages release proteases and oxygen metabolites into the external tissue environment.[3,4]

Several studies have demonstrated that lysosomal neutral proteases, especially elastase, can cause tissue injury such as in experimental models of emphysema. It has been postulated that the tissue effects of elastase may be in part responsible for the pathologic alterations at inflammatory sites.[5-7] However, recent studies have shown that tissue injury by activated phagocytic cells cannot be entirely inhibited by antiproteases, nor is tissue injury diminished in mice whose leukocytes are deficient in neutral proteases, suggesting that alternative mechanisms (involving nonprotease factors) are involved.[8-10] The focus of this review will be the current evidence implicating the role of oxygen-derived free radicals and their metabolites in neutrophil- and macrophage-mediated tissue injury.

Production of Oxygen Radicals by Neutrophils and Macrophages

In response to activation of neutrophils and macrophages by particulate and/or specific soluble inflammatory mediators, these cells undergo a respiratory burst. This is associated with a 2–20-fold increase in oxygen consumption and increased glucose metabolism via the hexose monophosphate shunt, depending on the cell and the nature of the stimulus.[11-16] In conjunction with an increase in oxygen consumption, neutrophils and macrophages have been shown to

Table 1 — Superoxide and Hydrogen Peroxide Production by Macrophages

			O_2^- Production†‡ (nmole/mg protein/30 min)		H_2O_2†‡ (nmole/mg protein/30 min)	
Cell	Source	Stimulus	Non-stimulated	Stimulated	Non-stimulated	Stimulated
Macrophage	Guinea pig	PMA	0.6	75.0	<1.0	49.9
	Oil-elicited [30]	Con A	0.7	19.5	<1.0	7.3
		PHA	1.0	3.2	–	–
		WGA	1.1	23.3	<1.0	70.0
		f-met-leu-phe	0.8	20.0	–	–
		Ionophore A23187	0.3	12.7	–	–
		Opsonized zymosan	1.0	18.3	–	–
Monocyte	Human	PMA	–	172.0	–	438
	Peripheral blood [70]	Opsonized zymosan	–	99.5	–	306
Macrophage	Mouse peritoneal [19]					
	Resident	PMA	1.0	16.3	–	–
		Opsonized zymosan	1.0	77.7	–	–
	Thioglycolate-elicited	PMA	2.0	164.3	–	–
		Opsonized zymosan	2.0	101.3	–	–
	LPS-elicited	PMA	0.0	42.3	–	–
		Opsonized zymosan	0.0	140.3	–	–
	BCG-elicited	PMA	0.3	172.6	–	–
		Opsonized zymosan	0.3	101.6	–	–
Macrophage	Mouse [12]					
	Resident	Opsonized zymosan	7.7	33.6	–	–
	Casein-elicited	Opsonized zymosan	4.9	15.2	–	–
	Listeria-activated	Opsonized zymosan	5.0	14.9	–	–
Polymorphonuclear leukocyte	Mouse [12]	Opsonized zymosan	12.1	25.3	–	–
	Guinea pig	Opsonized zymosan	4.8	29.3	–	–
Alveolar macrophage [208]*	Rabbit	Opsonized zymosan	0.58	1.44	0.4	0.28
Alveolar macrophage [209]*	Human					
	Nonsmoker	PMA	10.06	28.4	–	–
		Heat-killed bacteria	10.6	18.4	–	–
	Smoker	PMA	19.2	58.0	–	–
		Heat-killed bacteria	19.2	31.4	–	–

* Values expressed as nmoles/10^6 cells/30 min.
† Values are normalized /mg protein/30 min from several different experiments.
‡ Values may vary due to different experimental conditions.
PMA = phorbol myristate acetate; Con A = concanavalin A; WGA = wheat germ agglutinin.

Table 2—Superoxide and Hydrogen Peroxide Production by Polymorphonuclear Leukocytes

			O_2^- Production (nmole/10^6 PMN/15 min)		H_2O_2 (nmole/10^6 PMN/15 min)	
Cell	Source	Stimulus	Nonstimulated	Stimulated	Nonstimulated	Stimulated
PMN	Human[24]	Opsonized zymosan	1.2	6.9	–	–
		C5a	1.2	4.2	–	–
		IgG-aggregated	1.2	3.0	–	–
PMN	Human[23]	Opsonized *Staphylococcus aureus*	–	11.7	–	1.4
		Opsonized *Staphylococcus aureus* + Cytochalasin B	–	23.2	–	2.26
PMN	Rabbit[16]	F-met-leu-phe	0.3	4.8	0.0	5.4
		F-met-leu-phe + Cytochalasin B	0.5	19.0	0.3	7.5

All values are normalized per 10^6 PMN/15 min.
Values may vary due to experimental conditions.

secrete both superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2).[12-19] A partial summary of the various particulate and soluble mediators that have been shown to induce metabolic activation of phagocytic cells is shown in Tables 1 and Table 2. These activating substances include bacteria,[20-22] opsonized zymosan,[12,23] immunoglobulins and immune complexes,[19,23-26] the chemotactic peptide derived from C5, C5a,[24] synthetic oligopeptides such as N-formylmethyionyl-leucyl-phenylalanine, f-Met-Leu-Phe,[16,26-29] and phorbol myristate acetate, PMA, a nonspecific membrane activator.[30-33] The most potent of these activating agents are opsonized phagocytic particles and PMA. Each of these substances has been shown to induce secretion of nanomole quantities of O_2^- and H_2O_2 after stimulation of 1-10 million neutrophils and macrophages. The chemotactic peptides f-Met-Leu-Phe and C5a induce significant secretion of O_2^- and H_2O_2 into the extracellular environment only when cells are pretreated with cytochalasin B, an inhibitor of microfilaments.[16,23] In recent studies, potent chemotactic and leukoagglutinating activities have been associated with lipoxygenase products of arachadonic acid metabolism.[34,35] However, these biologically active lipids do not appear to stimulate either O_2^- or H_2O_2 production by phagocytic cells.[36,37]

Recent studies have shown that most (>90%) of the oxygen consumed by neutrophils after initiation of the respiratory burst can be accounted for by O_2^- secretion.[23,27,38] When cytochalasin-B–treated neutrophils are incubated with opsonized staphylococci in the presence of O_2^- scavengers (ferricytochrome or nitroblue tetrazolium), there is a marked reduction in H_2O_2 release from the cells. These results suggest that most of the H_2O_2 released during phagocytosis is directly derived by dismutation of O_2^-.

The enzyme system responsible for the increased oxygen consumption and O_2^- generation has been identified as a membrane-associated nicotinamide adenine dinucleotide (NADP[H], NAD[H]) oxidase (Figure 1).[39-43] There has been great debate in recent years concerning the location and nature of the membrane-associated O_2^- oxidase system. Although definitive data are not yet available concerning the precise location and biochemical characterization of the O_2^--forming oxidase system in phagocytic cells, there appear to be at least two identifiable components. The first is a substrate-binding moiety with specificity for reduced nicotinamide adenine dinucleotide; the second component oxidizes the reduced form in the presence of oxygen, generating a reduced form of molecular oxygen, O_2^-. Several investigators have suggested that the oxidase system of the neutrophil is located at least in part on the external surface of the plasma membrane, accounting for the release of substantial amounts of O_2^- into the external environment after cell stimulation. However, recent studies by Babior et al[42] indicate that the pyridine nucleotide binding site may be located on the cytoplasmic surface of the plasma membrane, while the O_2^--forming oxidase component may be located within the lipid bilayer with only a portion exposed to the cytoplasmic surface. This proposal is more attractive, because NADPH and NADH are present within the cytoplasm, and O_2^- appears to be generated either within or on the external surface of the plasma membrane. Additional studies have shown that the cofactor-binding site has a greater affinity for NADPH than NADH, and several authors have suggested that the intracellular ratio of NADPH to NADH may be critical in the regulation of O_2^- formation.[40]

Although the production by activated phagocytic cells of O_2^-, H_2O_2, and subsequent metabolites have been confirmed by several investigators, it is difficult to assess the biologic significance of the reactive molecules without an understanding of their chemical na-

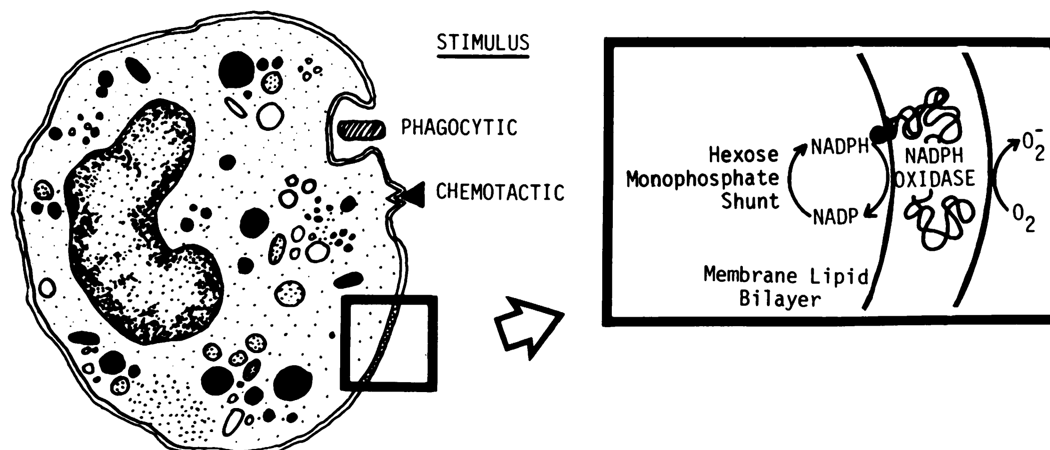


Figure 1 – Proposed mechanism of activation and location of membrane-associated NADPH oxidase in phagocytic cells.

ture and mechanisms of formation. When oxygen accepts an electron from a reducing agent, the chemical nature of the molecule may change to produce superoxide anion or a protonated form, $HO_2^{\cdot}$.[44,45] Since the pKa of ionization is 4.8, the reduced form of oxygen exists as the superoxide anion at neutral pH. However, within an acidic environment, such as a phagolysosome, the protonated form ($HO_2^{\cdot}$) of O_2^- would predominate.

