Oxy radicals and their clinical implications

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Free radicals are the major mediators of bactericidal and cytotoxic actions of polymorphonuclear leukocytes, macrophages and monocytes, and can stimulate lymphocytes mitogenically. Hydrogen peroxide can participate in reactions resulting in free radical generation, induces interferon production by human macrophages and activates natural killer cells; interferon can in turn enhance free radical generation in cells. Anti-cancer drugs can augment free radical generation causing tumour cell lysis. Our studies suggest that some polyunsaturated fatty acids can selectively kill tumour cells but not normal cells in vitro by virtue of their capacity to augment free radical generation in tumour cells. Our studies also reveal that free radical generation and plasma lipid peroxides are increased in patients with pneumonia, septicemia, hypertension and collagen vascular diseases. This increase in generation of free radicals may account for the inflammation seen in these conditions. Thus, free radicals have both harmful and beneficial effects.

It is now believed that oxygen and free radicals reacting with each other are toxic. It has been suggested that free radicals are involved in cellular ageing and several disease processes. Free radicals are implicated mainly in the pathogenesis of several inflammatory conditions, radiation injury and ischaemia. This review proposes to give an overview of the biology of free radicals with an outline of their possible role in various physiological and pathological conditions.

Definition and nomenclature

A free radical is defined as any atom, group of atoms or molecules in a particular state with one unpaired electron occupying an outer orbital. A few common molecules such as nitric oxide (NO) and nitrogen dioxide (NO₂) contain an unpaired electron in an outer orbital in their normal state and hence by definition are free radicals. Nitric oxide (NO), a gas, is known to be produced by endothelial cells, macrophages, some tumour cells and neuronal cells. The significance of NO lies in the fact that it can control blood pressure, has tumoricidal action and is a neurotransmitter. The beneficial significance of NO will be discussed in detail later. A biradical is a species containing two unpaired electrons in outer orbitals and molecular oxygen (O₂) is a good example of this.

Elemental oxygen will be referred to as molecular oxygen and the symbol (O₂) is used. The one-electron reduction of oxygen results in the formation of the superoxide anion radical. The two-electron reduction product of oxygen in fully protonated form is hydrogen peroxide, H₂O₂, while the three-electron reduction product of (O₂) is the hydroxyl radical, OH'. Hydroperoxides are represented as ROOH with ROO' and RO' representing the peroxide and alkoxy radical respectively.

Reactivity of free radicals and the protective and controlling mechanisms

The unpaired electron of the free radicals is generally very reactive. The complex system of radical reactivity has been discussed in detail by Del Maestro. Oxygen in its ground state as such is a relatively weak oxidant. The complete reduction of oxygen by the univalent pathway results in the formation of superoxide anion radical, hydrogen peroxide, and hydroxyl radical as the intermediates. These intermediates are too reactive and hence are not tolerated by living tissues. Therefore, several protective and controlling mechanisms, which can be enzymatic, hydrophobic, hydrophilic and structural groups have been developed in the body. For example, cytochrome oxidase prevents the release of superoxide anion, hydrogen peroxide, and hydroxy radicals into the cellular milieu. Superoxide dismutase is an enzyme which scavenges superoxide radical and catalyses and peroxidases catalytically decompose hydrogen peroxide. Recently, it has been shown that catalase also scavenges electrons besides hydroxyl radicals.

Vitamin E, intercalated in cellular membranes, β-carotenes and glutathione peroxidase, a selenium containing enzyme, reduce hydrogen peroxide to water and also reduce lipid hydroperoxides. Cell membranes with their large content of polyunsaturated fatty acids are the sustained hydrophobic regions of the cells and vitamin E, which is closely intercalated in cell membranes, is probably the best hydrophobic scavenger known. Ascorbic acid, caffeine, cysteine, reduced glutathione, and to some extent, plasma constituents such as ceruloplasmin and transferrin have the capacity to remove radicals in ionic environment(s). The structural integrity of living cells appears to play an important role in lending support to the protective
mechanisms since their disruption leads to rancidification. Cholesterol, by virtue of its size and structure, intercalated into cell membranes and the hydrophobic scavengers, vitamin E and beta-carotene, can protect the fatty acid double bonds from peroxidative injury.

