THE RELATIONSHIP BETWEEN NEUTROPHIL REACTIVE OXIDANT PRODUCTION, LUNG FUNCTION AND ANTI-OXIDANT NUTRIENTS

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A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy.

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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This research was approved by the Committee for Research on Human Subjects, University of the Witwatersrand (Protocol Nos 12/8/86; 1/6/87; 37/2/89).

This thesis is dedicated with love to all those I could not have done without, in particular, my parents who began my education, and my wife and children who continued it.

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SUMMARY

This thesis describes and discusses oxidants and anti-oxidants and their relation to human disease. Cigarette smoking is used as the model for the study of chronic inflammatory processes, firstly, to establish a causative link between oxidants and disease viz. lung function abnormalities in smokers, secondly to evaluate the interaction between hyperactive phagocytes and anti-oxidant defences and thirdly, to investigate the possibility that intervention with anti-oxidants may ameliorate the effects of chronic inflammation.

The major investigative work is summarised below.

Spirometric values determined from the flow-volume loops of 60 healthy young smokers (mean age 28 ± 0.6 years) were correlated with measurements of the release of extracellular and intracellular reactive oxidants (RO) as determined by luminol-enhanced chemiluminescence (LECL) from peripheral blood activated with the synthetic chemotactic tripeptide N-Formyl-N-Methionyl-L-Leucyl-L-phenylalanine (FMLP) combined with cytochalasin B (CB). Fractionation and reconstitution experiments revealed that LECL originated predominantly from polymorphonuclear leukocytes (PMNL). Circulating total leukocyte counts and serum thiocyanate levels were also determined. The data were analysed using Spearman's correlation coefficient and by multiple regression analysis. Cigarette smoking was associated with elevated intracellular and especially extracellular LECL

responses, the latter being strongly correlated ($p<0,0001$) with cigarettes smoked per day, serum thiocyanate levels, circulating leukocytes, and PMNL counts. Abnormalities of the spirometric parameters FEV_1/FVC, FEF_{50}/FVC, FEF_{25}, FEF_{25-75}, and FEF_{75-85} correlated best with extracellular LECL ($p<0.0002$ to $p<0.0001$), but also with pack-years ($p<0.006$ to $p<0.0001$), cigarettes smoked per day ($p<0.008$ to $p<0.0002$), thiocyanate levels ($p<0.04$ to $p<0.002$) and leukocyte counts ($p<0.03$ to $p<0.002$). According to stepwise multiple regression analysis of the data the combination of the independent variables extracellular LECL, pack-years, and numbers of circulating PMNL accounts for 35.6% of the variation in lung function in the group of cigarette smokers, with LECL being the most important contributor (26%). These findings indicate that cigarette smoking can increase the intracellular level of RO and their extracellular release from FMLP/CB-stimulated PMNL and that this increased LECL appears to be associated with the impairment of flow volume parameters.

Subsequently the plasma levels of the anti-oxidant vitamins C and E, and beta-carotene were correlated with smoking histories and the release of reactive oxidants from circulating phagocytes and spirometry in asymptomatic cigarette smokers. Smoking histories, the generation of reactive oxidants by activated phagocytes and spirometric abnormalities were strongly inter-correlated. However plasma levels of the anti-oxidant nutrients did not correlate with any of the other measured parameters. These findings indicate that plasma levels of

vitamins C and E, and beta-carotene are apparently not predictive of predisposition to oxidant-mediated-spirometric abnormalities in cigarette smokers.

Lastly, sixty asymptomatic cigarette smokers were randomly allocated into three treatment groups. Smokers in Group 1 received 900 international units of Vitamin E (VE) daily for 6 weeks, whereas 40mg of beta-carotene (BC) daily was administered to those in Group 2 for the same period. Subjects in Group 3 were treated with a matched placebo. Plasma levels of VE and BC as well as circulating leukocyte counts, and the LECL responses of blood phagocytes activated with phorbol myristate acetate (PMA) and FMLP with cytochalasin B (FMLP/CB) were measured prior to the administration of the anti-oxidants/placebo after 4 and 6 weeks of supplementation and 12 weeks after cessation of treatment. Leukocyte counts remained unchanged throughout the trial in all three treatment groups. Administration of VE for 4 weeks however was accompanied by decreased FMLP/CB-activated ($p<0.005$) and PMA-activated ($p<0.005$) LECL responses. With PMA as stimulant, the inhibition of LECL was transient, with partial recovery observed after 6 weeks despite continued administration of VE. Administration of BC was associated with progressive inhibition of both FMLP/CB-activated ($p<0.05$ and $p<0.01$ after 4 and 6 weeks respectively) and PMA-activated ($p<0.025$ after 6 weeks) LECL. No alterations in LECL responses were observed in Group 3 (placebo). VE appeared to inhibit the generation of oxidants by activated phagocytes, whereas BC scavenged oxidants generated by the myeloperoxidase/H_2O_2/halide system.

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LIST OF ABBREVIATIONS

AAP	Alpha-1 antiprotease
BC	B-Carotene
CB	Cytochalasin B
$C5^a$	Fifth component of complement
DAG	Diacylglycerol
FMLP	N-Formyl-N-Methionyl-L-Leucyl-L-phenylalanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
HLE	Human leukocyte elastase
HOCL	Hypochlorous acid
H_2O_2	Hydrogen peroxide
IL-I	Interleukin-I
IP_3	Inositol 1, 4, 5 triphosphate
LTB_4	Leukotriene B_4
MPO	Myeloperoxidase
NADP+	Nicotine adenine dinucleotide
NAP	Neutrophil activating peptide (IL-8)
$O_2^{\cdot -}$	Superoxide radical
O_2	Molecular oxygen
$OH^{\bullet}$	Hydroxyl radical
PMA	Phorbol myristate acetate
PMNL	Polymorphonuclear leukocyte
RO	Reactive oxidants
SOD	Superoxide dismutase
TNF	Tumour necrosis factor
VC	Vitamin C
VE	Vitamin E

LUNG FUNCTION PARAMETERS

FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
FEF_{50}	Forced expiratory flow rate at 50% of the FVC
FEF_{25-75}	Mean forced expiratory flow rate from 25% to 75% of the FVC
FEF_{75-85}	Mean forced expiratory flow rate at 75% to 85% of the FVC
FEV_1/FVC	The FEV_1 as a percentage of the FVC
FEF_{50}/FVC	The forced mid expiratory flow rate as a percentage of the FVC

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CHAPTER 1

INTRODUCTION

1.1 THE PROTEASE/ANTI-PROTEASE-IMBALANCE HYPOTHESIS OF EMPHYSEMA

It is well recognised that cigarette smoking is the major risk factor for both emphysema and lung cancer and that the epidemic of both has been only minimally influenced by the adverse publicity initiated by Doll and Hill in 1952 (Doll and Hill, 1952; Doll, 1981; Doll and Peto, 1978; Seeley et al. 1971). Despite the established causal risk, investigations into possible mechanisms have been hampered by the fact that not all people who smoke develop chronic obstructive pulmonary disease and that those that do may not develop it at the same rate (Fletcher and Peto, 1977; US Dept of Health and Human Services, 1984), and in addition, even fewer smokers will develop lung cancer. In their study in 1977, Fletcher and Peto described the presence and rate of development of airway obstruction in a smoking population aged 51 to 59 (Fletcher and Peto, 1977). Twenty eight percent of ex-smokers, 24% of light smokers (<15/day) and 46% of heavy smokers (>15/day) manifested airway obstruction as determined by decreased FEV_1. In addition, the rate of development of the disease appeared to be related to both an inherent susceptibility and to the total number of cigarettes smoked.

These epidemiological links have precipitated the development of new hypotheses regarding the pathogenesis of obstructive disease, viz. that the histologically noted inflammatory and structural changes (Berend et al. 1979) which

occur in the lungs of cigarette smokers are due to an imbalance between factors causing and those preventing proteolysis. It has also been postulated that the polymorphonuclear leukocyte (PMNL) is responsible for this imbalance because of the known effects of smoking on PMNL number, localisation and metabolism (Idell and Cohen, 1983).

1.2 THE EFFECTS OF CIGARETTE SMOKING ON PMNL

1.2.1 The Effects of Cigarette Smoking on PMNL Number

Cone and co-workers in 1971 and later Helman and Rubenstein in 1973 showed that smoking increased the peripheral PMNL count and that this increase was proportional to the amount of cigarettes smoked (Cone et al. 1971; Helman and Rubenstein, 1973). In addition to these observations it has been shown more recently that an inverse relationship exists between the peripheral leukocyte count and the FEV_1 irrespective of smoking history (Chan-Yeung and Dy Buncio, 1984; Chan-Yeung et al. 1988). Of note, however, is the fact that the initial PMNL count correlated significantly with the <u>decline</u> in FEV_1 in smokers only, and not in non- or ex-smokers.

1.2.2 The Effects of Cigarette Smoking on PMNL Localisation

The examination of cell suspensions isolated from bronchial lavage fluid in both smokers and non-smokers demonstrate significant increases in both PMNL and macrophages in the former. It is likely that this altered cellular

population results from secretion of chemotactic factors from macrophages which have been stimulated by a component of tobacco smoke (Hoidal and Niewoehner, 1982; Hunninghake and Crystal, 1983).

1.2.3 The Effects of Cigarette Smoking on PMNL Metabolism

Studies by Hoidal and colleagues demonstrated that, in addition to increased number, macrophages and PMNL have enhanced oxidative metabolism and that in vivo and in vitro this is associated with a decrease in the elastase inhibitory capacity (EIC) of alpha-1 anti-protease (Carp and Janoff, 1978; Carp and Janoff, 1980; Clark et al. 1981; Gadek et al. 1979; Janoff et al. 1983; Johnson and Travis, 1979). A decrease in the EIC supplies indirect evidence for oxidant release beyond the phagosome and, in addition, implies that the RO burden could be capable of overwhelming normal systemic anti-protease defence mechanisms. If this occurred persistently, chronic elastolysis and proteolysis would occur, inducing tissue damage which could culminate in emphysema.

To summarise, the PMNL is considered the major culprit in the aetiology of emphysema because:

1. smokers have a peripheral leukocytosis
2. lung phagocyte recruitment is induced by cigarette smoke, and
3. PMNL and macrophages have increased oxidative function in cigarette smokers.

These abundant hyperactive macrophages and PMNL in the lungs of smokers may be important not only in the aetiologic aspects of emphysema, but also bronchial carcinoma because of the potential mutagenic effects of reactive oxidants (Weitberg et al. 1983; Weitzman et al. 1985; Junod, 1987).

1.3 THE PHAGOCYTE POPULATION

There are two major groups of phagocytic cells, both derived from bone marrow, the PMNL and the mononuclear cells. The latter cells which have the potential to differentiate into tissue macrophages such as alveolar macrophages, provide a non-specific defence mechanism elaborating substances which, as described by Babior: "kill like Atilla the Hun, laying waste to both the targets and the nearby landscape with the subtlety of an artillery barrage" (Babior, 1984b). Exposure to a chemotactic stimulus initiates activity to ensure the proximity of the cell to the invader, ie. chemotaxis, adherence and aggregation. This is followed by degranulation and the release of preformed lysozymal elements and by activation of membrane associated NADPH-oxidase to form reactive oxygen species. Description of the processes of chemotaxis, recognition and ingestion are not wholly within the scope of this study save to demonstrate that the process is activated by similar stimuli, and that these initial steps result in the presence of phagocytic cells at the sites where they wreak destruction. They will be discussed briefly.

1.3.1 Chemotaxis

Chemotaxis involves the vectoring of cells towards a target by means of concentration gradients of chemotactic factors released in the environs of tissue damage or invasion (Stossel, 1974). Physiological inflammatory/chemotactic stimuli such as C_{5a} and bacterially derived N-formylated polypeptides as well as non-physiological stimuli such as phorbol myristate acetate (PMA) promote adhesion of the PMNL to endothelial surfaces of target organ capillaries, as well as chemotaxis. The former however requires higher concentrations of stimulant than the latter (Gamble et al. 1985; Charo et al. 1986; Schleimer and Rutledge, 1986).

The microtubule organising centres and Golgi apparatus are located on the same side of the nucleus as the chemoattractant (Malech, 1977). This placement and an intact microtubule system appear essential to determine the direction of PMNL locomotion, the orientation of pseudopodia and the positioning of the cytoplasmic granules on the same side as the stimulus (Malech, 1977; Zigmond, 1977; Zakhireh and Malech, 1980; Zigmond et al. 1987). PMNL chemotaxis and diapedesis are impeded initially by the endothelial cell barrier to which they adhere, requiring them to extend a leading lamella between the cells and thus to squeeze through. The PMNL undergoes considerable deformation during trans-endothelial passage, but its overall integrity and that of the endothelial cell remains intact (Hurley, 1963; Marchesi and Florey, 1960). The <u>in vivo</u> adherence of PMNL to the endothelium is greatly augmented by

tissue damage and other inflammatory stimuli and, with more intense inflammation adhesion between PMNL occurs, greatly enhancing the numbers localised to the site of damage.

1.3.2 Mechanisms of Adhesion

PMNL utilise a complex array of adhesive mechanisms that determine their locomotion to and localisation at sites of inflammation. The adhesion molecules on PMNL and endothelial surfaces are shown in Figure 1. Mac-I and LFA-I are glycoproteins belonging to a subgroup of adhesion molecules, the integrins. These share a ß2 subunit (CD18), but have differing α-subunits designated CD11a and CD11b respectively (Smith, 1990). Mac-I is found on all PMNL as is LFA-I, the former in an intracellular pool within the secondary granules that can be localised within minutes to the cell surface and the latter remaining on the cell surface with additional amounts synthesised as required. Mac-I mediates adhesion, spreading, H_2O_2 secretion and homotypic aggregation, whereas LFA-I appears to recognise the intracellular adhesion molecule-I (ICAM-I) on endothelial cells. The endothelial cell surface constitutively expresses ICAM-I but not its other major adhesion molecules, endothelial-leukocyte adhesion molecules (ELAM-I) and GMP-140 (Smith, 1990).