Superoxide anion may act as either an oxidant or a reductant, depending on the substrate with which it reacts. Reduction of ferricytochrome C or nitroblue tetrazolium represents a sensitive assay for the detection of the presence and the production of O_2^- by phagocytic cells.[18,46] It should also be noted that O_2^- may act as an oxidant, gaining an electron, to form H_2O_2, such as in the oxidation of epinephrine to adrenochrome. This chemical reaction represents a third method for detecting the presence of O_2^-.[47]

When two molecules of O_2^- react with each other, one is oxidized and the other is reduced, forming hydrogen peroxide and oxygen in a dismutation reaction. Spontaneous dismutation of O_2^- occurs most rapidly at a pH equal to the pKa of ionization.[45] At pH 4.8 and at equilibrium, $HO_2^{\cdot}$ and O_2^- are of equal concentration, and the rate of reaction is maximum. Since the nonprotonated form (O_2^-) of superoxide anion predominates in environments of neutral pH, very little H_2O_2 is formed as a result of spontaneous dismutation. At neutral pH, enzymatically catalyzed dismutation of O_2^- by (superoxide dismutase) is a more important mechanism for generating H_2O_2. The significance and location of superoxide dismutase activity is discussed in following sections.

Mechanisms for the generation of H_2O_2 from O_2^- have been described above. A third mechanism also exists by which H_2O_2 may be formed by phagocytic cells. This involves the direct double reduction of oxygen without the intermediate formation of O_2^-. An example of this reaction involves the formation of H_2O_2 by the reaction between glucose and glucose oxidase.[48] This is in contrast to formation of H_2O_2 by the xanthine–xanthine oxidase reaction, in which hydrogen peroxide may be formed either by direct double reduction of O_2 or by dismutation of superoxide anion.[49,50]

Several additional highly reactive oxygen derived metabolites have been identified or predicted to exist as a result of activation of phagocytic cells. These include hydroxyl radical ($OH\cdot$),[51–54] singlet oxygen ($^1O_2$),[55–60] and hypochlorous acid (HOCl).[61–63] The proposed mechanism of $OH\cdot$ formation is the result of the interaction of O_2^- and H_2O_2 in the modified Haber–Weiss reaction, also known as the Fenton reaction.[45,48,64] The classic Haber–Weiss reaction involves the direct reduction of H_2O_2 by O_2^- with the formation of $OH\cdot$, O_2, and OH^-.[65,66] However, this reaction occurs very slowly under most physiologic conditions, and it is an unlikely source of significant quantities of hydroxyl radical. In the Fenton reaction, O_2^- first reacts with an oxidized form of a trace metal, causing reduction of the metal and generation of O_2. The reduced form of the metal then reacts with H_2O_2, regenerating the initial oxidized metal and forming OH^- and hydroxyl radical. This reaction is depicted in Figure 2.

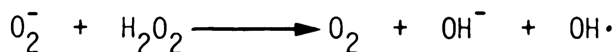
A recent study has shown that lactoferrin (an iron-binding protein present in specific granules of neutrophils) can increase $OH\cdot$ production *in vitro*, presumably by its ability to provide iron as a catalyst for the reduction of H_2O_2 by O_2^-.[67] Therefore, components of the lysosomal granules can potentially contribute to the generation of oxygen-derived free radicals; and, because lactoferrin is released into the extracel-

lular environment during activation of neutrophils, this may represent an important mechanism in the potentiation of cell and tissue injury by phagocyte-derived O_2^-.

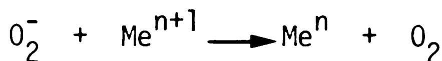
A second mechanism for the generation of OH· may result from the reaction of O_2^- with hydroperoxides formed by lipid peroxidation.[15] However, this source of OH· is dependent on the initial oxidation of lipids, resulting in formation of lipid peroxides, which then react with O_2^-. This process of OH· radical production would appear to behave as an amplification mechanism for the continued production of OH· after initial lipid peroxidation has occurred. Which of the two proposed mechanisms of OH· generation is more significant *in vivo* is uncertain.

Singlet oxygen is a strong electrophilic molecule and is formed when molecular oxygen absorbs a significant quantity of energy to cause a shift of one of its two unpaired electrons of similar spin to an orbital position of higher energy, with inversion of spin of one of the electrons.[68] When the excited electron forms an electron pair and occupies the same orbital, delta singlet oxygen ($\Delta\ {}^1O_2$) is formed. Delta singlet oxygen is relatively stable having a half-life ($t_{1/2}$) in water of approximately 2 microseconds. When the excited electron remains unpaired (occupying a different orbital), a second form of singlet oxygen is formed, designated as sigma singlet oxygen (Σ^1O_2). Sigma singlet oxygen is less stable than delta singlet oxygen and has a shorter $t_{1/2}$. The potent reactivity of singlet oxygen and its potential for indiscriminant interaction with other molecules is the result of its great instability. When singlet oxygen decays to the more stable form of molecular oxygen, energy is released as the excited electron returns to its thermodynamically stable configuration. This decay may result in chemical reactions with another molecule, release of thermal energy, or light emission at specific wave lengths.

Classical Haber-Weiss Reaction



Modified Haber-Weiss Reaction
(Fenton Reaction)



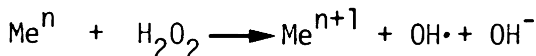


Figure 2—Mechanisms of generation of hydroxyl radical from superoxide anion and hydrogen peroxide.

Under certain conditions chemiluminescence can occur through the interaction of singlet oxygen with a specific molecule causing secondary excitation of that molecule. Subsequent decay of the excited molecule can result in light emission. This has been proposed as a mechanism for the chemiluminescence associated with activation of phagocytic cells.[55–59, 68, 69]

In vitro studies have shown that singlet oxygen can also be formed as a result of the interaction of H_2O_2 with hypochlorite (see below).[58,59] However, additional studies have failed to demonstrate definitively the generation of singlet oxygen by a myeloperoxidase (MPO) system. Because this reaction is not favorable under most physiologic conditions,[60] it is unlikely that significant generation and/or secretion of singlet oxygen by an MPO mechanism occurs either in phagolysosomes or in the extracellular environment.

Additional reactive metabolites can be formed as a result of the metabolism of H_2O_2 by cellular enzymatic systems. Both neutrophil- and macrophage-derived enzymes have been shown to metabolize H_2O_2 in the presence of halide, forming additional potent oxidants. The most important reaction involves leukocyte myeloperoxidase reacting with H_2O_2 to form an enzyme substrate complex. This complex has the capacity to oxidize various halides (eg, iodide [I^-], chloride [Cl^-]) producing highly reactive toxic products.[60–63] Because of the wide distribution of chloride ion in biologic systems, the formation of hypochlorous acid (HOCl) by the MPO–H_2O_2 complex is probably the most significant reactant.

The amount of O_2^- and H_2O_2 produced by neutrophils and macrophages is both species- and stimulus-specific and will vary with specific experimental conditions.[11–19] Recent studies have demonstrated significant variation in oxygen consumption and free radical production by macrophages, depending on their state of activation and site of isolation. Mouse alveolar macrophages have been shown to produce approximately 50% more O_2^- than resident peritoneal macrophages during phagocytosis of zymosan particles.[12] Whether this represents an adaptive response of the alveolar macrophage because it is exposed to higher O_2 tensions, compared with resident macrophages, is not known.

When circulating blood monocytes are cultured *in vitro*, there is a significant loss by 3 days in the production of O_2^- and H_2O_2.[70] After 2 weeks of culture *in vitro* there is only minimal production of O_2^- and H_2O_2 by these cells. This decrease in the ability of cultured monocytes to secrete reactive oxygen metabolites correlates with their differentiation into macrophages, epithelioid histiocytes, and giant cells, suggesting that oxygen-derived metabolites may play a

significant role in tissue injury only during the early period of macrophage activation. Further investigations have shown that isolated peritoneal macrophages in suspension produce significantly less O_2^-, compared with adherent cells.[71] In addition, after phagocytosis of zymosan particles, both thioglycollate and bacille Calmette-Guérin (BCG)-elicited peritoneal macrophages produce 2–3 times more O_2^- when compared with resident peritoneal macrophages. There is approximately a 15-fold increase in O_2^- production by these cells following stimulation with PMA.[19] Therefore, the production of oxygen metabolites by macrophages is dependent not only on the site of isolation but also on the state of activation and the extent of differentiation of the cells.