**Free radical induced injury and disease states**

Hyaluronic acid, the cementing substance of the extracellular space, and other glycosaminoglycans and collagen can be degraded by free radicals, which can significantly alter the permeability and structural characteristics of the tissues. In addition, free radicals disrupt lysosomal membranes and degrade DNA and thus play a role in mutagenesis and carcinogenesis.

Hydroxyl radical and singlet oxygen react with the polyunsaturated fatty acids (PUFAs) of cell membranes, resulting in the generation of lipid peroxide radicals, lipid hydroperoxides, and other products such as malondialdehyde (MDA). These products can influence vascular permeability and leucocyte chemotaxis, and alter prostaglandin (PG) formation and histamine release; thus free radicals are able to perpetuate inflammation. The action(s) of many anti-cancer drugs and the toxicity of paraquat and carbon tetrachloride can also be linked to their ability to alter the rate of generation of free radicals.

In the normally metabolizing aerobic cell oxygen undergoes a tetravalent reduction with acceptance of four electrons resulting in formation of $H_2O$. During this process, several free radical intermediates or highly reactive substances are formed. With the first electron transfer, the superoxide anion radical ($O_2^-$) is formed. Acceptance of second electron results in the generation of hydrogen peroxide, a reactive compound that is not a free radical but can participate in reactions resulting in free radical generation. With the third electron transfer, the very reactive hydroxyl radical (OH) is formed. The hydroxyl radical is widely believed to account for much of the cell damage caused by free radicals. Normally, cytochrome oxidase system of the mitochondria can detoxify the free radicals generated and thus prevent damage to the cells. It is also known that mitochondria generate a steady flux of free radicals which can be controlled by scavenging mechanisms available. A decrease in the intracellular scavenging mechanisms, an increase in the flux of free radicals and/or a combination thereof can lead to free radical induced injury to macromolecules and membranes.

It is now believed that inflammatory states and chronic diseases such as connective tissue disorders, rheumatoid arthritis, ulcerative colitis, vasculitis and some immunological disorders are due to increased extra-cellular generation of free radicals. Hyperoxenation syndromes such as retrolental hyperplasia and status epilepticus; hypoxenation syndromes like hypoxia;

**Beneficial actions of free radicals**

It is now well known that free radicals are the major mediators of the bactericidal actions of neutrophils, macrophages and monocytes. Free radicals can also stimulate lymphocyte mitogenesis. Both activated macrophages and NK (natural killer) cells liberate free radicals to bring about their lytic actions on bacteria, viruses, tumour cells and virus-infected cells. Interferon, PUFAs and some anti-cancer drugs enhance the production of free radicals in the tissues and this may be responsible for their tumoricidal actions and side effects.

**Oxidative burst in neutrophils**

When leukocytes ingest micro-organisms or come in contact with a variety of inflammatory stimuli, their capacity to utilize oxygen is substantially enhanced; this can be correlated with the production of hydrogen peroxide, increase in oxygen uptake and enhanced glucose oxidation via the hexose monophosphate shunt. Leukocytes can also emit light during the respiratory burst probably due to the decay of an excited form of oxygen called singlet oxygen. Oxygen-dependent mechanisms are important in the microbicidal activity of leukocytes (for review see Ref 21); this is supported by the following observations: (i) removal of oxygen radicals by chemical scavengers or specific enzymes prevent the destructive capacity of leukocytes to microorganisms; (ii) oxygen radicals produced by chemical or enzymatic generating systems are lethal to microbes; (iii) in conditions such as chronic granulomatous disease (CGD), hereditary myeloperoxidase deficiency and specific granule deficiency there is a pronounced defect in the ability of leukocytes to kill bacteria, and hence these are characterized by increased susceptibility to recurrent bacterial infections.