FIGURE 1

ADHESION MOLECULES INVOLVED IN CHEMOTAXIS

NEUTROPHILS	ENDOTHELIAL CELLS
LFA-I	ICAM-I
Mac-I	GMP-140
MEL-14 Antigen	MEL-14 Receptor

Both of these molecules are markedly upregulated following cytokine stimulation (Mantovani and De Jana, 1989; Smith, 1990). The major cytokines involved are interleukin-I (IL-I) and tumour necrosis factor (TNF). IL-I is a cytokine produced by many cell types, but primarily by the mononuclear phagocytes (Oppenheim et al. 1986; Dinarello et al. 1988). It is not directly chemotactic, but elicits PMNL extravasation by changing the adhesive properties of endothelial cells, as described above, and by inducing production of chemotactic cytokines (Bevilacqua, 1985; Sayers et al. 1988). TNF is a functionally related cytokine that exhibits pro-inflammatory responses in endothelial cells which overlap with those of IL-I. TNF also induces expression of adhesion structures for PMNL (Gamble et al. 1985) and the production of chemotactic cytokines and colony stimulating factors from endothelial cells and macrophages (Mantovani and De Jana, 1989; Seelentag et al. 1987). (The latter are of relevance in that they regulate the differentiation of haemopoietic precursors of myelomonocytic lineage (Clark and Kamen, 1987)).

Chemotactic stimuli for PMNL therefore promote the adhesive functions of Mac-I, the enhancement of H_2O_2 secretion and homotypic aggregation while cytokine stimulation of endothelial cells results in expression of ICAM-I which is recognised by LFA-I. Integrin (CD18) independent adhesion to endothelial cells appears to involve three related molecules. ELAM-I, MEL-14 antigen and GMP-140 (McEver et al. 1989; Smith, 1990), the genes for which appear to be located on chromosome I

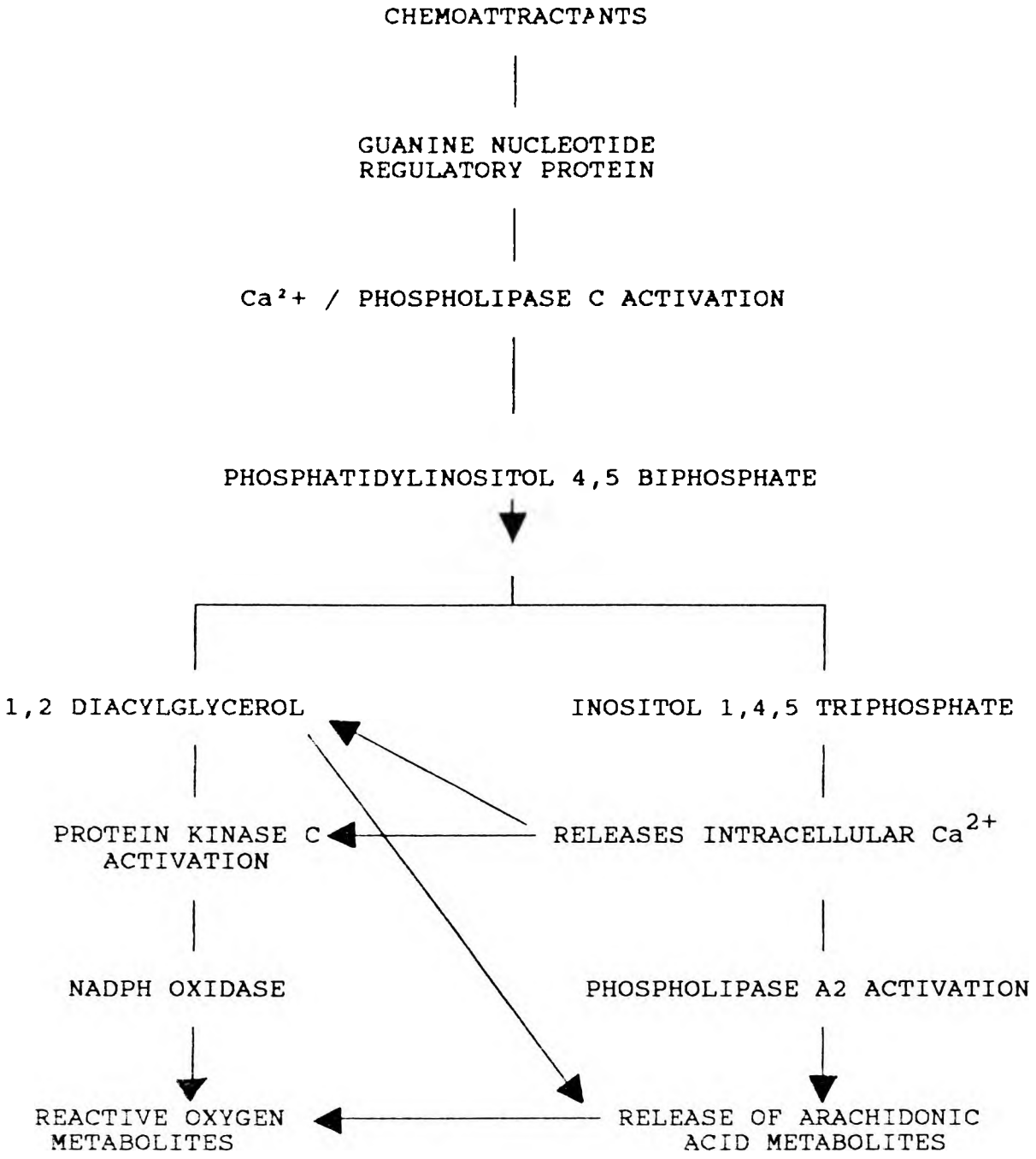
(Smith, 1990). Elam-I as noted above, is not constitutively expressed on the cell surface, but is synthesised and expressed over a period of 2-6 hours following cytokine stimulation. GMP-140 however is located in Weibel-Palade bodies and is rapidly shifted to the cell surface in response to histamine. These molecules are adhesive to unstimulated PMNL (Smith, 1990). The MEL-14 antigen which is constitutively expressed on the PMNL surface rapidly downregulates following cytokine stimulation, coincident to the upregulation of Mac-I. This probably prevents accumulation of unstimulated PMNL (bound to MEL-14) at the inflammatory site and promotes binding of stimulated PMNL via Mac-I and LFA-I (Smith, 1990). The relative importance of the various adhesion mechanisms remains uncertain at this stage.

1.3.3 Stimulus-Response Coupling

Studies of leukocyte chemotaxis have identified a number of factors which lead to the accumulation of inflammatory cells. Subsequent investigations have shown that these same chemotactic factors at high concentrations stimulate potentially cytotoxic or microbicidal responses by PMNL through degranulation of storage vesicles and the production of toxic oxygen products. The binding of these chemoattractants to specific receptors on the cell surface is followed by rapid transient increases in phosphoinositide metabolism and cytosolic calcium levels (Fantone et al. 1987) (Figure 2). Specific receptors have been characterised for the N-formylated peptides, C5a and leukotriene B4 (LTB4). All utilise a similar

FIGURE 2

BIOCHEMICAL CHANGES FOLLOWING STIMULATION OF INFLAMMATORY CELLS



transduction pathway but different receptors. Binding studies for FMLP demonstrate a single receptor with approximately 50,000 sites per cell, LTB4 has 2 receptors with 4,000 and 270,000 sites respectively and C5a one receptor with approximately 10,000 sites per cell (Williams et al. 1977; Koo et al. 1982; Kreisle and Parker, 1983; Goldman and Goetzl, 1984; Hugli, 1984; Schreiber, 1984).

Chemoattractant receptors appear to be coupled to a GTP binding protein, termed a guanine nucleotide regulatory protein or a G-protein. These are plasma membrane components that serve a transducing function between a variety of receptors and their effector systems (Gilman, 1984). Interaction of the chemoattractant receptor with the G-protein results in a high affinity of this complex for chemoattractants. Subsequent binding to the receptor stimulates the binding of GTP to the G-protein (G-GTP) which activates polyphosphoinositide-specific phospholipase-C (PLC), enabling it to express activity at physiological calcium concentrations. G-GTP dissociates from the receptor and hydrolysis of the GTP component terminates the reaction by producing inactive G-GDP (Snyderman and Pike, 1984; Smith et al. 1986; Fantone et al. 1987). Subsequent to PLC activation, enhanced turnover of membrane phosphoinositides results in the formation of inositol 1, 4, 5 triphosphate (IP3) and diacylglycerol (DAG) from the phosphodiesteric cleavage of phosphatidylinositol 4, 5-biphosphate a membrane phospholipid (Figure 2) (Litosch and Fain, 1986; Majerus et al. 1986; Fantone et al. 1987).

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IP_3 and DAG are messenger molecules for a diverse group of cellular functions (Fantone et al. 1987) (Figure 2). IP_3 induces the release of calcium from intracellular stores (Prentki et al. 1984) which results in the activation of phospholipase A2 (White et al. 1984) with the consequent formation and release of arachidonic acid (Fantone et al. 1987) and its derivatives. Diacylglycerol, however, binds to and activates protein kinase C in a similar manner to the tumour promoting phorbol esters such as phorbol-myristate acetate (Fantone et al. 1987). It appears that calcium is also involved in the activation of protein kinase C, and the fact that this is synergistic with the action of phorbol esters, indicates that both may act via increases in diacylglycerol (White et al. 1984). Interestingly, while the formyl peptide alone causes an increase in DAG, this is markedly enhanced by the inclusion of cytochalasin B (CB) (Honeycutt and Niedel, 1986) which suggests that CB may also increase levels of DAG in cells exposed to chemoattractants and that this increase may be via alternate sources not derived from phosphoinositide metabolism. (This would be similar to the action of PMA and calcium ionophores (Bennett et al. 1980; Truett et al. 1987)). DAG production in response to physiological chemoattractants appears to occur in two phases, the first due to phosphatidylinositol 4,5 biphosphate hydrolysis and the second as a result of calcium influx from extracellular stores (Truett et al. 1987).

1.3.4 Degranulation and Phagocytosis

Coincident with the onset of membrane associated oxidative metabolism is the ingestion of the organism by means of an active, energy-dependent event involving activation of ATP generating processes This is consistent with the idea that energy utilisation by myosin-actin interaction drives ingestion as well as migration. The opsonised microorganism is engulfed by the phagocyte and sequestered in a vesicle lined with what was plasma membrane which is termed the primary phagosome (Stossel, 1974; Babior, 1978; Fantone et al. 1987).

Phagocytosis results in the release of preformed granule (or lysozymal) contents to the outside of the cell without concomitant liberation of cytoplasmic materials (Henson, 1971; Leoni and Dean, 1983). Little is known of the actual molecular events that occur during fusion of secretory vesicles or granules with the plasma membrane in PMNL undergoing exocytosis, but the process can be broken down as follows:

(a) An intracellular signal causes translocation of secretory vesicles to the inner surface of the plasma membrane;

(b) Repulsive forces are overcome enabling contact of the two opposing membranes;

(c) The membrane bilayers undergo a focal destabilisation;

(d) Fusion of the secretory vesicle membrane and the plasma membrane occurs with re-establishment of the membrane bilayer structure (Henson et al. 1988).

An increase in intracellular ionised calcium appears to be the triggering event for many PMNL functions (Henson et al. 1988) and this is in agreement with the observed responses following stimulation of PMNL with chemoattractants.

Whatever the exact mechanisms, cytoplasmic organelles fuse with the phagosome membrane and discharge their contents. In PMNL the primary (azurophilic) granules contain, amongst others, myeloperoxidase (MPO), about 10% of total lysozyme, a number of acid hydrolases, elastase and cationic proteins, while the secondary (specific) granules contain lactoferrin, vitamin B12 binding protein, collagenase, gelatinase, lysozyme and alkaline phosphatase (Stossel, 1974). Contrary to the findings of previous investigators (Dewald et al. 1982), the main storage compartment of gelatinase has been found to be localised to the specific granules (Hibbs and Bainton, 1989). (By labelling with human lysozymal membrane glycoproteins another compartment has been identified within PMNL which is composed of small and large vesicles and multivesicular bodies (Bainton and August, 1988)). The recently described serine esterase enzyme proteinase-3 (see later) is also derived from PMNL granules, but although it is uncertain which one, it is probably the azurophilic granules (Kao et al. 1988).

PMNL therefore contain many enzymes, of which four, elastase, proteinase-3 and the two metalloproteinases, collagenase and gelatinase have the greatest destructive potential. These enzymes are able to attack the essential components of the extracellular matrix, (collagen, elastin,

proteoglycans and glycoproteins) which lie beneath epithelium and surround connective tissue cells (Kao et al. 1988; Weiss, 1989). The functional interaction between these enzymes and the products of oxidative metabolism will be discussed further.

Macrophage granules contain additional factors such as plasminogen activating factors and cathepsin but mature macrophages, as opposed to juvenile phagocytes, do not contain MPO (Nakagawara et al. 1981).