Several assays for measuring the production of O_2^- have been described. Perhaps the most common assay involves monitoring the rate of reduction of ferricytochrome C to ferrocytochrome C by O_2^-. The formation of ferrocytochrome C can be measured by its absorbance at either 550[18] or 418 nanometers,[72,73] which is approximately 3–4 times more sensitive but less specific. A second method for detecting the presence of O_2^- is the reduction of nitroblue tetrazolium as measured by absorbance at 516 nanometers.[74,75] One can determine the specificity of the reduction of each of these molecules by O_2^- by measuring the reduction of these reagents in the presence and absence of superoxide dismutase. The difference between the amount of reduction of substrate in the presence and absence of superoxide dismutase reflects the actual contribution of O_2^-. Each of these assays has been shown to be capable of detecting nanomole quantities of O_2^-.

H_2O_2 generation by phagocytes can be measured by several techniques. These include the generation of O_2 from H_2O_2 by catalse,[76] oxidation of formate ^{14}C- by catalase[77] or oxidation by horseradish peroxidase of several substrates (eg, phenol,[30] scopoletin[22]), and the oxidation of ferrocyanide to ferricyanide.[78] These assays appear to be capable of detecting nanomole quantities of H_2O_2.

Several assays for measuring OH· production by activated phagocytes have recently been reported. These include conversion of dimethylsulfoxide to methane,[53] detection of ethylene formation from methional 2-keto-4-methylthiobutyric acid by gas chromatography,[51,52] and electron spin trapping techniques with substrates such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).[54] The measurement of singlet oxygen production by the conversion of 2,5-diphenylfuran to cis-dibenzoylethylene has also been reported.[58] However, unlike the assays for O_2^- and H_2O_2, where specificity can be determined by inhibition with specific enzymes (superoxide dismutase [SOD] and catalase, respectively), each of the assays described above for hydroxyl radical and singlet oxygen is not necessarily specific. Special care must be taken to exclude the possibility that contaminating oxidants or free radicals may produce factitious values for rates of hydroxyl radical and singlet oxygen production.

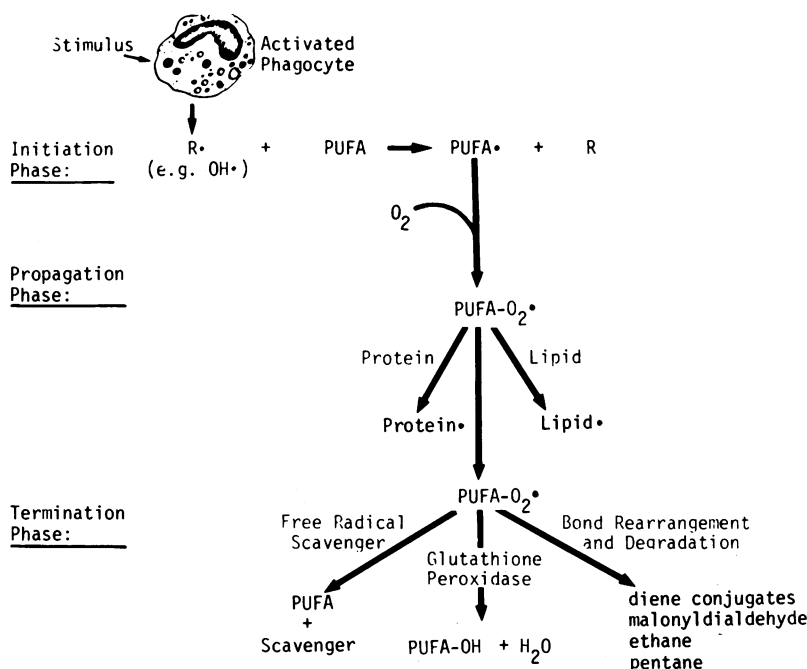


Figure 3—Free radical mediated lipid peroxidation, *PUFA*, polyunsaturated fatty acid.

Frequently free radical scavengers have been used in inhibition of the assays described above to implicate specific free radicals in chemical reactions.[51,52,54,58,59] These scavengers incliude thiourea, tryptophan, sodium benzoate, mannitol, ethanol, histidine, and 2,3-diphenylfuran. However, free radical scavengers generally are not specific, and conclusions derived from experiments with these substances are only valid under carefully controlled experimental conditions.

The precise mechanism by which oxygen-derived free radicals and their metabolites cause cell injury is not clear. However, experimental evidence suggests that lipid peroxidation reactions on the cell membrane may play an important role in radical-mediated cell injury. *In vitro* studies with purified membrane preparations have shown that lipid peroxidation of biologic membranes will cause both structural alterations and abnormal membrane functions.[74-83] The mechanism of free-radical–mediated lipid peroxidation involves at least three distinct phases. The initiation step occurs when a free radical (eg, OH·, $^1O_2$) interacts with a polyunsaturated fatty acid (PUFA) and extracts a proton, forming a fatty acid radical (Figure 3). This step is followed by the second or propagation phase, in which the fatty acid radical can react with oxygen, generating a fatty acid peroxy radical. The fatty acid peroxy radical can react with other lipids, proteins, or free radicals, perpetuating the transfer of protons with subsequent oxidation of substrates. The peroxidation reactions can be terminated in a number of ways. Glutathione peroxidase can enzymatically reduce lipid hydroperoxides to nonreactive hydroxy fatty acids (see below). In addition, bond rearrangement may cause formation of diene conjugates or degradation products such as malonyldialdehyde[84-87] and products of high molecular weight with fluorescent properties.[88] In addition, specific lipid hydroperoxides may also undergo metal-catalyzed beta-scission followed by a hydrogen abstraction generating volatile hydrocarbons such as ethane and pentane.[89,90] Tissue or cell content of diene conjugates and malonyldialdehyde are frequently used as indicators for the occurrence of lipid peroxidation. The measurement of exhaled pentane and/or ethane can be used in certain circumstances as a measure of *in vivo* lipid peroxidation. Anti-oxidants such as vitamin E or free radical scavengers can also terminate the radical chain reaction by reducing the level of peroxide radicals.[80,91]

Free radicals and lipid peroxides have also been shown to have potent effects on carbohydrates and proteins. Protein polymerization may either occur by incorporation of fragments of lipid oxidation products derived from the lipid peroxides into the protein structure or result from the direct transfer of free radicals to proteins.[92,93] A recent study has demonstrated the transfer of free radicals from peroxidized linoleic acid to lysozyme, with the formation of lysozyme oligomers. No incorporation of the lipid components into the protein occurs in this situation.[94] Finally, the capacity of free radicals to damage amino acids and carbohydrates by an iron-dependent mechanism has been demonstrated *in vitro*.[85,95]

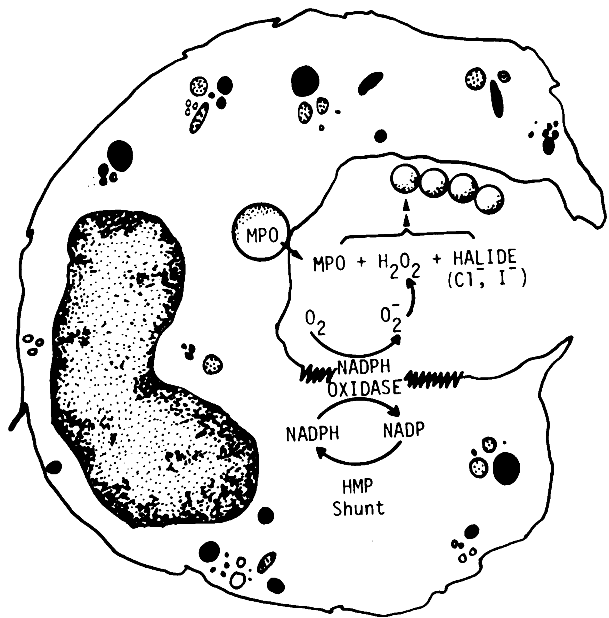


Figure 4 – Proposed mechanism of oxygen-metabolite – mediated bactericidal activity of phagocytes.

The Role of Oxygen Radicals in the Bactericidal Activity of Phagocytes

Since the mechanism of injury of bacteria and eukaryotic cells by phagocytic cells is thought to be similar, the mode of microbicidal activity of phagocytes will be reviewed (Figure 4). Extensive evidence exists showing that an intact respiratory burst and the production of O_2^- and other metabolites are essential for the destruction by phagocytic cells of ingested microorganisms. Initial observations indicate that both SOD and catalase are capable of inhibiting phagocyte-mediated bacterial killing.[18,96] However, *in vitro* studies have shown that O_2^- by itself has little bactericidal activity.[97,98] Current evidence suggests that the primary means of microbicidal activity in phagocytes results from production of H_2O_2 and subsequent metabolites. H_2O_2 has significant bactericidal activity by itself, but this activity is augmented in the presence of ascorbic acid and certain metals.[99] However, the most potent bactericidal agent is a product of the

reaction of H_2O_2 with myeloperoxidase and halide. In the presence of myeloperoxidase and a halide, H_2O_2 has potent bactericidal activities at a concentration of 10 μM, and comparable microbicidal activity of H_2O_2 in the absence of MPO and halide occurs at approximately 50-fold greater concentrations.[100-102] As discussed above, the most likely product of the MPO-H_2O_2 complex incriminated in this reaction is HOCl.

The mechanism of bacterial injury by the MPO–H_2O_2-halide system appears to result from halogenation and/or oxidation of the surface of the microorganism.[100,102-105] However, additional *in vitro* studies have shown that the MPO-H_2O_2 system can also decarboxylate amino acids, with the generation of CO_2, ammonia, and aldehydes.[106,107] Therefore, decarboxylation of cell walls and/or cell membrane proteins coupled with the local generation of toxic aldehydes may play a significant role in cell injury.