Further, leukocytes from patients with CGD do not show the normal phagocytosis-associated increase in NADPH oxidase activity which is necessary for free radical generation. A heterogenous variety of particulate and soluble stimuli can trigger the respiratory burst in neutrophils, suggesting that there is more than...
one mechanism for activation of the oxidase system. Several studies reveal that cyclic nucleotides, calcium, calcium-dependent protein kinases, arachidonic acid and its metabolites, and calmodulin can regulate free radical generation by human neutrophils

**Lymphocyte mitogenesis**

Studies by Novogrodsky et al. and Gallagher and Curtis demonstrated that scavenging of hydroxyl and other reactive oxygen radicals can inhibit lymphocyte mitogenesis. Activation of macrophages, neutrophils, mast cells, eosinophils, platelets or basophils causes a rapid increase in oxidative metabolic activity in these cell types. It is known that arachidonic acid (AA) released by stimulated lymphocytes can be metabolized to prostaglandins (PGs) and leukotrienes (LTs) by resident monocytes. Both PGs and LTs are known to modulate immune response (reviewed in Ref. 23). Further, free radicals may modify PG synthesis and release AA from the cell membrane lipid pool, and modulate immune response. Thus, human neutrophils, lymphocytes, monocytes, and their metabolic products such as PGs, LTs and free radicals may behave as a self-regulatory system to elicit and control the immune response.

**Macrophages, NK cells, lymphokines, PUFAs, and free radicals**

Activated macrophages and lymphocytes are known to produce lymphokines such as interleukin-1 (IL-1), interleukin-2 (IL-2), tumour necrosis factor (TNF), and interferon. Our own studies and those of others clearly show that these lymphokines (or cytokines) can augment free radical generation by neutrophils, macrophages, lymphocytes, and NK cells. Studies by Schlager et al. clearly demonstrated that development of tumoricidal activity by lymphokine (LK)-activated macrophages is associated with an increase in macrophage linoleenic acid content. The linoleenic acid-enriched macrophages showed a transient increase in free radical release. We showed that linoleic acid and other PUFAs can augment free radical generation in human neutrophils and tumour cells. PGs have a negative feedback control on immune response and can inhibit free radical generation by neutrophils, whereas LTs augment free radical generation by neutrophils and activate NK cells. Thus, there seems to be a close interaction between neutrophils, macrophages, and immunocytes and lymphokines, free radicals, PGs, LTs and PUFAs.

**PUFAs, free radicals collagen vascular diseases and cancer**

In a series of studies we showed that tumour cells can be selectively killed by PUFAs such as gamma-linolenic acid (GLA), AA and eicosapentaenoic acid (EPA), and that their tumoricidal action is mediated by free radicals. Anticancer drugs such as adriamycin, radiation and photochemotherapy using haematoxoporphyrin derivative are all capable of augmenting free radical generation and thus bring about their tumoricidal actions. Since lymphokines enhance free radical generation and neutrophils, macrophages, NK cells, and lymphocytes generate free radicals to kill bacteria, viruses, virus-infected cells, and tumour cells, it can be said that free radicals are not always harmful. A better understanding of the free radical system may pave the way for developing methods to suppress their production in inflammatory conditions such as autoimmune disease, ageing, radiation-induced injury, and their enhancement selectively in tumour cells to eliminate them without harming normal cells. In fact, it has now been shown that though PUFAs augment free radical generation they also possess anti-inflammatory actions due to their inhibitory actions on the production of IL-1, IL-2 and TNF which are potent stimulators of inflammation, and mediators of collagen-vascular diseases and chronic inflammation.

In fact, in a recent study we have shown that in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) there is an increase in superoxide anion and H2O2 generation and that plasma lipid peroxides levels are high with a concomitant decrease in the levels of PUFAs. Since PUFAs have a negative feedback control on ILs and TNF production, it was suggested that a fall in the levels of PUFAs may contribute to continued and excess production of cytokines which can lead to chronic inflammatory process as seen in these collagen vascular diseases. The clinical relevance of these studies is that PUFAs may be useful in the treatment of collagen vascular diseases. Such a study is now in progress in our Institute and the initial results are very encouraging.