1.3.5 Membrane Associated Activity

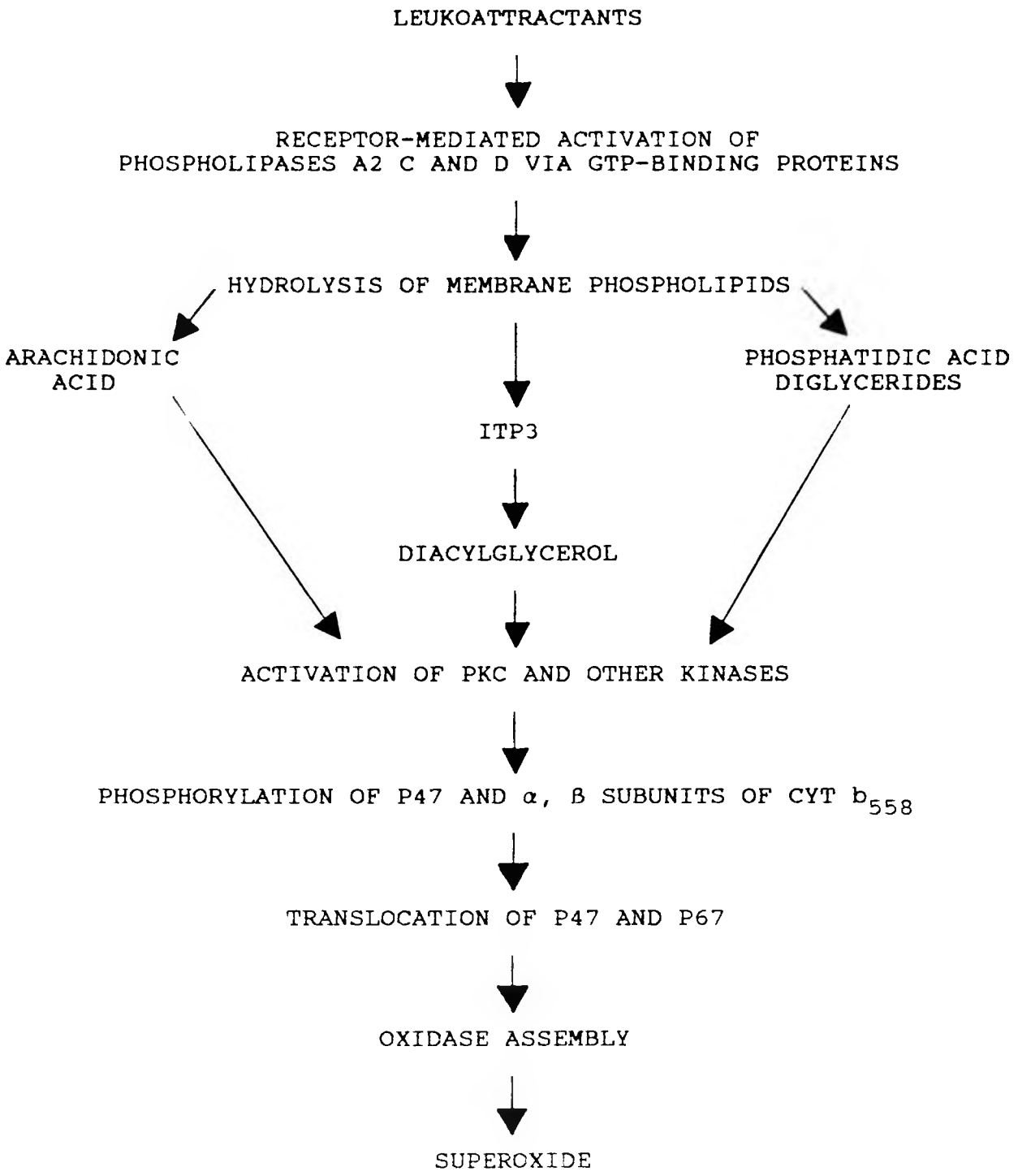
As can be noted from Figure 2, additional pro-inflammatory products are produced via calcium-dependent activation of phospholipase A_2 (PLA_2). The first involves the release of arachidonic acid from cell membrane phospholipids (which may also be derived from the metabolism of diacylglycerol by diacylglycerol lipase) and the second involves the release of reactive oxygen metabolites by activation of NADPH-oxidase (Fantone et al. 1987). (The products of arachidonic acid metabolism from both the 5'-lipoxygenase and cyclo-oxygenase pathways contribute to inflammation in diverse ways but will not be discussed in any detail in this discourse).

The respiratory burst is initiated by the activation of the enzyme NADPH-oxidase which catalyses the one electron reduction of oxygen to superoxide ($O_2^{\cdot-}$) at the expense of NADPH (Malech and Gallin, 1987).

Perturbation of the cell membrane therefore induces the rapid consumption of oxygen, its conversion to oxygen radicals, and the acceleration of the hexose-monophosphate shunt (HMPS) due to increased and continued oxidation of NADPH to NADP+ (Klebanoff, 1980) (see Figures 3 and 5). The mechanisms involved in this process are still being unravelled. NADPH-oxidase, which is dormant in the resting PMNL, exists as a membrane associated oxidase, binding NADPH in the cytosol and generating superoxide at the exterior in the stimulated cell (Babior et al. 1981). This oxidase consists of a number of components located in both the membrane and the cytosol which interact to accomplish the reduction of oxygen to superoxide (Segal, 1989; Clark, 1990).

The membrane-associated components comprise a unique cytochrome (cytochrome b_{558}), a flavin adenine dinucleotide (FAD) containing flavoprotein and possibly ubiquinone. Cytochrome b_{558}, because of its unusually low midpoint potential (-245mV) is capable of producing all the superoxide generated by the membrane oxidase (Cross et al. 1985; Segal, 1989; Clark, 1990; Baggiolini and Wymann, 1990). It consists of two subunits, the larger ß subunit, molecular mass approximately 91 kDa, is a glycoprotein (designated gp91) and the smaller, α-subunit molecular mass 22 kDa, is a peptide (designated p22). Most patients with classical X-linked chronic granulomatous disease lack both of these subunits (Segal, 1987; Parkos et al. 1989). It is possible that a flavoprotein is the component linking the substrate NADPH with cytochrome b_{558} and that the co-factor involved is flavin adenine dinucleotide (FAD) (Babior, 1987; Segal, 1989).

FIGURE 3 TRANSDUCTIONAL MECHANISMS INVOLVED IN THE ACTIVATION OF NADPH-OXIDASE BY PHYSIOLOGICAL SIGNALS



Whereas the only thoroughly characterised membrane component is cytochrome b_{558}, the cytosolic components consist of at least two PMNL proteins of 47 kDa and 67 kDa, designated p47 and p67 respectively (Volpp et al. 1988). Moreover, all autosomal recessive cytochrome b_{558} positive chronic granulomatous disease (CGD) patients have had a total deficiency of either p47 or p67 (mostly of the former) (Clark, 1989).

Activation is initiated by signal transduction elements described previously, ie. binding to a high affinity receptor, G-protein-coupled activation of phospholipase C, release of inositol phosphates, elevation of cytosolic Ca^{2+}, activation of protein kinase C and the phosphorylation of the cytosolic proteins. The link between the NADPH-oxidase system and the above is poorly understood at present, but a GTP binding protein located in the cytosol appears to be important because of the affinity of both p47 and p67 for GTP (Gabig et al. 1987; Volpp et al. 1988). In addition, phosphorylation of the cytosolic component p47 by a kinase (probably protein kinase C) (Clark, 1990) is associated with activation of the respiratory burst. How phosphorylation affects the functional properties of p47 is uncertain but it possibly facilitates movement of the protein to its membrane docking site on cytochrome b_{558} by neutralising the strong positive charge of its COOH-terminal region (Clark, 1990). The NADPH binding component has not been precisely identified, although it is possible that it is the FAD - flavoprotein. Electrons are shuttled from NADPH via proximal

carriers including p67 and p47 to the heme group of cytochrome b_{558} and thence to O_2. The transductional mechanisms involved in the activation of NADPH-oxidase by physiological signals are seen in Figure 3.

1.3.5.1 The chemistry of free radicals

Free radicals have abundant sources both endogenous and exogenous (Table 1). Oxygen radicals are unstable forms of oxygen which, though capable of independent existence, due to the presence of one or more unpaired electrons are amongst the most reactive substances known to chemistry. The oxygen molecule is, by definition, itself a radical (diradical), having two unpaired electrons, each located in differing orbitals. Its reactivity is limited, however, by the fact that the electrons in its outer orbitals have parallel spin instead of the necessary opposite spin. This imposes a restriction on electron transfer, allowing acceptance of only single electrons and therefore resulting in its sluggish reaction with non-radicals (Britigan et al. 1988).

One electron reduction of ground state oxygen produces the superoxide radical ($O_2^{\cdot-}$), which when it itself is reduced produces the peroxide ion ($O^{2-}{}_2$). Despite the fact that the latter exists in biological systems as H_2O_2 (hydrogen peroxide) and is therefore not a radical it is, however a potent oxidising agent (Babior, 1984; Fantone et al. 1987; Klebanoff, 1980; Malech and Gallin, 1987; Reilly and Chapman, 1988).

TABLE 1

THE SOURCES OF FREE RADICALS

ENDOGENOUS	• *MITOCHONDRIAL ELECTRON TRANSPORT CHAIN*
	• *MICROSOMAL ELECTRON TRANSPORT CHAIN*
	• *OXIDANT ENZYMES - CYCLOOXYGENASES*
	- LIPOXYGENASES
	- DEHYDROGENASES
	- PEROXIDASES
	• *PHAGOCYTE MEMBRANES - NADPH-OXIDASE*
	• *ENDOTHELIAL CELL MEMBRANES*
EXOGENOUS	• *TOBACCO SMOKE*
	• *POLLUTANTS (OZONE)*
	• *ORGANIC SOLVENTS (CARBON TETRACHLORIDE)*
	• *IONIZING RADIATION*
	• *PESTICIDES (PARAQUAT)*
	• *DRUGS (PARACETAMOL, BLEOMYCIN, DAUNORUBICIN etc)*

The reactions are written as follows:

$$2O_2 + NADPH \longrightarrow 2O_2^{\cdot} + NADP^+ + H^+$$

In practice H_2O_2 is formed in a dismutation reaction, in which one molecule of O_2 is oxidised by another in the presence of the enzyme superoxide dismutase (SOD) which is particularly active at neutral or alkaline pH where spontaneous dismutation is slow.

$$HO_2^{\cdot} + HO_2^{\cdot} \xrightarrow{SOD} H_2O_2 + O_2$$

(Babior, 1978; Babior, 1984; Fantone et al. 1987).

It has been postulated that the further reduction of H_2O_2 in the presence of a transition metal catalyst could generate an even more powerful oxidant - the hydroxyl radical ($OH^{\cdot}$) [the Haber-Weiss Reaction] (Babior, 1978; Fantone et al. 1987; Klebanoff, 1980; Malech and Gallin, 1987).

$$O_2^{\cdot} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \qquad Fe^{3+} + OH^- + OH^{\cdot}$$

or $$O_2^{\cdot} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot}$$

This hydroxyl radical, which is extremely reactive has, however, not been shown to be generated by neutrophils (Malech and Gallin, 1987). In addition, even if the reaction is triggered in the presence of high concentrations of iron as catalyst, little if any $OH^{\cdot}$ is formed. Innate cellular protective mechanisms divert H_2O_2 to alternative chemical reactions and also limit the availability of iron by releasing lactoferrin which renders it unavailable to the reaction (Britigan et al. 1988).

It has also been postulated that singlet oxygen, an electrically excited molecule possessing energy above ground state (22 Kcal and 38 Kcal respectively for the 2 forms found ΔO_2^1 and ΣO_2^1) may participate in the development of tissue damage. It appears however, that only insubstantial amounts of this oxidant are generated under physiological conditions (Kanofsky et al. 1984).

1.3.6 The Myeloperoxidase Halide System

The majority of the H_2O_2 formed is catabolised rapidly in the presence of a halide (which due to its ubiquitous nature is usually chloride) and the granule derived enzyme myeloperoxidase (MPO), to form hypohalous acids, or more specifically hypochlorous acid (HOCl) (Klebanoff, 1980; Malech and Gallin, 1987; Test and Weiss, 1986).

$$H_2O_2 + X^- + H^+ \xrightarrow{MPO} HOX + H_2O$$

$$x = Cl^-;\quad Br^-;\quad I^-;\quad SCN \text{ (thiocyanate)}$$

It has recently been demonstrated that the major product of oxidative metabolism is HOCl and that 10^6 PMNL can produce approximately 2×10^{-7}mol (equivalent to about 200 μM) of HOCl during a two hour incubation (Test and Weiss, 1986).

HOCl is an extremely powerful oxidant and has many potential targets - amines, thiols, thioethers, nucleotides, hemoproteins and polyenoic acids. Such is its reactivity that most of the HOCl formed immediately disappears in attacks on biological molecules containing these moieties (Test and Weiss, 1986).

Importantly, a substantial portion reacts with low molecular weight amines to yield chloramines which though less powerful than HOCl are available to chlorinate or oxidise a wide range of targets.

$$R - NH2 + HOCl \longrightarrow R\ NHCl + H_2O$$

Because HOCl and O_2^- are rapidly utilised it would be expected that all oxygen metabolites would disappear rapidly from the serum. It has been shown, however, that depending on the R group, the N-chloramines so formed may have either a long or short half life. As opposed to HOCl therefore they have selective reactivity, stability and long half life (up to 16 hours) which allows their accumulation at inflammatory sites and their diffusion over large distances (Test et al. 1984; Weiss et al. 1983).

The well characterised products therefore resulting from PMNL oxygen metabolism are O_2^- and H_2O_2 via NADPH-oxidase activation, HOCl from interaction of these products with MPO and chloride and the N-chloramines from the interaction of HOCl with PMNL or serum derived amines (Table 2).

While this reaction is long lasting, with MPO generating additional HOCl from its substrate H_2O_2, it is ultimately self limited (Nathan, 1987). Once the pool of extracellular and cellular targets are depleted, both the chemotactic agent and the MPO are themselves oxidatively inactivated (Test and Weiss, 1986).

TABLE 2

DERIVATION OF REACTIVE OXIDANT METABOLITES

$$2O_2 + NADPH \xrightarrow[OXIDASE]{NADPH} 2O_2^{\cdot-} \text{ (SUPEROXIDE)} + NADP^+ + H^+$$

$$HO_2^{\cdot} + HO_2^{\cdot} \xrightarrow{SOD} H_2O_2 \text{ (HYDROGEN PEROXIDE)} + O_2$$

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl \text{ (HYPOCHLOROUS ACID)} + H_2O$$

$$R - NH_2 + HOCl \longrightarrow R - NHCl \text{ (N CHLORAMINES)} + H_2O$$

HABER-WEISS

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$$

1.3.7 Which Radicals are Toxic?

Both O_2^- and H_2O_2 can react with a number of substrates but have constraints placed on their ability to directly mediate tissue damage. Almost all the O_2^- produced is rapidly and preferentially converted to H_2O_2 and, in addition, most H_2O_2 is rapidly consumed by the PMNL in reactions mediated by MPO. Much of the remaining H_2O_2 is metabolised by both catalase and glutathione peroxidase.