Additional evidence has suggested that hydroxyl radical may also play an important role in bacterial killing. Superoxide dismutase and catalase have been shown to inhibit independently the bactericidal activity derived from either phagocytes or a xanthine and xanthine oxidase system *in vitro*, suggesting that the presence of both O_2^- and H_2O_2 is necessary for cell killing.[18,96] Several authors have interpreted these findings to suggest that hydroxyl radical or possibly singlet oxygen resulting from the interaction of O_2^- and H_2O_2 is the toxic agent for microorganisms. In addition, mannitol, a scavenger of hydroxyl radical, inhibits the bactericidal activity of an acetaldehyde-xanthine oxidase system.[108,109] Further, *in vitro* studies have shown that staphylococci grown in iron-rich environments have increased susceptibility to killing by H_2O_2.[110] H_2O_2-induced lysis of bacteria under these conditions is inhibited by thiourea, sodium benzoate, and dimethylsulfoxide (DMSO), suggesting that OH· is the active agent. Thus, there is substantial evidence for the bactericidal cell activity of hydroxyl radical derived from leukocytes.

The significance of an intact respiratory burst and production of O_2^- by phagocytic cells is emphasized in patients with chronic granulomatous disease (CGD).[111-117] Patients with this genetic disorder have an increased susceptibility to bacterial infections. This susceptibility is apparent during early childhood. Bacterial infections occur frequently and tend to be severe, with the development of atypical granulomatous inflammatory responses. Almost any organ may be affected. Generalized lymphadenopathy and splenomegaly occur frequently. Leukocytes from patients with CGD have the ability to phagocytize bacteria; however, when activated, the cells do not exhibit a respiratory burst and are unable to kill catalase-negative bacteria. Cell motility, lysosomal degranulation, and hexose-monophosphate shunt activity are intact. It appears that the membrane-associated oxidase enzyme (described above) is either not activated or absent. As a result of the failure of leukocytes to generate O_2^- nitroblue tetrazolium (NBT) reduction and chemiluminescence do not occur when cells are phagocytically stimulated. A selective ability of leukocytes from patients with CGD to kill certain phagocytized bacteria has been associated with the ability of the bacteria to produce H_2O_2.[113] Organisms such as streptococci and pneumococci, which excrete H_2O_2 into the extracellular environment, are killed by CGD leukocytes, while bacteria that do not excrete H_2O_2 (eg, *Staphylococcus aureus*) are not killed. If H_2O_2 is provided to CGD leukocytes, either directly or by a glucose–glucose oxidase system, H_2O_2 will be taken up into the phagolysosome with the bacteria, and effective bacterial killing will occur.[114-116] In similar studies, using endothelial cells and red blood cells as target cells, neutrophils from patients with CGD activated by PMA fail to cause significant cytotoxicity, compared with normal neutrophils.[117,118] These studies have emphasized the importance of the respiratory burst and its metabolites in leukocyte-dependent bactericidal activity and tissue injury by activated leukocytes.

A second, rare genetic deficiency has been identified that results in a decreased respiratory burst and increased susceptibility to bacterial infections.[119,120] Patients deficient in glucose-6-phosphate dehydrogenase (G-6-PD), which catalyzes the initial reaction of the hexose-monophosphate shunt, have decreased capacity for generating NADPH. Decreased levels of NADPH results in loss of substrate for the membrane oxidase system and decreased O_2^- production. Therefore, although the enzyme defects are different for individuals with CGD and G-6-PD-deficient individuals, the net result of a decreased respiratory burst and that of impaired bactericidal and cytolytic capacity are the same.

Although there is abundant evidence suggesting that products of the MPO–H_2O_2–halide system play an important role in phagocyte-mediated bacterial killing and tissue injury, the significance *in vivo* of this system in humans is uncertain. Patients deficient in leukocytic MPO do not experience recurrent bacterial infections as compared with CGD and G-6-PD patients and only occasionally have MPO-deficient individuals been reported to suffer from fungal infections, in particular, *Candida* species.[121-124] However, *in vitro*, phagocytes from MPO-deficient patients show partial impairment of bacterial killing, and chemi-

luminescence is decreased, indicating at least a partial dependence on MPO products for these two cellular functions. Although the production of MPO products is decreased, O_2 consumption and the production of O_2^- and H_2O_2 by MPO-deficient leukocytes are increased and may represent a compensatory mechanism of the leukocyte to restore the bactericidal activity.[125,126] A recent report in which 28 patients with either partial or complete MPO deficiency were evaluated suggests that only 2 of these patients experienced recurrent systemic infections.[127] *In vitro* assay of neutrophil function of 3 patients with a complete MPO deficiency has shown only a 10% decrease in killing of *Staphylococcus aureus* and a greater than 90% decrease in *Candidicidal* activity. Therefore, it appears that either the MPO–H_2O_2–halide system and its metabolic products are not necessary for the bactericidal activity of phagocytic cells or compensatory mechanisms may come into play to circumvent this potentially lethal metabolic defect.

The Role of Oxygen Radicals in Cell and Tissue Injury

Since most bactericidal activity of phagocytes occurs in the phagolysosome, where relatively high concentrations of oxygen metabolites and myeloperoxidase develop, it would appear that extracellular injury of nonphagocytized cells would occur less efficiently. However, several investigators have reported *in vitro* studies demonstrating that oxygen metabolites released from activated neutrophils and macrophages may be toxic to a wide variety of eukaryotic cells, including erythrocytes,[124,128,132] endothelial cells,[119,133] fibroblasts,[134] tumor cells,[135–140] leukocytes,[141–143] platelets,[144] and spermatozoa.[145] It appears that the toxic effects of activated phagocytes on eukaryotic cells are mediated in a similar manner to the bactericidal effects. Each of the various oxygen metabolites (including O_2^-, H_2O_2, HOCl, $OH\cdot$, and $^1O_2$) have been implicated in extracellular cytolysis of host cells. Severa *in vitro* studies using scavengers of free radicals and specific enzymes (SOD, catalase) have implicated products of either the interaction of H_2O_2 and O_2^- or the MPO–H_2O_2–halide system as generating the most potent cytolytic agents.[130,132,137,141]

The ability of activated neutrophils and macrophages to kill specific cells appears to be dependent on several factors, including species of effector cell, target cell, and the activating stimulus. Murine BCG-elicited macrophages or peritoneally derived neutrophils, when activated with PMA, have the ability to lyse murine lymphoma cells through an oxygen- and glucose-dependent mechanism.[136] Catalase is effective in inhibiting this reaction. However, SOD, scavengers of singlet oxygen and/or hydroxyl radical, and inhibitors of heme enzymes, such as azide and cyanide, have no inhibitory effects, suggesting that H_2O_2 is the toxic metabolite mediating cell lysis. In contrast, human neutrophils activated by either concanavalin A or opsonized zymosan cause cytolysis of lymphoma cells by a mechanism that is inhibited by azide, cyanide, and catalase, and is dependent on the presence of halide.[137,146] Neutrophils deficient in MPO had decreased cytotoxic activity, suggesting that a product of the MPO–H_2O_2–halide system is the cytolytic agent.[134] Similar *in vitro* studies have shown that fibroblasts and endothelial cells[118,133] appear to be most sensitive to H_2O_2 and products of the MPO–H_2O_2–halide system, while erythrocyte lysis appears to be mediated by both O_2^- and H_2O_2 (see below). However, additional studies have shown that neutrophils isolated from myeloperoxidase-deficient individuals, when stimulated with PMA, show the normal capacity to cause endothelial cell cytolysis. Addition of purified myeloperoxidase to the deficient cells does not enhance cytotoxicity. Unfortunately, it is not known whether the capacity of myeloperoxidase-deficient neutrophils to mediate cytotoxicity is the result of increased levels of O_2^- and H_2O_2 produced. Therefore, without serious consideration of the target cell, it is not possible to identify a rank order to potency between hydroxyl radical, singlet oxygen, HOCl, O_2^-, H_2O_2, or other as yet unidentified oxygen metabolites in the injury and/or destruction of intact cells.