In another set of investigations the in vitro observation that some PUFAs are selectively cytotoxic to tumour cells has now been extended to animal tumour models. It was observed that PUFAs can also regress ascitic tumours and inhibit the development of skin papillomas in mice induced by benzo[a]pyrene and croton oil (unpublished data). Extending these studies in a limited clinical study, we have also shown that 1 mg/day intratumoral administration of gamma-linolenic acid (GLA) can regress human gliomas without any side-effects. Injection of GLA into the brains of normal dogs did not produce any changes in their behaviour, no clinically detectable adverse signs were observed and thorough histological examination of these brains did not reveal any cytotoxic effects on the normal dog neuronal cells (unpublished data). Thus, from the results of both in vitro and human studies it can be concluded that GLA has selective tumoricidal action without harming the normal cells at the concentrations used.
Nitric oxide and its clinical significance

Nitric oxide (NO or EDRF) is a recently identified free radical produced by endothelial cells, neuronal cells, macrophages and some tumour cells. It has potent vasodilator and platelet anti-aggregator actions and also has neurotransmitter function. Similar to other free radicals, NO has bacterial and tumoricidal actions as well. In view of its wide-ranging actions, NO is believed to have a significant role in several physiological and pathological processes. For example, NO may participate in insulin secretion, and is one of the mediators of the tumoricidal action of T cells, NK cells and macrophages. Since NO is a potent vasodilator, it is now believed that a deficiency in the production of NO may contribute to the development of systemic hypertension, primary pulmonary hypertension, atherosclerosis and peripheral vascular disease(s). It has been clearly demonstrated that inhaled NO can decrease pulmonary hypertension and endothelial dysfunction seen in hyperlipidemias may at least in part be due to the inability of endothelial cells to produce adequate amounts of NO on demand since oxidized lipoproteins are known to inactivate EDRF and NO. Hence, oral supplementation of L-arginine may form a new therapeutic approach to induce vasodilatation and prevent atherosclerosis in high risk patients. Further, NO is known to cause damage to DNA and thus may participate in some human genetic diseases.

Excess production of NO may be a key factor in the pathogenesis of endotoxaemia and septic shock and cytokines known to be involved in septicemia are also capable of augmenting the synthesis of NO. Hence, selective inhibition of NO synthesis may form a novel approach in the management of septicemia and septic shock. Similarly, NO is believed to play a role in the pathogenesis of other clinical conditions such as cirrhosis of the liver and hepatic encephalopathy, in the induction of diabetes and in systemic essential hypertension. In a recent study, we observed that in patients with essential hypertension there is a significant increase in superoxide anion, hydrogen peroxide and lipid peroxide levels with a concomitant decrease in NO levels when the hypertension is uncontrolled. Superoxide anion, hydrogen peroxide, lipid peroxides and NO levels returned to control values when the hypertension was controlled with various anti-hypertensive drugs. Since superoxide anion can inactivate NO, it is possible that the low levels of NO observed in uncontrolled hypertension may be a result of enhanced levels of superoxide anion. These results suggest that modulation of NO synthesis may be a novel approach in the treatment of hypertension.

Another condition in which NO may have a significant role is collagen vascular diseases. These are a group of immune complex diseases characterized by arthralgias and arthritis, skin rash, vasculitis, renal and pulmonary involvement. Rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren’s syndrome, vasculitis, Behçet’s syndrome and progressive systemic sclerosis (PSS) are some of the conditions included in this category. Lymphokines and free radicals are known to play a role in these conditions. Raynaud’s phenomenon, which is characterized by intense spasm of the digital vessels, is particularly a very distressing symptom of SLE, PSS and rarely in RA. Studies have demonstrated that the plasma endothelin levels are elevated in Raynaud’s phenomenon and that endothelin can induce digital arterial spasm. Since NO is the natural antagonist of endothelin, we recently studied the possible beneficial effect of L-arginine, the precursor of NO, in Raynaud’s phenomenon occurring in patients with SLE and obtained highly gratifying results. No side-effects due to L-arginine therapy were observed. The beneficial effect of L-arginine in SLE observed can be attributed to an increase in NO synthesis, the ability of NO thus formed to inhibit release of endothelin and inhibition of neutrophil superoxide anion production.