As has been previously mentioned, the hydroxyl radical has not been shown to be produced as part of PMNL oxygen metabolism, whereas large volumes of HOCl (up to 2500 - 5000nmol per $25x10^6$ cells) have been shown to be produced (Wasil et al. 1987) and that this is highly reactive with many biological targets and may also produce a generation of longer lasting oxidants, the N-chloramines.

1.3.8 Tissue Injury Mediated by Reactive Oxidants

It is likely that any <u>direct</u> injury from oxidants that occurs is mediated by these latter substances. However it is possible that they are not as important as had been thought since HOCl preferentially reacts with amines to yield chloramines which, while strongly microbicidal, apparently do little tissue damage (Weiss, 1989). Weiss also draws attention to the fact that large quantities of both substances (ie. buffered HOCl in high concentration and synthetic chloramines) were used as wound disinfectants in the First World War without adverse results (Weiss, 1989). He does, however, make three provisos: firstly, these substances have been shown to be toxic

in vitro in simple buffer systems that do not contain amine moieties; secondly, that it is possible that the toxic effect of HOCl is effected through the prior binding of MPO to the target cell and thirdly, that it is possible that lung and glomerular tissue are specifically sensitive to the toxic effects of chloramines (Weiss, 1989). Whatever the final toxic product of oxygen metabolism, injury is mediated either directly, through lipid peroxidation and DNA injury or indirectly by depriving cells of both energy and of the anti-proteolytic screen or by activation of PMNL derived metalloproteinases (Table 3).

DIRECT MECHANISMS

1.3.8.1 Lipid peroxidation

A primary mechanism proposed to explain free radical-mediated cell and tissue injury involves the formation of lipid peroxides within cell membranes and organelles. These interfere with the function and integrity of the membrane and, in addition the lipid-peroxy compounds so formed function as intermediates in the propagation sequence (Halliwell and Gutteridge, 1985.)

Cellular membranes consist of a lipid bilayer with protein moieties interspersed, with the content of the latter increasing in relation to the activity of the membrane, eg. a myelin sheath contains 20% protein and the mitochondrial membrane 80%. The fatty acids of lipid membranes are located in the hydrophobic regions of the bilayer and consist of long unbranched chains with even numbers of carbon atoms which are inbound to the polar portion of the lipid layer.

TABLE 3

TISSUE TOXICITY MEDIATED BY OXYGEN METABOLITES

Direct	*1.*	*lipid peroxidation of cell membrane components (Halliwell and Gutteridge, 1985; Logan and Davies, 1980)*
	2.	*DNA damage (Weitberg et al. 1983; Weitzman and Stossel, 1982; Weitzman et al. 1985; Jackson et al. 1989)*
Indirect	*1.*	*Cellular energy depletion (Dallegri et al. 1988; Baker et al. 1989)*
	2.	*oxidative inactivation of the anti-protease screen (Carp and Janoff, 1980; Clark et al. 1981; Gadek et al. 1979; Janoff et al. 1983; Johnson and Travis, 1979)*
	3.	*oxidative auto-activation of PMNL derived metalloproteinase enzymes (Desroches et al. 1988; Weiss et al. 1985)*
	4.	*proteolytic inactivation of Alpha-1-Antiprotease (AAP) (Desroches et al. 1988; Wasil et al. 1987; Weiss et al. 1985).*

The sequence of events culminating in lipid peroxidation involves three phases: initiation, propagation and termination (Halliwell and Gutteridge, 1985).

Initiation is the abstraction of a hydrogen atom from a methyl group by any agent of sufficient reactivity. The free radical so derived undergoes molecular rearrangement to form a diene which can react with oxygen to form a peroxy-radical.

$$CH_2 \longrightarrow H - C\cdot + H^+$$

$$H - C\cdot + O_2 \longrightarrow R\text{-}OO^\circ \text{ (peroxy-radical)}$$

Propagation. The peroxy-radical extracts a hydrogen atom from an adjacent lipid molecule, thereby giving rise to the lipid hydroperoxide R - O - O - H and another free radical:

$$R\text{-}OO\cdot + R\text{-}H \longrightarrow R\text{-}OOH + R\cdot$$

This reaction becomes a self-propagating chain reaction terminated by bond rearrangements to form diene conjugates which include cyclic peroxides and cyclic endoperoxides in addition to the lipid hydroperoxides mentioned earlier. These substances undergo further degradation to form aldehydes such as malondialdehyde and also metal catalysed ß-scission reactions to form volatile hydrocarbons such as ethane, pentane and ethene.

Aldehydes may react with the amine and sulphydryl groups of membrane proteins, thereby inactivating surface receptor molecules such as Na^+ and K^+-ATPases and membrane bound enzymes. In addition, continued fragmentation of the fatty acid side chains results in alterations in membrane permeability and cell death.

Free radical chain reactions may be terminated by naturally occurring anti-oxidants such as Vitamin E or by the enzymatic reduction of lipid hydroperoxides by glutathione peroxidase. These latter protective mechanisms will be discussed later.

1.3.8.2 DNA damage

Stimulated human PMNL can, through production of oxygen metabolites, induce DNA strand-scission, mutations in bacteria, and mutations and sister chromatid exchanges in cultured hamster ovary cells (Brawn and Fridovich, 1981; Weitberg et al. 1983; Weitzman and Stossel, 1981).

The mechanisms by which these changes occur have not been precisely elucidated but the predominant extracellular oxidant responsible appears to be H_2O_2 (Jackson et al. 1989). Following diffusion into the cell H_2O_2 generates OH· in the presence of iron which oxidatively damages adenine, guanine, thymine and cytosine and induces DNA strand breaks (Weitzman, 1985). In addition, poly ADP ribose polymerase, an enzyme associated with DNA repair, shows enhanced activity post hyperoxic exposure, and after exposure of DNA to H_2O_2 at concentrations of 20 μM and greater, correlating with the previously noted DNA damage (Junod, 1987; Anderson and Theron, 1990). Although DNA is subject to repair by this enzyme, it may be incomplete or incorrect resulting in gene modification which could promote cellular transformation (Jackson, 1989). Potential but unproven mechanisms of injury include an effect on the key enzymes responsible for DNA replication and an effect at ribosomal level.

INDIRECT MECHANISMS

1.3.8.3 Cellular energy depletion

Evidence is accumulating that oxidant induced endothelial injury is mediated by depletion of intracellular ATP. This concept was suspected following the observation that cyanide potentiates endothelial damage caused by activated PMNL *in vitro* (Sacks et al. 1978). Subsequently it was shown that HOCl and H_2O_2 can inhibit ATP generation and that this results in severe and sometimes permanent cellular malfunction (Anderson et al. 1990). HOCl oxidatively inactivates the enzyme glyceraldehyde-3- phosphate dehydrogenase (GAPDH) thereby inhibiting glycolytic metabolism (Dallegri et al. 1988; Jackson et al. 1988; Baker et al. 1989). H_2O_2 however depletes ATP by two distinct mechanisms. Low concentrations damage DNA, as described above, resulting in activation of the DNA repair enzyme poly ADP ribose polymerase which utilises nicotine adenine dinucleotide (NAD) as a cofactor. The consequent intracellular depletion of this cofactor inhibits the function of NAD-requiring enzymes such as GAPDH as well as the Krebs cycle enzymes isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase (Anderson et al. 1990). High concentrations of H_2O_2 however may directly oxidatively inactivate GAPDH (Hyslop et al. 1988; Anderson et al. 1990).

Oxidant stress applied under physiologic conditions does not cause gross morphological cellular abnormality (Holman and Maier, 1990) in contrast to the effect of higher oxidant doses (Hinshaw et al. 1986). Cells depleted of ATP do however

demonstrate focal gaps between cells, loss of prominence of cell margins and disruption of microfilament organisation and shortening (Spragg et al. 1985; Hinshaw et al. 1988). These abnormalities may result in increased permeability (Holman and Maier, 1990), decreased synthesis of DNA, proteoglycan and hyaluronic acid by chondrocytes (Baker et al. 1989) and contractile dysfunction in ventricular myocytes (Goldhaber et al. 1989).

1.3.8.4 Oxidative inactivation of alpha-1-anti-protease and human leukocyte elastase (HLE) induced injury

The elastase of PMNL is a serine proteinase which functions to attack the carbonyl carbons of peptide bonds (Janoff, 1985). HLE has a serine residue at position 195 of its primary sequence. This amino acid contributes a nucleophilic hydroxyl group which preferentially attacks the carbonyl carbons of valine or alanine residues situated at the p1 position of target substrates. It has a MW of 33,000, is synthesised in promyelocytes and stored in the azurophilic granules. The destructive potential of this enzyme is immense, having the capacity to degrade almost all components of the cellular matrix (collagens [type III and IV], elastins, proteoglycans and glycoproteins) and, in addition, immunoglobulins, complement and clotting factors (Weiss, 1989). Although HLE may be required to mediate the physiological turnover of the connective tissue framework, where hydrolysis of the matrix macromolecules does occur, it is probably a pathological process (Janoff, 1977; Janoff, 1985; Weiss, 1989). Other proteinases have also been

shown to contribute to the total elastolytic burden. Cathepsin G may act synergistically in the solubilisation of elastin *in vitro* (Boudier et al. 1981; Reilly et al. 1984) and Proteinase-3 (PR-3) is also capable of degrading elastin and of causing emphysema in laboratory animals (Kao et al. 1988). These latter authors suggest that qualitative differences exist between these three enzymes. Whereas HLE attacks peptide bonds on the carboxyl side of valine and alanine, cathepsin G primarily attacks bonds on the carboxyl side of tyrosine and phenylalanine (Blow and Barrett, 1977). It is probable that PR-3 attacks sites different from both these enzymes, but is nevertheless a true elastase, with the ability to degrade ligamentum nuchae elastin more efficiently than HLE (Kao et al. 1988). The ability of PR-3 to produce emphysema is probably related to this property. However, like HLE which can degrade many connective tissue architectural components, it is possible that other components may also be vulnerable to the proteolytic activity of PR-3.

Powerful regulatory mechanisms comprising systemic and locally produced inhibitors, exist to restrict the activity of these enzymes to their major physiological role of bacterial digestion and killing. AAP and alpha-2-macroglobulin are the major systemic antagonists, while anti-leukoprotease is found in interstitial fluids and mucous secretions (Janoff, 1985). For elastase to be of importance in the aetiology of tissue injury, it would have to be capable of bypassing this anti-proteolytic screen. AAP is the most important protective agent, as evidenced by the high incidence of chronic obstructive pulmonary disease

present in those patients with a genetically determined deficiency of this inhibitor. The enzyme contains a critical methionine residue (met 358), which under physiological conditions is attacked by the serine hydroxyl contained in the active site of PMNL elastase (Janoff, 1985, Travis and Solnesen, 1983). This results in cleavage of the adjacent bond and the development of strong forces between AAP and elastase which render the latter incapable of acting on other substrates (Janoff, 1985).

In order to escape from inhibition elastase concentrations must exceed those of the inhibitor or be released at sites where AAP has been inactivated. While the former is unlikely as neutrophils are immersed in vivo in fluids containing huge excesses of AAP relative to their elastase content, it has been repeatedly demonstrated that AAP is inactivated by oxidation of met 358 (to a methionine sulphoxide), and specifically by products of the H_2O_2-MPO-halide system, ie. HOCl (Carp and Janoff, 1979; Matheson et al. 1981; Schechter et al. 1975; Wasil et al. 1987; Zaslow et al. 1985). This oxidation of met 358 causes a 2000 fold decrease in the rate of association between AAP and elastase (Janoff, 1985; Travis and Solnesen, 1983).

Recombinant DNA technology has been employed to develop AAP with valine substituted for methionine in position 358. This variant which is functionally active, but resistant to oxidative inactivation (Janoff, 1985; Jallat et al. 1986) underlines the mechanism of inactivation as well as the therapeutic potential of genetically engineered oxidation-resistant variants of AAP.

1.3.8.5 Metalloproteinases and oxidative metabolism

Recent reports have documented other mechanisms whereby oxygen metabolites potentiate proteolysis. The metalloproteinases, collagenase and gelatinase which are released from PMNL secondary granules in a latent form, can be activated by a wide variety of substances in vitro. These include alkylating agents, organomercurials and heavy metals, all of which do so by interaction with thiol groups contained in the enzymes. None of these agents however are likely to be endogenous activators. Chlorinated oxidants have also been shown to interact rapidly with thiols and it has been demonstrated that the endogenous activator is HOCl (Peppin and Weiss, 1986; Shah et al. 1987; Weiss and Peppin, 1986; Weiss et al. 1985). The precise mechanism of activation is unknown, but probably represents a molecular rearrangement whereby the active sites of the enzymes are exposed.