Perhaps the most extensively studied model of oxygen-metabolite-mediated eukaryotic cell injury is the erythrocyte (Figure 5). Initial *in vitro* studies have demonstrated that products of an acetaldehyde-xanthine oxidase system are capable of causing erythrocyte lysis that is inhibited by SOD and catalase, suggesting that O_2^- and H_2O_2 are necessary components for optimal toxicity.[131] However, since scavengers of hydroxyl radical and singlet oxygen also prevent cell lysis, the mechanism for cell injury is not clear. Additional studies have revealed that red cell ghosts can be injured by a similar enzyme system and that SOD is protective in this model.[147] Catalase and scavengers of hydroxyl radical and singlet oxygen have minimal protective effects. These data suggest that O_2^- has toxic effects on the erythrocyte membrane, while other metabolites may react with intracellular components, possibly hemoglobin, to mediate cell lysis indirectly. Other studies have shown that murine macrophages activated with PMA cause cell lysis by an H_2O_2-dependent mechanism that is independent of O_2^-, hydroxyl radical, and sin-

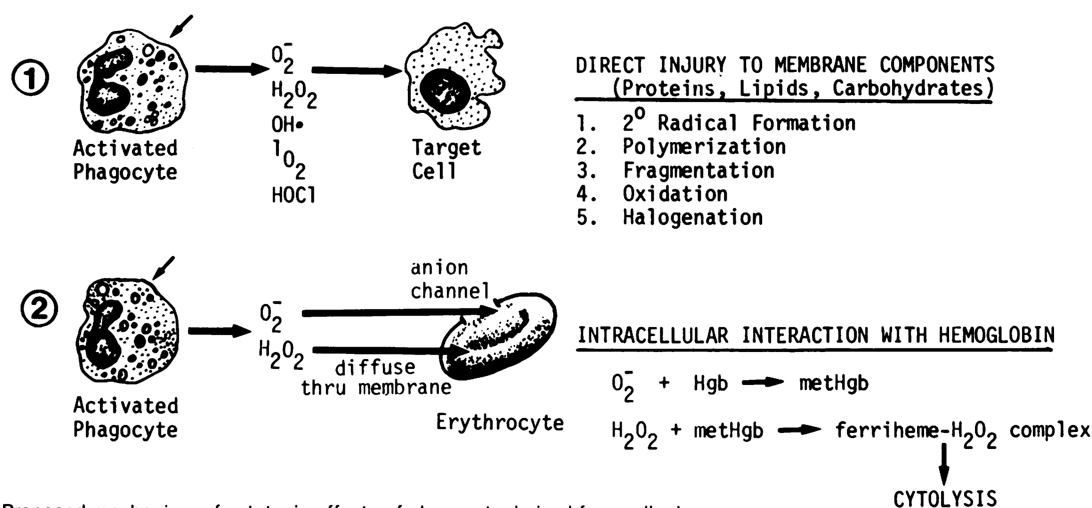


Figure 5 – Proposed mechanism of cytotoxic effects of phagocyte-derived free radicals.

glet oxygen.[129] These data demonstrate the capacity of oxygen metabolites generated by either enzymatic mechanisms or by activation of phagocytic cells to cause erythrocyte lysis. However, the ability of an individual oxygen metabolite to injure erythrocytes appears to be dependent on the source of O_2^- and H_2O_2 generation.

Recently, Weiss proposed a mechanism of neutrophil-mediated erythrocyte injury that in part explains several of the previous experimental observations (Figure 5). Human neutrophils stimulated with PMA cause lysis of human erythrocytes by a mechanism dependent on both O_2^- and H_2O_2.[148] Erythrocyte lysis is associated with the oxidation of oxyhemoglobin to methemoglobin, a process that is inhibited by the presence of SOD and catalase. Nitrite-treated erythrocytes (which are rich in methemoglobin) are susceptible to lysis by activated neutrophils in the presence of SOD. Blockade by sulfonated stilbenes of anionic membrane channels in the intact erythrocytes inhibits O_2^--induced oxidation of hemoglobin. This is consistent with the observations of Lynch and Fridovich, who have reported diffusion of enzymatically generated O_2^- out of granulocytes through anionic channels and into the extracellular environment.[147] These data suggest that O_2^- enters the intracellular compartment and directly oxidizes oxyhemoglobin to methemoglobin. However, the possibility that O_2^- reacts either directly or indirectly (possibly via products of the Fenton reaction) with membrane components forming products that subsequently oxidize hemoglobin cannot be excluded.

The second proposed phase of neutrophil derived and oxygen-dependent injury of erythrocytes involves the diffusion of H_2O_2 through the cell membrane and its reaction with methemoglobin, forming an H_2O_2-methemoglobin complex.[148] Subsequent to this phase is the formation of a cytoxic peroxide-ferriheme complex. Support for this mechanism comes from the observation that catalase protects nitrite-treated cells from neutrophil-mediated cytolysis. Furthermore, either inhibition of intracellular catalase and glutathione peroxidase or addition of exogenous SOD potentiates erythrocyte cytolysis. In addition, several authors have demonstrated potent oxidant activity of products of H_2O_2-methemoglobin and H_2O_2-metmyoglobin complexes.[150-153] How these reactive products interact with other cellular elements of the erythrocyte to cause cell lysis awaits further study. This proposed mechanism of cell injury by phagocyte-derived oxygen metabolites offers a novel mechanism by which reactive oxygen species diffuse into the target cell and act on intracellular components, causing cell injury. The relative importance of this intracellular mechanism versus a mechanism featuring the effects of oxygen metabolites on cell membrane components cannot be determined at this time.

Opsonized particles such as zymosan- or immunoglobulin-sensitized cells show increased phagocytosis by macrophages and neutrophils as a result of binding to Fc and/or C3b receptors of the phagocytic cell.[154] It has been demonstrated that Fc- and/or C3b-mediated uptake of sensitized cells plays an important role in their clearance from the circulation. Because the generation of oxygen metabolites occurs concurrent with phagocytic uptake, it is possible that the cytolytic effects of neutrophils and macrophages via oxygen metabolite generation may represent an important mechanism of cell injury in certain autoimmune diseases. Additional *in vivo* studies are necessary for us to determine whether the rate of clear-

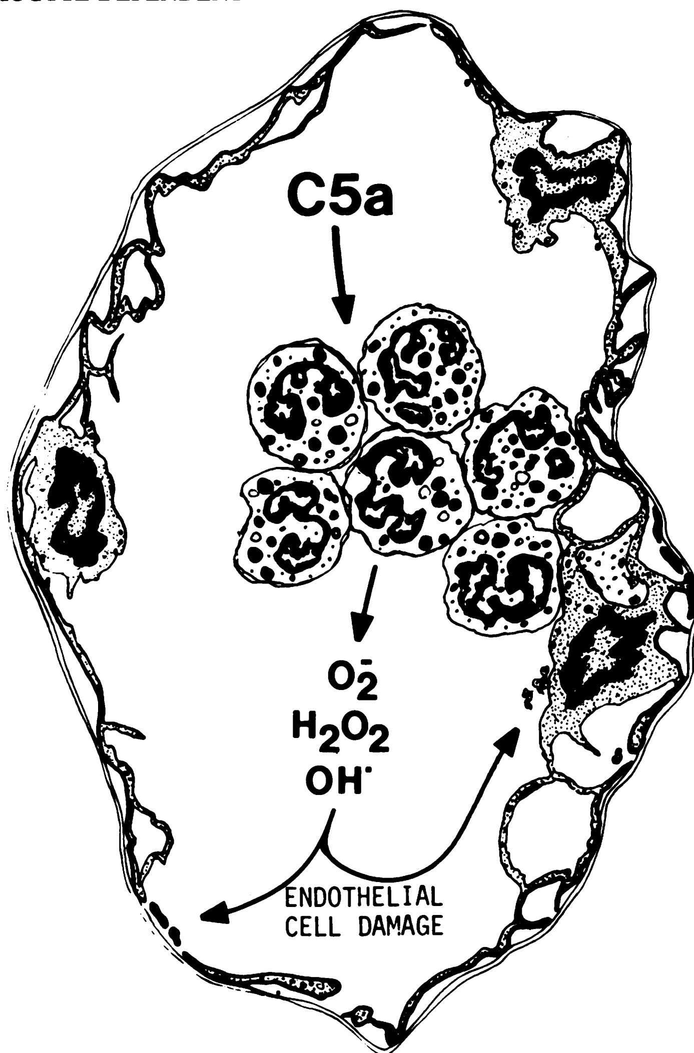


Figure 6 — Proposed mechanism of endothelial cell injury by polymorphonuclear leukocytes following intravascular complement activation and O_2^- generation.

ance of sensitized cells by phagocytic cells may be modulated by treatment of individuals with either specific enzymes or free radical scavengers which would inhibit oxygen-metabolite–mediated cytolysis.

Because oxygen metabolites have been shown to cause damage of endothelial cells *in vitro*, it is possible that the local production of these metabolites by neutrophils is responsible for the respiratory distress syndrome observed in many clinical conditions. It has been proposed that in patients undergoing extracorporeal hemodialysis[155] or those with burn or trauma injury,[156] activation of plasma mediator systems (including complement) occurs, resulting in the sequestration of neutrophils in the pulmonary capillary bed and the subsequent local release of O_2^- and H_2O_2 (Figure 6). Recent experiments have shown that infusion of zymosan-activated serum into sheep or infusion of cobra venom factor into rabbits will produce marked pulmonary leukostasis and pulmonary edema.[157,158] It has been postulated that the mechanism of pulmonary edema results from injury of the capillary endothelial cells by oxygen metabolites derived from activated leukocytes. Preliminary evidence in rats following intravascular infusion of cobra venom factor indicates that leukocytic sequestration occurs within the pulmonary vasculature and that this is associated with an increase in lung permeability and focal intraalveolar hemorrhage (Figure 7). This is associated with evidence of endothelial cell damage which can be prevented by prior treatment of animals with catalase. Therefore, it appears that the injury of vascular endothelial cells is related to the generation by neutrophils of H_2O_2. Further studies are necessary to define the precise mechanism of oxygen metabolite injury to endothelial cells *in vivo* and the role of these metabolites in the initiation of lung injury.