Conclusions

It is evident from the preceding discussion that free radicals including NO have both harmful and useful actions and participate in the pathobiology of several physiological and pathological processes. A better understanding of the various factors that control their synthesis, release and detoxifying mechanisms is necessary so that the free radical system can be manipulated for the benefit of the body. For example, our original hypothesis and data suggesting that free radicals are involved in the pathogenesis of essential hypertension is supported by the observation of Nakazono et al. that intravenous administration of human recombinant Cu/Zn-type SOD with high affinity for heparan sulphate on endothelial cells can prevent the development of hypertension in SHR (spontaneously hypertensive) rats. This study lends direct evidence to our free radical theory of hypertension. Hence, it is possible that in future, a recombinant human SOD with high affinity for vascular endothelial cells may be employed in the treatment of essential hypertension. Similarly, our studies with PUFAs and cancer suggested that free radical generation induced by GLA can be easily handled by normal cells but not by tumour cells and hence, tumour cells are eliminated by GLA administration. This led us to employ GLA in the treatment of human gliomas successfully. A better understanding of the interactions between endothelin, NO and lymphokines has paved the way for use of L-arginine in the treatment of Raynaud’s phenomenon and eventually in SLE by us. Based on these fruitful results, we are now studying the role of lymphokines, growth factors, free radicals, NO and PUFAs in other
clinical conditions such as diabetes mellitus, septicaemia, glomerular diseases of the kidney, myocardial infarction and rheumatoid arthritis. It is our strong belief that understanding the basic pathobiology of these diseases would eventually lead to the development of new therapeutic strategies in these clinical conditions which are likely to be less expensive, less toxic and highly fruitful.
Modern vaccinology — From empiricism to a science

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Vaccination has undoubtedly remained the most effective means of combating infectious diseases. Today the death toll from such ailments is down to just 1–2% in industrialized countries. In developing countries however the mortality from infectious diseases is still needlessly high and vaccination offers the only real way of controlling and ultimately preventing this condition. Indeed the most expensive health care policy in relation to many infections is to do nothing to prevent it. To take an example, the cost of treating just one child with tuberculosis is the same as the cost of vaccinating 700 children against this disease. To vaccinate every child in the world against diphtheria, whooping cough, tetanus, poliomyelitis, measles and tuberculosis costs as little as US $5–15 per child. In spite of the widely acknowledged cost effectiveness of a mass vaccination program, the fact remains that the basic price of many vaccines is still out of reach of many developing countries and there is a pressing need to develop even cheaper versions.

Origin and development

The foundation for immunoprophylaxis was laid in ancient China where it was observed that small pox sufferers who recovered were protected against future attacks of the disease. As a result people were encouraged to develop a mild form of infection by inhaling a powder made from grinding the dried crusts of pustules from small pox patients. As an active science however vaccinology had its advent with the landmark demonstration in late-eighteenth century by Edward Jenner that inoculation with the cowpox virus could protect humans against small pox. This was conceptually extended by Louis Pasteur almost a century later who instead of using live virulent material to confer immunity developed attenuated strains of the rabies virus as an anti-rabies vaccine. The use of attenuated non-pathogenic strains as vaccines has achieved spectacular success and accounts for many of the vaccines commercially available today e.g. oral polio vaccine, BCG, measles and influenza. An alternative to live vaccines has been to use inactivated vaccines which consist of suspensions of killed microorganisms. Examples of these include pertussis, tetanus, and the injectable polio vaccine. While these two strategies have proved enormously successful in controlling and perhaps even eliminating (as in the case of small pox) many infectious diseases there are other pathogens for which such a straightforward approach is unlikely to bear fruit. This is particularly true of those microorganisms which have learnt to evade the immune defense mechanism of the host.

One way of looking at an infection is as an interactive competition for dominance between the pathogen and host. On the one hand pathogens in general have an evolutionary advantage due to their high multiplication rates in host which may result in progeny that are even more adept at eluding immune surveillance. At the other end of the spectrum however is the immune system of the host which, though evolutionarily static over the course of an infection, is nevertheless extremely adaptive and versatile with an enormous repertoire capable of recognizing diverse protein sequences. This potential is accentuated by the fact that the repertoire is dynamic in nature and the existing repertoire can be further amplified by appropriate stimulation. It is this expanse of the repertoire coupled with its adaptability that allows our bodies to eventually prevail over most infections encountered in everyday life.

In spite of the formidable immune defense mechanism presented by the host some pathogens have nevertheless acquired the ability to confound it. Two notable examples of this are the malaria parasite Plasmodium falciparum (PF) and the etiologic agent of AIDS the human immunodeficiency virus type-1 (HIV-1). Polymorphism within the antigenic determinants of PF serves as an effective evasive mechanism. More recently the possibility of separating parasite chromosomes on