1.2.8.6 Proteolytic inactivation of AAP

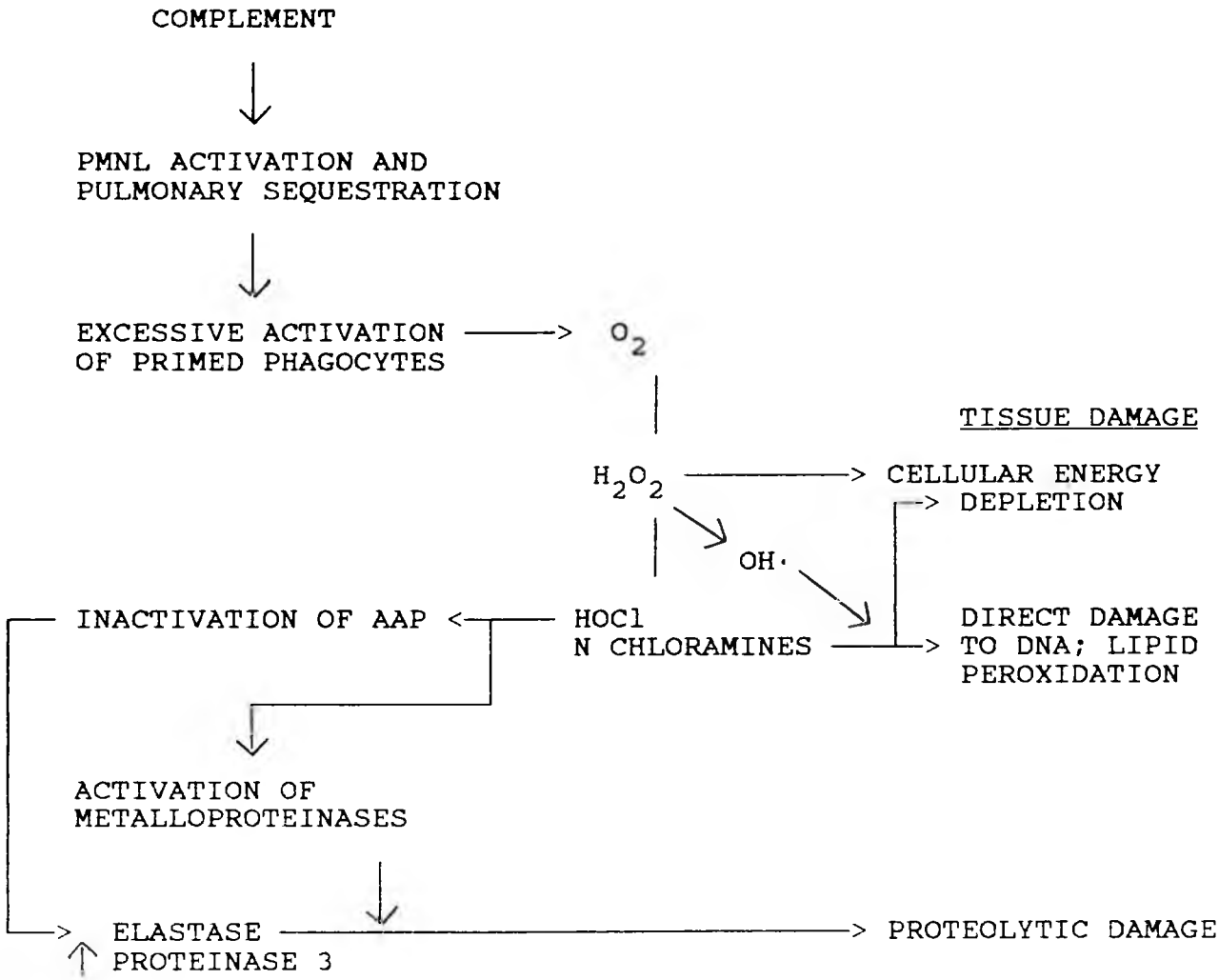
Activated metalloenzymes are capable of further reducing the anti-protease screen by proteolytic cleavage of AAP between Phe 352 and Leu 353 (Des Roches and Weiss, 1988; Wasil et al. 1987; Weiss, 1989).

In summary, tissue damage represents a complex interaction of oxidants, elastase, AAP and metalloenzymes. Oxidants are also capable of inducing direct damage by inducing lipid peroxidation, DNA damage and depletion of intracellular

ATP. Whether or not damage occurs will depend upon the site of release and the relative concentrations of these agents and on the presence of the anti-oxidant screen.

These mechanisms of tissue damage are depicted graphically in Figure 4.

FIGURE 4



1.4 PROTECTION AGAINST OXIDANT STRESS

Considerable evidence is accumulating that naturally occurring anti-oxidants (Table 4) may ameliorate oxidant damage when administered in excess (White and Repine, 1985) or may enhance these effects when depleted (Machlin and Bendich, 1987).

Although plasma and tissue levels of naturally-occurring anti-oxidant nutrients, particularly ß-carotene, vitamins A, C and E and selenium have been related to the subsequent development of cancer (Marklund, 1982) (which is not surprising as excessive free radical production is related to mutagenesis) (Fridovich, 1983), a direct relationship between nutritional status and smoking-induced disease such as emphysema has not been described.

1.4.1 The Endogenous Enzymatic Anti-oxidant Systems

Superoxide dismutase (SOD), catalase and glutathione peroxidase are the primary preventative intracellular anti-oxidant systems and have specific subcellular locations, organ distribution and different metal catalysts at their active sites.

1.4.4.1 The superoxide dismutases

These are metal-containing enzymes that cause superoxide to react preferentially with another superoxide anion to form hydrogen peroxide. The predominant SOD in mammalian cells is a 32,000 Dalton Cu^{2+}/Zn^{2+} SOD while an 86,000 Dalton Mn^{2+} SOD is found predominantly in mitochondria. In

TABLE 4 ENZYMATIC AND NON-ENZYMATIC ANTI-OXIDANTS

A. Intracellular enzymatic anti-oxidants

1. *Superoxide dismutase (O_2^-)*
2. *Catalase (H_2O_2)*
3. *Glutathione peroxidase (H_2O_2 + lipid peroxides)*
4. *Lipid peroxidases (lipid peroxides)*

B. Endogenous non-enzymatic anti-oxidant

Vitamin C:	*Water soluble, chain breaking: (O_2^-, lipid peroxides; MPO-H_2O_2halide products) regenerates tocopherol*
Vitamin E:	*Major membrane bound, lipid soluble anti-oxidant*
ß-Carotene:	*Lipid soluble extracellular scavenger of MPO-H_2O_2-halide system and singlet oxygen*
Zinc:	*Constituent of cytosolic SOD*
Selenium:	*Constituent of glutathione peroxidase*
Copper:	*Constituent of cytosolic SOD and ceruloplasmin*
Iron:	*Constituent of catalase*
Manganese:	*Constituent of mitochondrial SOD*

addition a 134,000 Dalton Cu^{2+}/Zn^{2+} SOD has been isolated from human lung and may be an important scavenger in extracellular locations (Marklund, 1982).

These enzymes catalyse the dismutation of $O_2^{\cdot}$ to H_2O_2 in a reaction 10^4 times faster than the spontaneous rate, and their ability to do so is attributed to the capacity of the active sites to overcome the electrostatic repulsion of negatively charged O_2^{-} ions (Fridovich, 1983). Theoretically SOD is not an anti-oxidant in that it <u>could</u> result in the generation of excess H_2O_2 which under certain circumstances would be available to interact with either PMNL MPO to form HOCl or with Fe^{3+} to form OH°. The overall effect of the enzyme is therefore dependent on:

(a) the availability of iron;

(b) the presence of activated PMNL, or

(c) the adequacy of catalase and GSH peroxidase reserves (see later).

If tissue iron is compartmentalised and unavailable to react, and if MPO is not present, catalases and peroxidases can reduce the H_2O_2 generated to non-toxic products (Scott et al. 1987).

1.4.1.2 Catalase

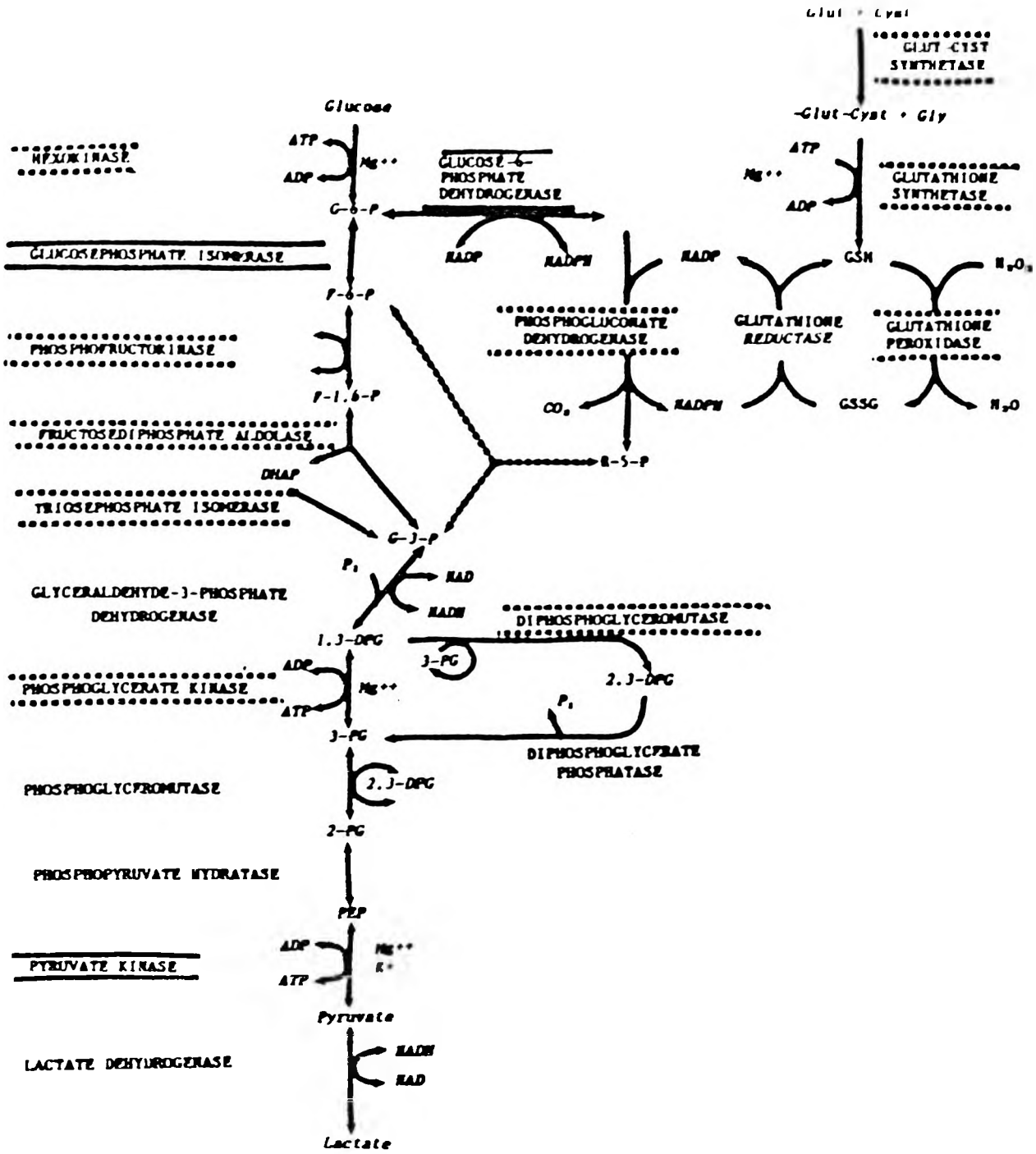
This is an ubiquitous anti-oxidant enzyme found primarily in cellular peroxisomes which is specific for small molecules such as H_2O_2 due to the nature of its binding site. Catalase activity increases linearly with H_2O_2 concentration until inactivated at concentrations above

100mM (Reidt et al. 1987; White and Repine, 1985). This enzyme is a 240,000MW tetrameric hemoprotein which, by alternate divalent oxidation and reduction of its non-heme active site in the presence of H_2O_2, catalyses the reaction $2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$ (Heffner and Repine, 1989).

1.4.1.3 The Glutathione redox cycle (Figure 5)

The glutathione redox cycle, like catalase, reduces H_2O_2, but is more efficient than catalase at low H_2O_2 concentrations. This system also exceeds catalase in its ability to reduce intracellular lipid peroxides including those generated by lipoxygenase or cyclo-oxygenase catalysed reactions (Ross et al. 1985). The key enzyme in the cycle responsible for reduction of hydroperoxides is glutathione peroxidase (GSH-P) which is an 85,000 Dalton tetrameric cytosolic protein with four atoms of selenium (Se) bound as selenocysteine moieties that confer its activity (Heffner and Repine, 1989). Hence the apparent relationship between selenium deficiency and cancer (Willet, 1988).

Glutathione, the substrate for GSH-P is a tripeptide, of glutamate, cysteine and glycine and exists in both the thiol reduced (GSH) and disulphide oxidised (GSSG) forms (Flenley, 1987). A high ratio of GSH/GSSG is maintained to ensure availability of GSH when required (Sies, 1987). A dual mechanism exists to maintain these high GSH/GSSG intracellular ratios. Firstly, only GSSG can be transported out of the cell whereas GSH is retained. Secondly, GSSG can be reduced back to GSH by the enzyme GSH reductase (see Figure 5) (Necotera et al. 1985).



IGURE 5 THE GLUTATHIONE REDOX CYCLE AND ITS RELATION TO THE EMDEN MEYERHOF PATHWAY

Glutathione synthesis is largely determined by the availability of its precursor L-cysteine which is derived from diet or protein breakdown, but levels can also be increased by supplying amino acid precursors, such as N-acetyl cysteine (Caplowitz et al. 1985). The regeneration of reduced GSH however requires the presence of NADPH supplied through the activity of the hexose monophosphate shunt which is markedly increased in conditions of oxidant stress. It is probable that the GSH redox cycle is the most important mammalian anti-oxidant peroxidase, in that systems in which catalase is inactivated can still be protected by GSH redox activity - but not vice versa (Caplowitz et al. 1985; Suttorp et al. 1986). The reasons for this are that GSH-redox enzymes are found throughout the cytosol and not just in peroxisomes, that the GSH-redox cysteine can interact with H_2O_2 at very low concentrations and that it can also react with hydroperoxides other than H_2O_2 (Suttorp et al. 1986), especially lipid peroxides.

Theoretically, the enzyme G-6-PDH is also an important anti-oxidant in that the whole redox cycle would be rendered inactive without sufficient NADPH. This is of course particularly manifest in the sensitivity of G-6-PDH deficient red cells to oxidant stress (Cohen and Hochstein, 1961).

1.4.1.4 Other peroxidases

There are also various non-selenium-dependent GSH peroxidases such as GSH transferases that may partially compensate for anti-oxidant deficiencies in selenium depletion states. These enzymes, however, metabolise only low molecular

weight organic hydroperoxides and not H_2O_2 (Lawrence and Burk, 1976). Another selenium containing protein has been described as a small 23,000 Dalton enzyme which catalyses the reduction of lipid hydroperoxides independently of phospholipase activity and which has been called phospholipid hydroperoxide GSH peroxidase (Heffner and Repine, 1989; Ursini et al. 1985).