Additional *in vitro* studies have shown that locally produced oxygen metabolites have the capacity to generate chemotactic factors. When human plasma is

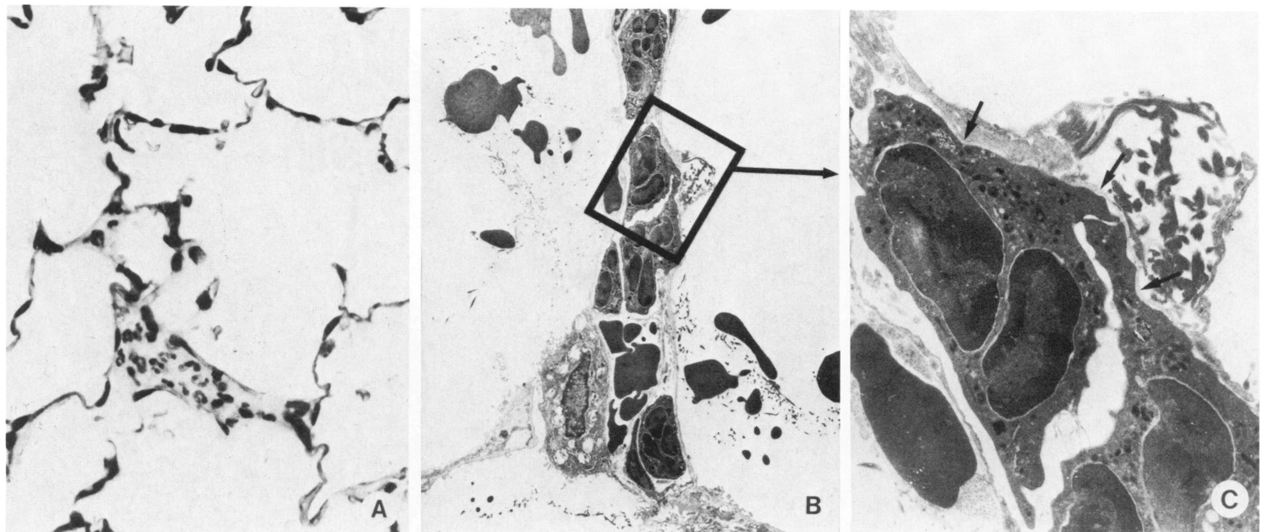


Figure 7—Histologic and ultrastructural alterations in pulmonary capillaries after intravascular complement activation. **Figure 7A** shows sequestration of neutrophils in alveolar capillaries 30 minutes after intravenous infusion of cobra venom factor. (H & E, × 120). **Figure 7B** (× 600) and **7C** (× 2,000) show accumulation of neutrophils in alveolar capillaries with blebbing of capillary endothelium and destruction of the vascular basement membrane. *Arrows* indicate areas of direct contact of neutrophils with vascular basement membrane. An extravascular accumulation of fibrin is also evident in **Figures 7B** and **7C**.

incubated with a xanthine–xanthine oxidase system a potent chemotactic agent is generated.[159] This substance has been tentatively identified as a heat-labile lipid that is bound to albumin. Further studies have demonstrated the production of a chemotactic lipid from arachadonic acid following incubation with an O_2^-- and H_2O_2-generating system.[160] This chemotactic factor is active at a concentration of 3.0 ng/ml and is distinct from arachadonic acid. Thus, it appears that oxygen metabolites from phagocytic cells may function as a positive feedback mechanism to potentiate the inflammatory response through the generation of chemotactic factors.

An additional mechanism by which phagocyte-derived free radicals may augment the inflammatory responses is by the inactivation of normal serum inhibitors of leukocytic proteases, thereby causing an increased destruction of the structural components of tissue (such as elastin).[161,162] *In vitro* studies have shown that reactive oxygen metabolites generated from human neutrophils that are phagocytizing immune complexes will inhibit α1-antiprotease activity, the primary inhibitor of leukocyte elastase. This inactivation appears to result from the oxidation by OH· of methionyl residues on the antiprotease molecule. Oxygen metabolites also have been shown to be directly capable of altering structural components of tissue. *In vitro* studies have shown that O_2^- produced either by neutrophils stimulated by PMA or by the xanthine–xanthine oxidase system will cause depolymerization of hyaluronate, a glycosaminoglycan and major component of synovial fluid, thus increasing its susceptibility to degradation by β-N-acetylglucosaminidase A.[163–165] In addition, cartilage proteoglycans and collagen have been shown to be degraded by O_2^- and its metabolites.[166] This represents direct evidence that oxygen metabolites either by themselves or in conjunction with lysosomal proteases can injure the structural matrix of tissues.

Although most reports suggest a proinflammatory role for oxygen metabolites, a recent report has shown that when either the complement derived chemotactic peptide, C5a, or the synthetic chemotactic peptide f-Met-Leu-Phe are incubated with an MPO–H_2O_2–halide system, chemotactic activity is lost as a result of oxidation of methionyl residues.[167] Therefore, the precise *in vivo* effects of oxygen metabolites on biologic substrates is not clear and awaits further definition.

In recent studies, two groups have shown that systemic administration of superoxide dismutase to rats will inhibit the reversed passive Arthus reaction in the skin.[159,164] This reaction is dependent on the availability of complement and neutrophils. Immunofluorescence studies have shown that suppression of the acute inflammatory response occurs despite the local formation of immune complexes and activation of complement (Figure 8). Further evidence implicating a role for O_2^- and H_2O_2 in acute immune-complex–induced tissue injury has been demonstrated by catalase-induced inhibition of acute inflammatory reactions in the lung.[169] In this model, catalase is

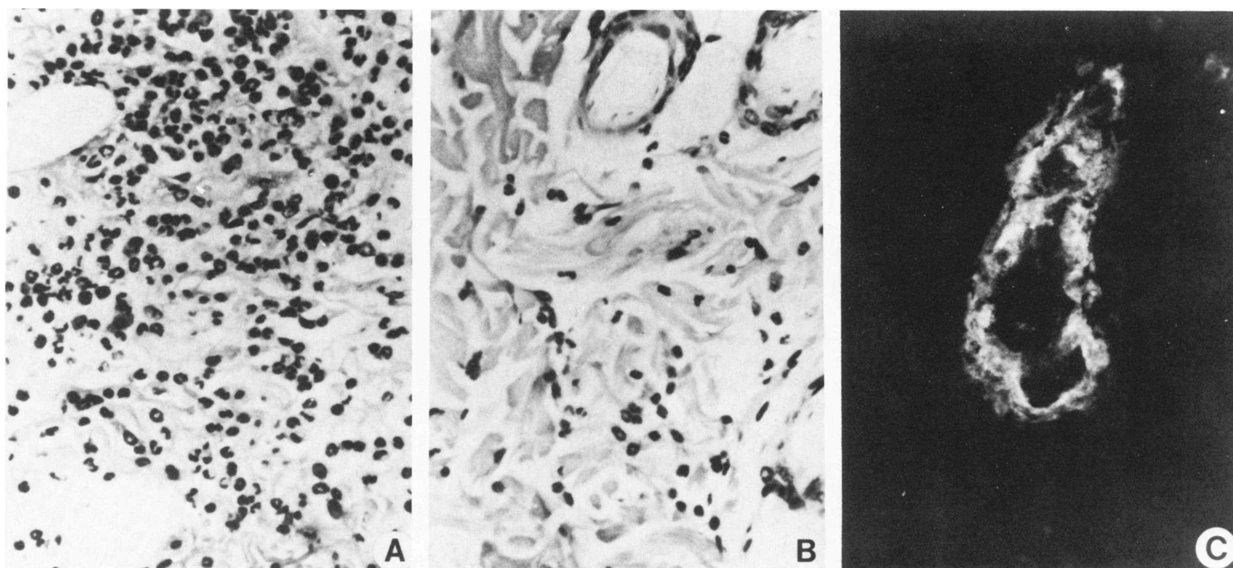


Figure 8—Histologic appearance of reverse passive Arthus reaction (immune-complex inflammation) in the skin of untreated (**A**) and SOD-treated (**B**) rats. Bovine serum albumin (10 μg) was administered intravenously simultaneously with the intradermal injection of rabbit anti-BSA, IgG (250 μg abN). The animals were sacrificed 6 hours later. In SOD treated rats, the neutrophil infiltrate was markedly decreased despite the presence of immune complexes, as represented by the presence of rabbit IgG (**C**), BSA, and C3 in the reaction site. (**A** and **B**, H & E, ×430; **C**, immunofluorescence, fluorescein-conjugated goat anti-rabbit Ig)

more effective than SOD, while antiproteases have little if any suppressive effects on lung injury. The suppressive effects of SOD on the inflammatory reaction are brief in duration (2 hours) and associated with the lack of influx of neutrophils (Figure 9). This would support the concept mentioned above that O_2^- is involved in the generation of an amplifying product, a chemotactic lipid. The protective effects of catalase in these reactions are not associated with blocking of the influx of neutrophils. This observation is in keeping with the idea that catalase destroys H_2O_2 generated by immune-complex–activated cells and so protects the tissue from injury but does not interfere with the generation of chemotactic mediators.

Initial *in vivo* studies have demonstrated the ability of SOD to suppress carrageenan-induced inflammation.[170] When carrageenan is injected into the pleural cavity of normal and SOD-treated animals, the amount of pleural effusion is reduced by approximately 50%, and the number of neutrophils is decreased by 30% in the SOD-treated animals, suggesting that O_2^- or a metabolic product is necessary for the full expression of the inflammatory response. In addition, systemic treatment with SOD has been shown to suppress carrageenan-induced foot pad edema in the rat by inhibiting the delayed phase of carrageenan-induced swelling.[171] Neutrophil depletion and inhibition studies with free radical scavengers and catalase suggest that macrophage-derived O_2^- is the toxic metabolite in this model.

However, other investigators, using a model of kaolin-induced inflammation in the rat, have shown a dependence on the production of both O_2^- and H_2O_2 for the development of a granulomatous inflammatory response.[170] The generation of H_2O_2 is associated with increased levels of malonyldialdehyde, a byproduct of lipid peroxidation (see above). Since scavengers of OH· are not effective in inhibiting kaolin-induced inflammation, it would appear that in this model H_2O_2 is the toxic metabolite.

Additional experiments have studied chronic adjuvant arthritis in rats (a proposed animal model of rheumatoid arthritis) and autoimmune glomerulonephritis in NZBxW F_1 hybrid mice (a proposed model of systemic lupus erythematosis) by systemic SOD treatment.[170] Rats treated with SOD show an apparent 30% decrease in inflammatory changes of joints after daily injections of SOD, while mice treated with SOD on alternate days demonstrate decreased mortality, decreased antibody titer, and decreased immunoglobulin deposition in glomeruli. However, in each of these animal models, further characterization of the active oxygen metabolite has not been pursued. Although there appear to be conflicting reports regarding the potency and significance of specific oxygen metabolites in inflammatory reactions, the data clearly support an important role of these metabolites in both acute and chronic inflammation. The reports cited above represent the first *in*

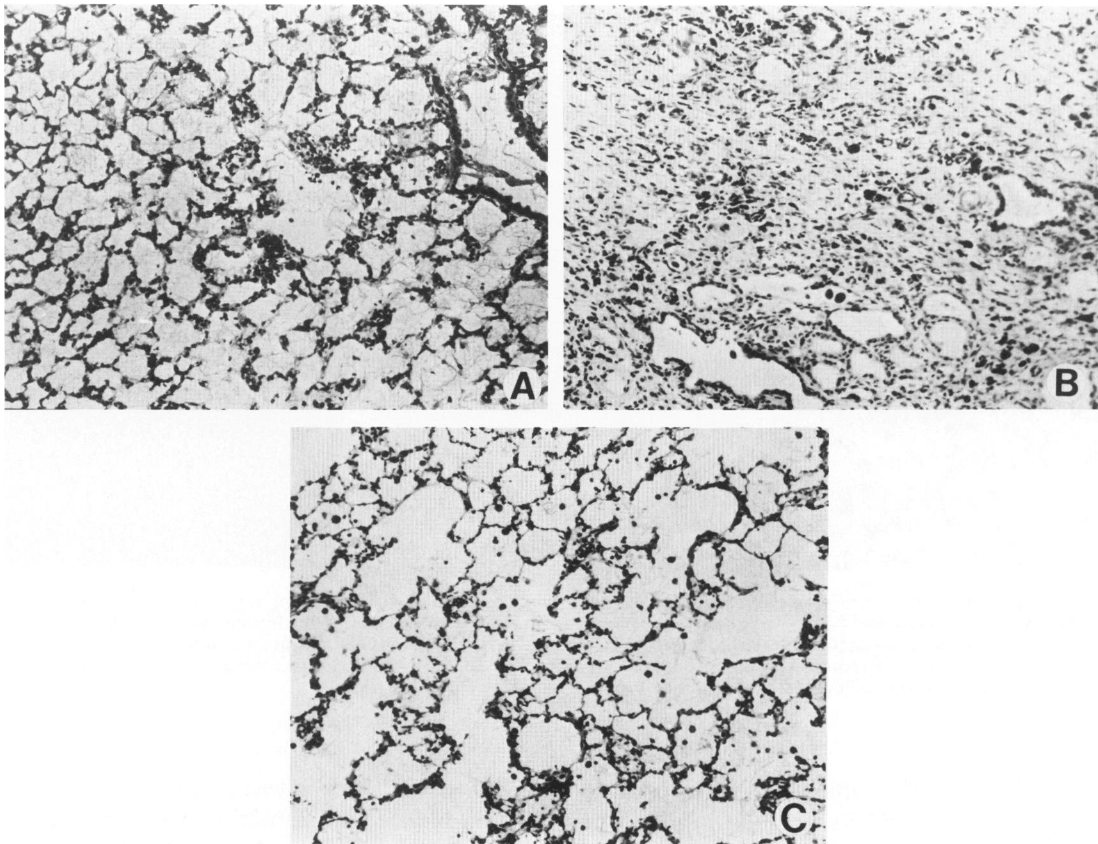


Figure 9—*In vivo* lung damage by enzymatically generated oxygen radicals. A—Section of rat lung 4 hours after intratracheal instillation of glucose (1 mg), glucose oxidase (28 μ), and lactoperoxidase (1.7 μ). There is smudging of alveolar walls with neutrophil infiltration, minimal hemorrhage, and intraalveolar fibrin deposition. B—Section of lung from rat similarly treated 14 days after instillation. Sections show collapse of lung tissue, loss of alveolar structure, and extensive pulmonary fibrosis. C—Section from the lung of a rat treated with glucose (1 mg) and glucose oxidase (70 μ) but without lactoperoxidase. The lung changes show neutrophils with minimal hemorrhage and fibrin accumulation. At 14 days, animals treated with glucose-glucose oxidase did not show extensive fibrosis.

vivo evidence implicating a role for O_2^- and hydrogen peroxide in neutrophil and macrophage-mediated inflammatory response.

A recent report has shown that the intratracheal installation of specific enzyme-substrate systems known to generate oxygen metabolites will cause acute lung injury (Figure 9).[172] When xanthine and xanthine oxidase are administered intratracheally, there is increased vascular permeability with only minor edema formation and focal hemorrhage at 4 hours. These pathologic changes can be inhibited with simultaneous instillation of SOD. When glucose and glucose oxidase (which generates H_2O_2) are instilled into the airways, there is a marked increase in vascular permeability, edema, hyaline membrane formation, hemorrhage, and a pronounced neutrophil influx. These changes are consistent with the pathologic changes observed in humans defined as diffuse alveolar damage and associated with adult respiratory distress syndrome (ARDS).[156,173] As expected, these pathologic alterations are inhibited with catalase but not SOD or chemically inactivated catalase (Figure 9). When either lactoperoxidase (LPO) or myeloperoxidase (MPO) is instilled with the glucose-glucose oxidase system, severe lung injury occurs, frequently progressing to diffuse pulmonary fibrosis by 4 days. These data suggest that a product of the MPO(LPO)-H_2O_2-halide system (perhaps HOCl or $^1O_2$) may play a significant role in the development of pulmonary fibrosis. These studies demonstrate that oxygen-derived free radicals and their metabolites have the capacity to cause acute lung injury and suggest that under certain conditions progressive lung injury with pulmonary fibrosis results.

The direct toxic effect of H_2O_2 and products of the xanthine-xanthine oxidase system on lung parenchyma have been supported with the use of an *in vitro* system. ^{51}Cr-labeled lung explants show cytotoxicity (as monitored by ^{51}Cr release and increased extracellular levels of lactate dehydrogenase (LDH) (a cytoplasmic enzyme) in culture supernatants after exposure to either H_2O_2 or a xanthine-xanthine oxidase

system.[174] In both systems catalase but not SOD is effective in blocking the cytotoxic effects, suggesting that H_2O_2 is the significant toxic agent. In addition, rat and human lung explants appear to be more susceptible to injury when compared with rabbit lung explants, indicating a species difference in the susceptibility of lung parenchyma to injury by toxic oxygen metabolites. Additional study is necessary for us to define the precise targets of the oxygen metabolites and the mechanisms responsible for the decreased susceptibility to injury observed in some species.

Antioxidant Protective Mechanisms

The ability of macrophages and neutrophils to injure cells and host tissues is dependent on the production of oxygen-derived free radicals and their metabolites and the ability of the target cells and tissues to detoxify the reactive metabolites. The balance between the production and catabolism of oxidants by cells and tissues is critical for the maintenance of their biologic integrity. We have previously discussed the mechanisms for production of oxygen-derived metabolites by phagocytic cells and in this section will address the protective mechanisms against oxygen metabolite-induced injury.

Early investigations have demonstrated potent antioxidant activity in human plasma separate from its vitamin E content.[175-178] The majority of the antioxidant activity in serum is dependent on the levels of the copper-containing protein ceruloplasmin, an α_2-glycoprotein with an approximate molecular weight of 160,000. Neither ceruloplasmin depleted of copper nor copper alone shows significant antioxidant activity.[179] The precise mechanism by which ceruloplasmin inhibits oxidant injury is not clear. Several authors have suggested that ceruloplasmin scavengers O_2^- via a dismutase reaction similar to superoxide dismutase.[180,181] However, other investigators dispute the ability of ceruloplasmin to dismutate O_2^- and suggest that O_2^- is scavenged by the reduction of copper within the protein.[182] Additional *in vitro* studies have shown that ceruloplasmin will also scavenge hydroxyl radical and, possibly, singlet oxygen by as yet undefined mechanisms.[183]

A third proposed mechanism for the antioxidant activity of ceruloplasmin is a result of its ferro-oxidase activity converting reduced iron (Fe^{2+}) to oxidized iron (Fe^{3+}). Since Fe^{2+} ions may initiate lipid-peroxidation reactions through their reaction with O_2^-, ceruloplasmin could inhibit these reactions by the enzymatic oxidation of Fe^{2+}.[184,185] This is supported by a recent study demonstrating the ability of ceruloplasmin to inhibit Fe^{2+}-mediated lysis of rat red blood cells, and ascorbic acid and iron-mediated lipid autooxidation.[186] Ceruloplasmin is one of the acute phase reactants whose serum concentration is increased in a variety of disease states. However, its definitive role in modulating specific inflammatory or immune responses awaits further study.