Other peroxidases such as lactoperoxidase, myeloperoxidase, prostaglandin synthetase, the cytochrome P-450 system and haemoglobin cannot be classified as anti-oxidant because of their ability to form pro-oxidants. The cytochrome P-450 system can however supply additional anti-oxidant function if induced prior to oxidant injury (Mansour et al. 1988). It is possible that inducers of Cyt P-450 may alter its spin state, thus determining whether it functions as an oxidant or a peroxidase (Heffner and Repine, 1989; Turrens et al. 1984; White and Coon, 1980).

1.4.1.5 Therapy with enzymatic anti-oxidants

The delivery of exogenous anti-oxidants has been receiving considerable attention of late as a means of at least ameliorating oxidant injury. Administration of the enzymes catalase and SOD alone is minimally effective because of their inability to cross cell membranes to act in the intracellular environment (Turrens et al. 1984). Encapsulation within liposomes however promotes increased intracellular concentrations of these enzymes (Heffner and Repine, 1989). In this regard, exposure to both SOD- and catalase-containing

liposomes has been shown to result in increased levels of these enzymes in cultured aortic endothelial cells and Type II pneumocytes respectively (Buckley et al. 1987; Freeman et al. 1983) and thus to confer enhanced in vitro cellular resistance to hyperoxia (Freeman et al. 1983). In addition, animals treated in this way demonstrate delayed toxicity to hyperoxia (Buckley et al. 1987; Turrens et al. 1984). Attachment of SOD and/or catalase to polyethylene glycol also prolongs survival of exogenously administered anti-oxidant enzymes and has been shown to decrease pulmonary toxicity in rats (White et al. 1989).

Intracellular anti-oxidant enzyme systems can also be augmented by stimulants or by increasing substrate. Corticosteroids increase intracellular SOD activity by 72% and GSH-P activity by 94% in foetal lung cells exposed to hyperoxia, but this effect does not occur in adult animals and is presumed to be of little therapeutic benefit (Tanswell et al. 1986). Exogenously administered GSH is incorporated into many tissues and may supply protection by increasing the intracellular GSH pool. However organ specific limitations to uptake may impose constraints on its use in all oxidant mediated diseases. Indirect evidence for a role for replacement therapy is found in the fact that vitamin E is deficient in the epithelial lining fluid of the lower respiratory tract in diseases such as idiopathic pulmonary fibrosis (Cantin et al. 1989). Chemical precursors can increase intracellular GSH formation and availability of sulphur-containing amino acids, particularly cysteine, appears to be the rate limiting step for its

synthesis. Cysteine per se is toxic at high concentrations, but can be administered in other forms, eg. as N-acetyl cysteine which is a well recognised mode of therapy for certain forms of oxidant injury. N-acetyl cysteine is able to mimic GSH reactivity toward electrophiles, to serve as a precursor for GSH synthesis and to scavenge H_2O_2 directly with the loss of a free thiol due to NAC-disulphide formation (Cosgreave and Moldeus, 1987).

1.4.2 The Non-Enzymatic Nutrient Anti-Oxidants

Anti-oxidant enzymes are primarily intracellular and thus extracellular free radicals must be inactivated by circulating anti-oxidants such as vitamins or ceruloplasmin. Whereas the alterations in enzyme systems are dictated by developmental changes, genetic factors or environmental oxidant stresses, the nutrient level is directly influenced by dietary intake - low intake of one or more of these factors could influence the body's anti-oxidant defence mechanisms. Only three essential nutrient vitamins directly scavenge free radicals, these are Vitamin E, (α-tocopherol), Ascorbate, and ß-carotene - the major carotenoid precursor of Vitamin A. There is evidence however that vitamin A may also perform an anti-oxidant role (Dogra et al. 1982).

1.4.2.1 Vitamin E (VE) (Figure 6)

This is the major lipid soluble anti-oxidant present in all cellular membranes and as such effectively protects against lipid peroxidation and mutagenesis (Weitberg et al. 1985). The

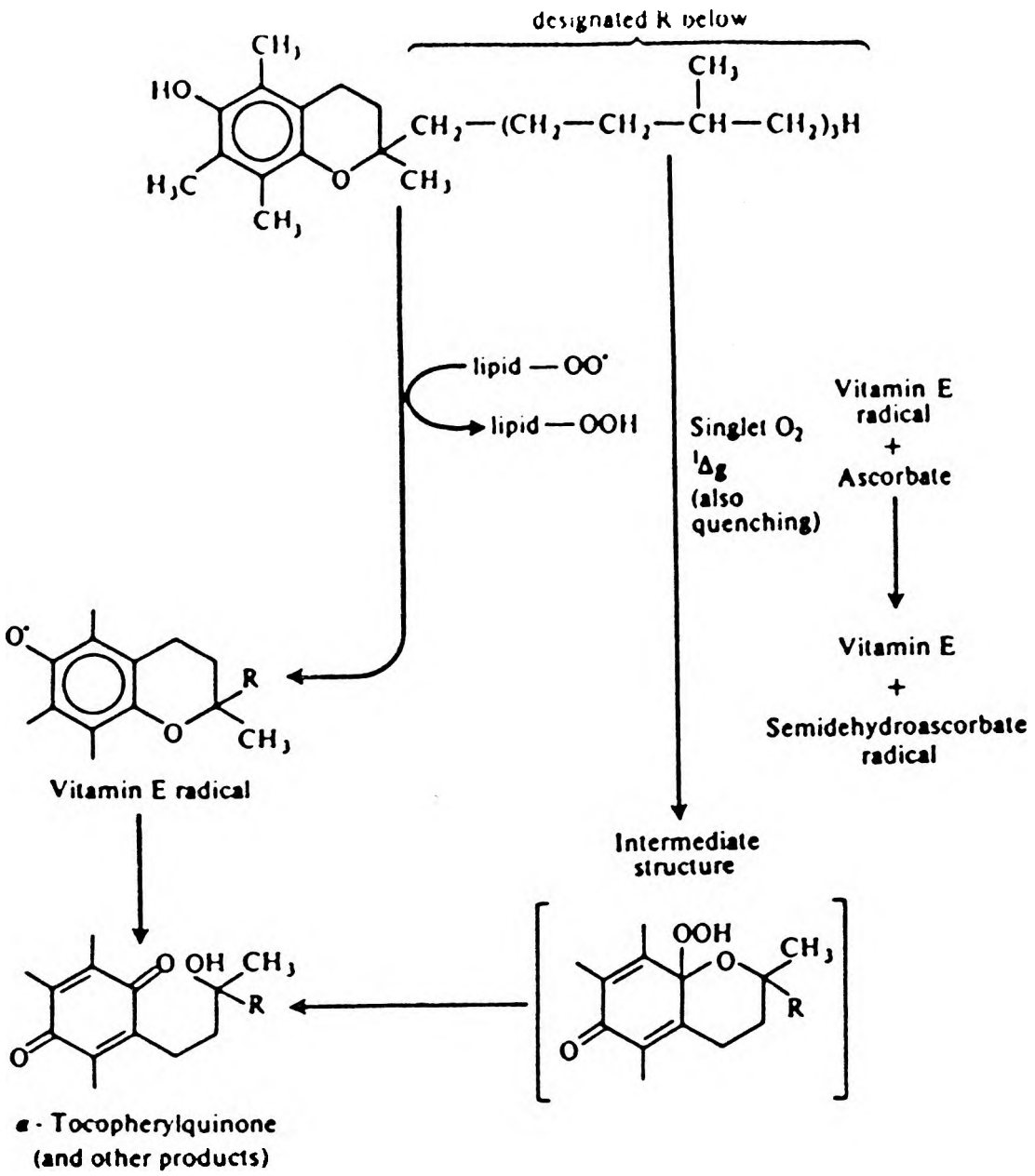


FIGURE 6 Structure of vitamin E and its reaction with vitamin C. The long, hydrophobic side-chain makes the molecule lipid-soluble whereas the ring structure allows reaction with radicals. During peroxidation of cell membranes <u>in vitro</u> some vitamin E becomes converted into the quinone form. (Adapted from Halliwell and Gutteridge, 1985).

mechanism of action is not entirely clear, but by virtue of its hydrophobic lipid solubility, it inserts into lipid membranes where it can function optimally in the protection of the cell (Bieri et al. 1983).

VE has been reported to function by converting lipid peroxy-radicals to less reactive forms in the reaction

$$\alpha TH + RO_2^{\cdot} \longrightarrow RO_2H + \alpha T^{\circ} \text{ and } \alpha\text{-tocopherol } (\alpha T^{\circ})$$

The α-tocopherol (αT°) radical so formed does not participate further in the reaction, either because of resonance stability, whereby the reactive energy of the extra electron is stored between several sites of the same molecule (Heffner and Repine, 1989) or by interaction with Vitamin C which functions as a water soluble electron transport system in extracellular fluids and cytosol (Packer et al. 1979) (see Figure 7).

However, this natural oxidant scavenging effect of VE is weak and most reports now indicate that to be effective it is necessary for it to interact over prolonged periods with oxidant-generating systems <u>in vivo</u>, which implies more than a direct scavenging effect. For example, while it <u>does not</u> prevent cytogenetic damage when added to eukaryotic cells during experimental exposure to oxidants (Weitberg et al. 1985), the administration of a daily dose of 1600 IU for 2 weeks <u>does</u> inhibit the release of H_2O_2 and the activity of the hexose monophosphate shunt of human PMNL (Baehner et al. 1982).

Baehner and colleagues also report that although VE suppresses both H_2O_2 and the products of its interactions with MPO/halide system it does not influence oxygen consumption or O_2^- production (Baehner et al. 1982). If this is correct, the implication is that VE functions as an inhibitor of SOD, for which there is no evidence, or that it acts purely as an intracellular scavenger of H_2O_2 which is unlikely as the co-incubation of α-tocopherol acetate at concentrations of up to 40 μg/ml with cell free and with neutrophil oxidant-generating systems in vitro, does not inhibit either the generation or reactivity of superoxide, or of oxidants generated by the peroxidase/H_2O_2/ halide system. ß-carotene on the other hand which is also lipid soluble, at concentrations of 0,4 μg/ml functioned as an extracellular and intracellular scavenger of oxidants - again specifically those of the H_2O_2/MPO/halide system (Anderson and Theron, 1990).

A third possible mechanism is that VE, by incorporation into the membrane, alters function by stabilisation of membrane structures and by influencing permeability. This could limit release of RO from, and generation of lipid peroxides within the cell in a nonspecific fashion unrelated to any anti-oxidant properties. It is perhaps the uncertainties as to its mode of action that have rendered its therapeutic use controversial. For example, it may be beneficial in rodents depleted of VE and then exposed to oxidant insult (Yoshikawa et al. 1982), but there is little evidence that it is of benefit in those not depleted (Halliwell, 1987). Human diseases likewise have not been shown

consistently to respond to VE therapy unless a pre-existing deficiency exists, eg. in retrolental fibroplasia or inborn errors of fat metabolism (Bieri et al. 1983).

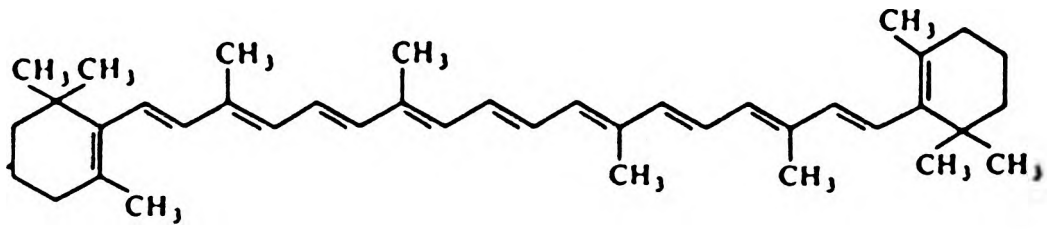
The limitations to effective therapy with this agent are possibly determined by the fact that VE is not immediately incorporated into cells in any substantial amount by which time acute injury might have already occurred and could not be influenced by a belated increase in VE tissue levels.

1.4.2.2 ß-Carotene (BC) (Figure 7)

Despite the fact that BC is the major carotenoid precursor of Vitamin A, Vitamin A itself has no known major anti-oxidant function. Carotenoid pigments are widely distributed in nature, in both photosynthetic organisms and in human cellular membranes including those of lysozomes (Machlin and Bendich, 1987). BC quenches singlet oxygen and can also function as a chain breaking anti-oxidant in the lipid phase by neutralising peroxy-radicals (Burton and Ingold, 1984; Krinsky and Deneke, 1982). Co-incubation of eukaryotic cells with BC prior to or during exposure to activated phagocytes, or to a cell-free oxidant-generating system prevents oxidant-induced chromosomal damage and a biological role for this agent in cytoprotection has been proposed (Weitberg et al. 1985). In addition, as mentioned earlier, previous work has shown that BC at concentrations of 0,4 ug/ml and greater, functions as an extra- and intracellular scavenger of oxidants generated by the MPO/H_2O_2/halide systems (Anderson and Theron, 1990). Despite the fact that BC did not scavenge superoxide, nor did it

FIGURE 7 The structure of ß-carotene

(From Halliwell and Gutteridge, 1985)



influence the <u>production</u> of this oxidant by phagocytes or cell-free oxidant generating systems (Anderson and Theron, 1990), it has the potential to play an important role in protecting lipids from peroxidation <u>in vivo</u>. In addition, the chain breaking action is complementary to that of VE as BC is effective at low oxygen concentrations and VE is effective at high concentrations (Burton and Ingold, 1984), suggesting that BC would be most useful if concentrated in those tissues where oxygen tensions are lowest and VE where tensions are highest.