A second antioxidant activity in serum has been associated with the iron-free fraction of transferrin.[177] As discussed above, free iron can function as a catalyst in lipid peroxidation reactions. The antioxidant ability of transferrin has been attributed to its ability to bind free iron. When either purified transferrin or plasma was added to auto-oxidation reactions, the anti-oxidant activity is directly proportional to the percentage of iron-free transferrin.[178] Iron saturated transferrin has no significant antioxidant activity.

Perhaps the oldest recognized antioxidant in biologic systems is α-tocopherol (vitamin E). *In vitro* evidence suggests that this lipid-soluble molecule functions as a free radical scavenger and plays an important role in protecting and maintaining the integrity of cell membranes against lipid peroxidation due to free radicals.[187] Vitamin E has been identified in relatively high concentrations in red cell membranes as well as mitochondrial membranes and endoplasmic reticulum.[188,189] In contrast to the effects of SOD and catalase, which function by inhibiting the primary initiation of lipid peroxidation by O_2^- and H_2O_2, and glutathione peroxidase, which inhibits both primary and secondary initiation, α-tocopherol functions by terminating free radical reactions by competing for peroxy free radicals ($ROO^{\cdot}$). This results in the formation of tocopherol dimers or quinones. Deficiency of vitamin E in animals is manifest by increased susceptibility to free radical oxidative injury, which can be inhibited by administration of other antioxidants.[189] Red blood cell hemolysis in animals deficient in vitamin E has been reported.[90,140] However, a definite protective (anti-oxidant) role for vitamin E in humans has not been well documented. In a recent report of a hemolytic-thrombocytopenic anemia in a premature infant, the patient was reported to respond with clinical improvement following vitamin E therapy.[191] However, the mechanism of the vitamin E effect has not been determined. Therefore, definition of the role which vitamin E plays in the protection of free radical oxidative reactions, particularly in man, awaits further study.

In 1969, McCord and Fridovich isolated an enzyme from bovine erythrocytes that catalyzes the dismutation of superoxide ion to hydrogen peroxide and oxygen.[192] This enzyme was previously referred to as hemocuprein. Its counterpart in the red cell, erythrocuprein, is found within all aerobic organisms and is

generally absent or present in very low concentrations in anaerobes.[193,194] Several forms of superoxide dismutase have been identified: a manganese-containing SOD present in mitochondria and in prokaryotic cells (molecular weight of 80,000, with four identical subunits, each containing 1 atom of manganese), a copper-zinc-containing SOD present in the cytosol of eukaryotic cells (molecular weight of 32,000, with two identical subunits, each containing 1 atom of copper and 1 atom of zinc) which is inhibited by cyanide and diethyl dithiocarbamate (a metabolite of disulfiram),[195,196] and an iron-bound enzyme associated with the plasma of *Escherichia coli*.[197] Early studies by Carson et al have demonstrated the anti-inflammatory properties of a protein termed orgotein, derived from bovine liver.[198] This was later shown to be identical to SOD.

Additional studies have shown that SOD has a protective effect in bacterial injury caused by oxidative metabolites.[199] SOD has also been shown to play a protective role in cell injury in several eukaryotic systems. Under conditions of hyperoxia, in which cells and tissue are subject to increased oxygen tension, there is an increase in the levels of SOD in neutrophils,[200] alveolar macrophages,[201] and granular pneumocytes (Type 2 alveolar epithelial cells).[202] Inhibition of SOD by disulfiram (or its active metabolite diethyl dithiocarbamate) results in increases in susceptibility to hyperoxia-mediated lung injury in rats.[196,197] Also, addition of SOD to actively phagocytizing human neutrophils prevents "cell suicide" (lysis of these cells). Exogenously added SOD has also been shown to prevent cell and tissue injury induced by activated phagocytes or O_2^--generating enzyme systems (see above). Therefore, it appears that SOD plays an important protective role in both prokaryotic and eukaryotic cells against O_2^--induced injury.

During the respiratory burst H_2O_2 is secreted into the extracellular environment and may diffuse back into the cytoplasm of the phagocytic cell, potentially injuring that cell. Two enzymatic mechanisms for the cellular detoxification of H_2O_2 have been identified, catalase and glutathione peroxidase. Catalase, a cytoplasmic heme-enzyme, catalyzes the divalent reduction of H_2O_2 to water. Several *in vitro* experiments have demonstrated a protective role of cellular catalase against H_2O_2-mediated cell and tissue injury (see above). At low concentrations of H_2O_2 catalase can function as a peroxidase when suitable electron donors (alcohols, nitrites, and formate) are present.[203] However, *in vivo* co-substrates have not been identified, and the biologic significance of catalase-associated peroxidase activity is questionable. Under certain hyperoxic conditions, in a manner similar to changes in tissue levels of SOD, catalase activity may be increased in alveolar macrophages and granular pneumocytes.[201,202] This elevation of catalase and SOD under hyperoxic conditions has been postulated to play a role in oxygen-induced tolerance.

Glutathione peroxidase, a selenium-dependent enzyme (molecular weight of approximately 85,000) present in significant concentrations in the cytoplasm of cells, detoxifies H_2O_2 to H_2O through the oxidation of reduced glutathione (Figure 10).[203,204] A selenocysteine amino acid has been identified as the active site of the enzyme. The oxidized form of glutathione may then be reduced by a second enzyme, glutathione reductase, with NADPH as the reducing agent. In addition, glutathione peroxidase can metabolize lipid hydroperoxides to less reactive hydroxy fatty acids.[83,203] Therefore, the ability of glutathione peroxidase to reduce H_2O_2 or other hydroperoxides is dependent on the activity of glutathione reductase as well as the availability of NADPH. NADPH gener-

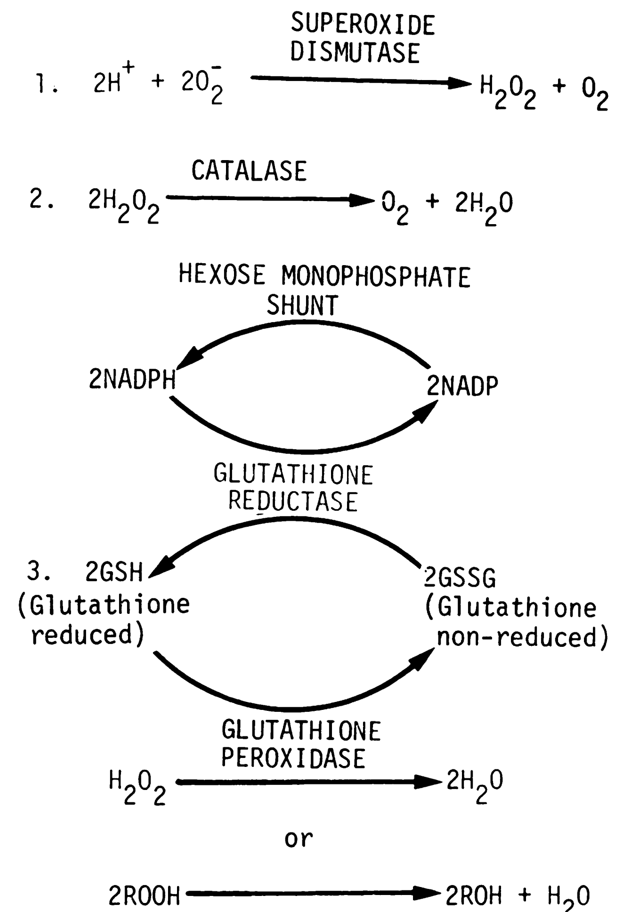


Figure 10—Anti-oxidant enzyme systems.

ated from activation of the hexose monophosphate shunt thus plays a regulatory role in oxygen metabolism as both the electron source for the reduction of O_2^- by the membrane-associated NAPH oxidase as well as the intermediate electron donor for the ultimate reduction of H_2O_2 or other hydroperoxide.

Recently, a selenium-independent glutathione peroxidase activity has been identified. This enzyme can detoxify organic peroxides but does not metabolize H_2O_2.[204,205] The distribution of selenium-independent glutathione peroxidase activities is both species- and tissue-dependent.[205] Almost all of the total glutathione peroxidase in guinea pig liver is selenium-independent while less than 40% activity in rat liver is selenium-independent. In addition, rat lung activity is totally selenium-dependent, while rat intestine contains 91% selenium-independent glutathione peroxidase activity.

As glutathione peroxidase is effective at low concentrations of H_2O_2 and has the capacity to metabolize certain hydroperoxide products of oxygen metabolite-lipid reactions, it is thought that it may play a more significant role than catalase in the protection of cells against oxygen injury. In addition, in humans the biologic significance of glutathione peroxidase versus catalase is emphasized when one studies individuals who are genetically deficient in the respective enzymes. Acatalasemic individuals are relatively normal except for an increased susceptibility to microorganisms that produce H_2O_2, while patients with glutathione peroxidase deficiency have been reported to suffer symptoms of chronic granulomatous disease.[206,207] Thus it appears that glutathione peroxidase plays a more significant role in protecting cells against a variety of oxidants, while catalase has an effective *in vivo* role only under the conditions of high H_2O_2 production.

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