1.4.2.3 Vitamin C (VC) (Figure 8)

Humans, together with guinea pigs, Indian fruit bats and several varieties of Bulbuls are amongst the few mammals unable to synthesise ascorbic acid from glucuronic acid and therefore acquire this agent from dietary sources (Levine, 1986). Ascorbate is a powerful reducing agent which not only provides reducing equivalents to many enzymes but can also directly scavenge O_2^- + $OH^{\cdot}$ with the formation of the semidehydro-ascorbate free radical (Levine, 1986). This substance is metabolised to oxalate which is cytotoxic unless it is in turn reduced in the cytosol by a GSH dependent dehydro-ascorbate reductase that regenerates ascorbate and GSSG (Halliwell and Gutteridge, 1985; Levine, 1986). Because of its reducing capability, an important role for this substance in the extracellular space is the reduction of the Vitamin E radical (α-T°) to regenerate membrane bound reduced VE (α-TH) which can function once more as a chain breaking anti-oxidant (McCay, 1985; Packer et al. 1979).

Acceptance of the efficacy of VC as a protective anti-oxidant has however been tempered by the realisation that it has two important pro-oxidant functions, firstly that it may interact with O_2^- to convert Fe^{3+} to Fe^{2+}, which in the "Haber-Weiss" reaction interacts with H_2O_2 to form the hydroxyl radical (Table 2) (Halliwell and Gutteridge, 1985) and secondly, that it appears capable of enhancing PMNL chemotaxis, phagocytosis and antimicrobial activity (Anderson et al. 1990). The former is demonstrated graphically in the worsening of

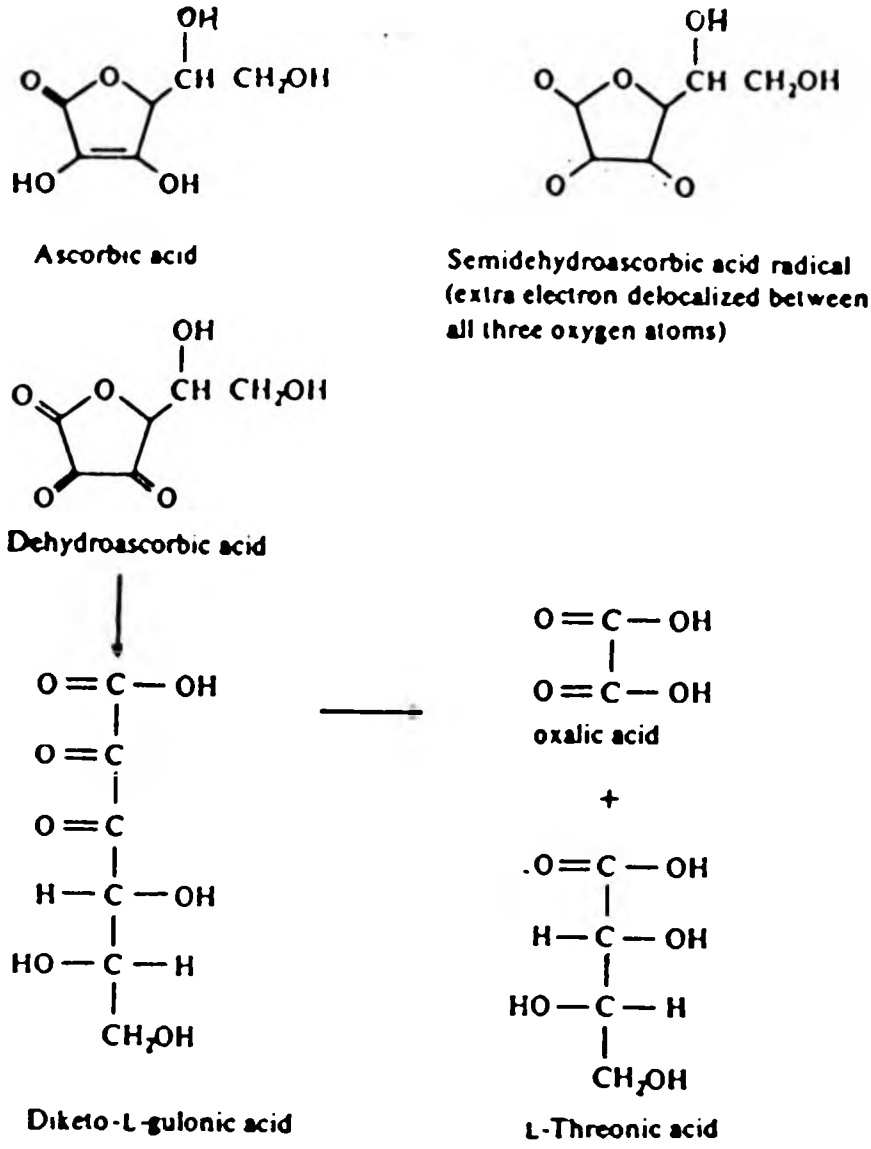


FIGURE 8 The structure of ascorbic acid and its oxidised forms.
(From Halliwell and Gutteridge, 1985).

cardiomyopathy in haemochromatotic patients administered VC and their subsequent improvement after administration of an iron chelator (Rowbotham et al. 1984). In the latter, because PMNL derived extracellular RO are autotoxic to the cell itself (Baehner et al. 1977), the scavenging of such substances would be likely to protect neutrophil function and in fact experiments have shown immunostimulant effects in vivo and in vitro which are probably related to the scavenging of extracellular products of the MPO/halide/H_2O_2 system (Anderson et al. 1990). The latter study demonstrated the sensitivity of human PMNL to HOCl mediated cellular dysfunction and postulated that ascorbate is protective by preventing the inactivation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, thereby sustaining intracellular ATP generation in the face of oxidant stress (Anderson et al. 1990).

1.5 STUDY OBJECTIVES

The studies that follow were designed to investigate the relationship between PMNL derived RO and lung function and to determine if this RO production could be influenced by exogenously administered nutrient anti-oxidants.

CHAPTER 2

THE INVESTIGATION OF THE RELATIONSHIP BETWEEN PRODUCTION OF REACTIVE OXIDANTS BY ACTIVATED PHAGOCYTES AND LUNG FUNCTIONS IN CIGARETTE SMOKERS

2.1 INTRODUCTION

The natural history of chronic obstructive pulmonary disease is such that not all persons who smoke develop this disease and that those who do may not develop it at the same rate (Fletcher and Peto, 1977). Current theories relating to the development of emphysema have emphasised the protease-antiprotease imbalance hypothesis (Idell and Cohen, 1983), ie. that an absolute or functional deficiency of alpha-1-antitrypsin, which inhibits polymorphonuclear leukocyte (PMNL) neutrophil elastase and other proteinases, may potentiate elastase activity leading to chronic elastolysis, which culminates in emphysema (Boudier et al. 1983; Campbell et al. 1983; Stone et al. 1983; Sibille et al. 1986). At present, much attention has focused on macrophages and PMNL, because it has been shown that smokers have a peripheral leukocytosis (Cone et al. 1981; Helman and Rubenstein, 1975), that lung phagocyte recruitment is induced by cigarette smoke (Hoidal and Niewoehner, 1982; Hunninghake and Crystal, 1983), and that PMNL and macrophages have increased oxidative function in cigarette smokers (Hoidal et al. 1981; Hoidal and Niewoehner, 1982; Ludwig and Hoidal, 1982). These abundant, hyperactive macrophages and PMNL in the lungs of smokers may be important in the aetiologic aspects of both emphysema and bronchial carcinoma since phagocyte-derived reactive oxidants (RO) mediate oxidative, functional inactivation of alpha-1-protease inhibitor (Carp and

Janoff, 1978; Gadek et al. 1979; Johnson and Travis, 1979) and are also potentially carcinogenic (Weitberg et al. 1983; Weitzman et al. 1985; Jackson et al. 1989).

In this study the possible relationship between PMNL activation and spirometric parameters in a group of 60 asymptomatic young cigarette smokers (mean age, 28 ± 0.6 years) has been investigated.

2.1.1 The Measurement of RO Production

In order to assess this relationship, it is necessary to employ a reproducible measurement of RO production. In this trial a micro-method using whole blood (De Chatelet and Shirley, 1981; Faden and Maciejewski, 1981) as opposed to pure PMNL since it has the advantage of minimising cellular trauma and retains them in their natural environments.

This method, found to be convenient, rapid and inexpensive, involved chemiluminescence (CL), the quantitative assessment of light emission from stimulated PMNL. Chemiluminescence is a phenomenon which was first described by Allen, whereby activated PMNL emit light in a reaction shown to be linked to the production of reactive oxidant species. This reaction can be enhanced by the addition of luminol (5-amino-2,3 -dihydro-1,4 pthalazinedione) (Allen and Loose, 1976) or lucigenin (bis-N-methylacridinium nitrate) (Allen, 1987) which act as bystander substrates for oxidation by RO metabolites. Whereas the unenhanced reaction requires a large number of cells and is dependent upon the interaction of some reactive species with a

phagocytosed particle, the use of secondary emitters permits the use of far fewer cell numbers, obviates the need for working under dark adapted conditions and can, as mentioned previously, be used to measure CL in samples of whole blood (De Chatelet and Shirley, 1981; Faden and Maciejewski, 1981). When a cell is stimulated with the chemotactic tripeptide FMLP in the presence of luminol, the cells respond and produce CL, ie. luminol enhanced CL (LECL) in a characteristic bimodal pattern (Bender and van Epps, 1983; Briheim et al. 1984), the first peak of which derives from extracellular and the second from intracellular products of the MPO-H_2O_2- halide system (Bender and van Epps, 1983; Briheim et al. 1984). In contrast, lucigenin, which is a larger molecule and therefore is unable to enter the intracellular environment, manifests only a single peak which results from the interaction of extracellular superoxide with the lucigenin (Dahlgren et al. 1985; Minkenberg and Ferber, 1984).

The use of the whole blood method, however, does have the disadvantage of the precise identification of the origin of the LECL and, in addition, negative or positive contributions of the various cellular and hormonal components of blood would be difficult to assess.

In this study the contributory or modifying effects of these components were assessed by fractionation and reconstitution experiments.

Cellular Stimulation: The extracellular release of destructive granule constituents and oxygen metabolites can be triggered by a variety of non-ingestible large particles (antigens, antibody complexes, etc), as well as by soluble stimuli, which include physiological reactants such as bacterial products (N-formylated peptides) or products of complement activation (C5a), and non physiological substances such as phorbol esters and calcium ionophores (Boxer and Curnutte, 1985; Klebanoff, 1982; Test and Weiss, 1986). In the case of receptor-mediated cellular activation, the ligand-receptor complex so formed appears to enhance the activity of a guanine nucleotide regulatory protein, which results in increased membrane phosphoinositide turnover, increased intracellular calcium, activation of protein kinase C and subsequently NADPH oxidase activity (see Figure 3 and Figure 4) (Becker, 1985; Koo et al. 1983). The phorbol ester, phorbol 12-myristate 13-acetate (PMA) is one of the most potent stimuli of phagocyte associated NADPH oxidase, and its activity is attributed to its ability to directly activate protein kinase C activity (Babior, 1984; Becker, 1985; De Chatelet et al. 1976; Koo et al. 1983; Nishizuka, 1984). Both of the above are used routinely as cellular stimulants *in vitro* and have also been employed in the following experiments. Chemoattractants appear to activate the same respiratory burst enzyme in human PMNL, but their transductional mechanism is different, accounting for the more immediate response induced by FMLP and the time lapse following PMA stimulation (McPhail and Snyderman, 1983).

Activation of NADPH oxidase by chemoattractants is both time and dose dependent and unlike other soluble stimuli such as PMA, FMLP requires the presence of cytochalasin B (CB) during the stimulation phase to potentiate the response. CB inhibits micro-filament function and thus inhibits a variety of cellular functions including chemotaxis and phagocytosis, which suggests that the state of actin filament assembly may regulate NADPH oxidase activation induced by chemoattractants (Estensen and Plageman, 1972; Hawkins, 1973; McPhail and Snyderman, 1983). The fact that cytochalasins enhance not only secretion, but most of the stimulated responses of the PMNL including oxygen metabolism (Goldstein et al. 1974), leukotriene production and adhesion (Cradock et al. 1978) emphasises the likelihood that cytochalasins may enhance the stimulus-response coupling process in general. Reports that their presence is associated with increased production of diacylglycerol may relate to such a process (Honeycutt and Neidel, 1986).

2.1.2 The Measurement of Lung Function

Lung functions were measured by spirometry. Many complex measures of lung function have been postulated to be useful in the measurement of early, smoking-related abnormalities, eg. closing volumes (McCarthy et al. 1972), maximal expiratory flow volume curves (Lord et al. 1969), frequency dependence of compliance (Woolcock et al. 1969) and the response of the maximal expiratory flow to helium (Dosman et al. 1975).

However, these tests require taxing subject cooperation and expensive apparatus, and do not necessarily contribute more information. Although it is apparent that one reading from the terminal position of the forced expiratory curve may not be relevant for an individual, correlates of these parameters with RO are of value when applied to the groups as a whole. Today rigid standardisation is employed and, when performed according to these criteria, can be particularly useful in determining early changes in pulmonary function (Lim, 1973; Marrero et al. 1986; Morris et al. 1975; Walter et al. 1979).

The more detailed methods and the results follow.

2.2 METHODS

2.2.1 Smoking Subjects

Sixty asymptomatic, young, cigarette smokers (mean age 28 ± 0.6 years) were recruited to the study after informed consent and after clearance by the Committee for Research into Human Subjects of the University of the Witwatersrand. Thirty five were male and twenty-five were female. All were current smokers with smoking histories ranging from 1 to 49 pack-years. All subjects, except two who were Asian, were Caucasian and resident in Johannesburg, South Africa, in similar environmental circumstances. None were involved in occupations potentially damaging to the lungs. Subjects were excluded if they had current evidence of coryza or a history of asthma or any other serious respiratory, cardiac, or systemic disease. Subjects known to be current marijuana users were not included.

2.2.2 Nonsmoking Control Subjects

Thirty young, nonsmoking Caucasian control subjects (mean age 27 ± 1.2 years), comprising 12 males and 19 females, were recruited using similar exclusion criteria in order to determine normal LECL values in nonsmokers.

2.2.3 Reactive Oxidant Estimation

A whole blood method was used in which 50 µl of heparinised blood (5 units of preservative-free heparin per millilitre blood) were added to 850 µl of Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, NY, USA) supplemented with 0.1% bovine serum albumin and 0.1mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St Louis, MO, USA) and incubated at 37°C for 45 minutes. Blood PMNL were then activated by the addition of 100 µl of FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine; Sigma Chemical Co.) and CB (cytochalasin B); Sigma Chemical Co.). The final concentrations were 1 µM and 1 µg/ml, respectively.

Spontaneous and FMLP/CB-activated LECL were measured at 10 second intervals for 1 minute and thereafter at 1-minute intervals for 25 minutes using a Lumac® Biocounter (Lumac Systems Inc., Titusville, FL, USA). These results are expressed in relative light units (rlu). These results were also corrected for leukocyte count and expressed as rlu/10^6 leukocytes. It was found that this whole blood method gives the typical bimodal FMLP/CB-activated LECL response observed with purified PMNL (Bender and van Epps, 1983, Briheim et al. 1984; Dahlgren et al.

1985), this being an early extracellular response and a late intracellular response (see Section 2.1.1). No LECL responses are elicited from blood from children with chronic granulomatous disease, suggesting that the detected LECL responses were exclusively phagocyte-derived and not caused by interactions of other cellular and plasma components within the assay system. However fractionation and reconstitution experiments were performed to investigate the following: (1) the origin of LECL in FMLP/CB-activated blood, and (2) the existence of possible cellular and/or humoral factors, which by their interactions with PMNL may modulate LECL responses. Five millilitres of heparinised whole blood were layered onto Percoll® gradients, and the various components (plasma, platelets, mononuclear leukocytes, PMNL, and erythrocytes) were separated by centrifugation (Hjorth et al. 1981). Measurements of FMLP/CB-activated LECL were then performed as above on reconstituted blood specimens selectively depleted of a single component in a serial fashion. Mixing experiments were also performed in which purified, numerically standardised PMNL from cigarette smokers were added to nonsmokers' blood from which PMNL had been removed, and FMLP/CB-activated LECL responses were then measured. The reverse experiments were also performed, ie. the mixing of nonsmokers' PMNL with PMNL-depleted blood from smokers. Additionally, correlates of intensity of LECL were performed on whole blood and numerically standardised purified PMNL from 18 cigarette smokers.

2.2.4 Other Investigations

Circulating total leukocyte counts and thiocyanate levels were also measured in the cigarette smokers. Thiocyanate estimation was performed using colourimetry. Serum proteins were precipitated using trichloroacetic acid and the supernatants treated with a reagent containing ferric nitrate. The resultant colour was compared with potassium thiocyanate standards and control serum samples consisting of pooled serum to which thiocyanate had been added. The developed colours were read at 560nm and then compared with the standards and controls (Bowler, 1944).

2.2.5 Lung Functions

Smokers were required to abstain from smoking for at least 2 hours before testing to eliminate any acute effects of tobacco smoking. A Jaeger Transfer Screen II with disc drive computer was used. The same machine was used throughout, and tests were performed at the same time every day over a 2 week period. The machine was calibrated prior to each operation according to ambient temperature, atmospheric pressure, and humidity and these were corrected to BTPS. Standing heights were recorded with the subjects barefoot, and the tests were performed standing and with noses clipped. Three flow-volume loops were performed on each patient, and the one with the largest forced vital capacity was used, provided that there was less than 5% variation between the two best curves. Parameters and predicted values were calculated according to ECCS (new

version) standards (Spirometric Tables, 1983). The following parameters were obtained: FEV_1, FEV_1/FVC, FEF_{50}/FVC, FEF_{25}, FEF_{25-75}, and FEF_{75-85}. The LECL and the lung functions were performed in two different laboratories, and the results were analysed independently by the Biostatistics Institute of the South African Medical Research Council.

Using the aforementioned parameters (excluding FEV_1), the smokers were arbitrarily divided into three categories of graded spirometric response: A. All test results >85% of predicted. B. Three of five parameters <85% of predicted. C. At least three of five parameters <80% of predicted.

2.2.6 Expression and Statistical Analysis of Results

The results of each series of investigations are expressed as the mean values ± SEM. Correlations between age, pack-years, circulating leukocyte counts (both PMNL and total), extracellular and intracellular LECL responses, and spirometric values were calculated using Spearman's correlation coefficient. Differences in these same parameters between Groups A, B, and C were analysed using the Kruskall-Wallis one-way analysis of variance (Lehmann, 1975). Identification of the best predictor of lung function requires the utilisation of a single representative measurement of lung function or a statistical composite of a number of measurements. Recent literature indicates that FEV_{25} and FEF_{50} may best discriminate between health and early obstructive disease (Marrero et al. 1986), although the discriminatory power for most parameters derived

from the terminal portion of the flow loop varies minimally (Marrero et al. 1986). In this study, each spirometric parameter was analysed individually and in addition, principal component analyses (Tabachnick and Fidell, 1983) identified a best variable retaining 80.2% of the available information. This variable was termed "Lung Function" and unifies the five variables. Stepwise multiple regression analyses were then performed to identify the best predictors for FEF_{25}, FEF_{25-75}, FEF_{75-85}, FEF_{50}/FVC, and FEV_1/FVC and "Lung Function".

2.3 RESULTS

2.3.1 Origins of LECL Responses in FMLP/CB-Activated Blood as Determined by Fractionation and Reconstitution Experiments

These results, using blood from a nonsmoker are shown in Table 5. Selective depletion of PMNL from blood caused almost total loss of FMLP/CB-activated LECL. Doubling and tripling the PMNL concentration increased the FMLP-activated LECL responses by approximately 100% and 200%, respectively. These results clearly show that PMNL are the predominant source of LECL in blood. Identical results were obtained using fractionated and reconstituted blood from a cigarette smoker (not shown).

TABLE 5

Fractionation and subtraction experiments demonstrating the origin of LECL

	Extracellular LECL Responses			
	Patient 1	Patient 2	Patient 3	Mean
MNL + PMNL Depleted Blood	27	25	17	23.0 ± 3.1
PMNL Depleted Blood	31	32	23	28.7 ± 2.8
PMNL *(1x) MNL + Blood	1,014	872	1,017	967.7 ± 48
PMNL *(2x) MNL + Blood	1,901	1,350	2,258	1,843 ± 265
PMNL (3x) MNL + Blood	2,799	2,175	3,263	2,746 ± 316

LECL = luminol-enhanced chemiluminescence;
MNL = mononuclear leukocytes;
PMNL = polymorphonuclear leukocytes

* The results are expressed as the mean values ± SEM in relative light units of triplicate determinations of a single representative experiment. Blood was fractionated on Percoll gradients as described in the text and reconstituted to the original volume and relative proportions of each component with selective depletion of a single component or combination of components as indicated. The FMLP/CB-stimulated extra-cellular and intracellular LECL responses of unfractionated whole blood were 1,078 ± 1,163 ± 142, respectively.

2.3.2 Smoking Histories, Laboratory Investigations, and Spirometric Parameters for the Group of Cigarette Smokers

The mean age (± SEM), cigarettes smoked per day, pack-years, serum thiocyanate levels, leukocyte and PMNL counts, and FMLP/CB-activated peak extracellular and intracellular LECL responses for the entire group of cigarette smokers were 28 ± 0.6 years, 22.7 ± 1.4 cigarettes, 12.5 ± 1.3 pack-years, 89.4 ± 0.6 μmol/L, 8.5 ± 0.32 and 5.7 ± 0.3 x 10^3/ul, and 1,390 ± 87 and 1,730 ± 106 rlu, respectively. The corresponding spirometric parameters FEV_1, FEV_1/FVC, FEF_{50}/FVC, FEF_{25-75}, and FEF_{75-85} were 104 ± 1.6, 91 ± 1.1, 92 ± 3.5, 99 ± 4.0, 103 ± 13.7, and 109 ± 4.7 of the predicted values, respectively. The mean age and FMLP/CB-activated peak extracellular and intracellular LECL responses for the group of nonsmokers were 27 ± 1.2 years and 975 ± 83 and 1,413 ± 121 rlu, respectively.

2.3.3 Analysis of Correlations Between Age, Smoking History, Laboratory Investigations, and Spirometric Parameters in Cigarette Smokers

These results are shown in Tables 6 and 7 for all 60 cigarette smokers as a single group. As shown in Table 6, the best correlates of extracellular LECL, in descending order of statistical significance, were with leukocyte counts = thiocyanate levels = PMNL counts = cigarettes smoked per day >pack-years. Correlates of the spirometric with nonspirometric

TABLE 6

CORRELATIONS BETWEEN AGE, SMOKING HISTORIES, CIRCULATING LEUKOCYTE AND PMNL COUNTS, THIOCYANATE LEVELS, AND LECL RESPONSES IN THE SIXTY ASYMPTOMATIC CIGARETTE SMOKERS *

	Age	Leukocyte Count	PMNL Count	Extracellular LECL	Intracellular LECL	Cigarettes per day	Pack-years	Thiocyanate
Age	1.00	0.22	0.11	0.09	0.29	0.06	0.50	0.11
	0.00	0.10	0.40	0.48	0.02	0.65	0.0001	0.43
Leukocyte Count	0.22	1.00	0.87	0.66	0.63	0.48	0.40	0.54
	0.10	0.00	0.0001	0.0001	0.0001	0.0001	0.0016	0.0001
PMNL Count	0.11	0.87	1.00	0.57	0.63	0.45	0.31	0.42
	0.40	0.0001	0.00	0.0001	0.0001	0.0003	0.02	0.0012
Extracellular LECL	0.09	0.66	0.57	1.00	0.62	0.47	0.30	0.58
	0.48	0.0001	0.0001	0.00	0.0001	0.0001	0.02	0.0001
Intracellular LECL	0.30	0.63	0.63	0.62	1.00	0.37	0.31	0.40
	0.02	0.0001	0.0001	0.0001	0.00	0.0038	0.01	0.0022
Cigarettes per day	0.06	0.48	0.45	0.47	0.37	1.00	0.77	0.56
	0.65	0.0001	0.0003	0.0001	0.0038	0.00	0.0001	0.0001
Pack-years	0.50	0.40	0.31	0.30	0.31	0.77	1.00	0.37
	0.0001	0.0016	0.02	0.02	0.01	0.0001	0.00	0.0046
Thiocyanate	0.11	0.54	0.42	0.58	0.40	0.56	0.37	1.00
	0.43	0.0001	0.0012	0.0001	0.0022	0.0001	0.0046	0.00

LECL = luminol-enhanced chemiluminescence;

MNL = mononuclear leukocytes;

PMNL = polymorphonuclear leukocytes

* The results are expressed as the correlation coefficients (uppermost) with the paired corresponding p values below

TABLE 7

CORRELATION OF SPIROMETRIC PARAMETERS WITH AGE, SMOKING HISTORY, CIRCULATING LEUKOCYTE AND PMNL COUNTS, THIOCYANATE LEVELS, AND LECL RESPONSES IN THE SIXTY ASYMPTOMATIC CIGARETTE SMOKERS *

	FEF_{50}/FVC	FEV_1/FVC	FEF_{25}	FEF_{25-75}	FEF_{75-85}	FEV_1
Age	-0.24 0.06	-0.11 0.42	-0.24 0.06	-0.17 0.19	-0.20 0.14	-0.08 0.55
Leukocyte Count	-0.40 0.002	-0.27 0.03	-0.35 0.005	-0.36 0.006	-0.34 0.009	-0.11 0.40
PMNL count	-0.32 0.01	-0.13 0.33	-0.24 0.07	-0.19 0.15	-0.19 0.15	-0.11 0.94
Extracellular LECL	-0.48 0.0001	-0.46 0.0002	-0.48 0.0001	-0.50 0.0001	-0.48 0.0001	-0.19 0.15
Intracellular LECL	-0.29 0.03	-0.21 0.10	-0.33 0.009	-0.35 0.008	-0.37 0.004	-0.19 0.15
Cigarettes per day	-0.39 0.002	-0.34 0.008	-0.44 0.0004	-0.41 0.002	-0.47 0.002	-0.08 0.54
Pack-years	-0.39 0.002	-0.35 0.006	-0.51 0.0001	-0.41 0.002	-0.53 0.0001	-0.14 0.28
Thiocyanate	-0.41 0.002	-0.28 0.04	-0.39 0.003	-0.33 0.02	-0.35 0.01	-0.16 0.25

LECL = luminol-enhanced chemiluminescence;
MNL = mononuclear leukocytes
PMNL = polymorphonuclear leukocytes

* The results are expressed as the correlation coefficients (uppermost) with the paired corresponding p values below.

parameters are shown in Table 7 and clearly demonstrate a lack of association with FEV_1 in these asymptomatic young smokers. However, the following significant correlates of the nonspirometric parameters (in descending order of importance) with the terminal portion of the flow-volume loop were observed.

1. FEF_{50}/FVC: extracellular LECL >thiocyanate levels = leukocyte counts = pack-years = cigarettes per day >PMNL counts >intracellular LECL.
2. FEV_1/FVC: extracellular LECL >pack-years = cigarettes per day >thiocyanate levels = leukocyte counts.
3. FEV_{25}: pack-years = extracellular LECL >cigarettes per day >thiocyanate levels >leukocyte counts >intracellular LECL.
4. FEV_{25-75}: extracellular LECL >pack-years = cigarettes per day >leukocyte counts >intracellular LECL >thiocyanate levels.
5. FEF_{75-85}: pack-years = extracellular LECL >cigarettes per day >intracellular LECL >leukocyte counts >thiocyanate levels.

2.3.4 Multiple Regression Analysis

Using stepwise multiple regression analyses with extracellular LECL as the dependent variable and intracellular LECL, pack-years, cigarettes per day, serum thiocyanate levels, leukocyte and PMNL counts and age as the independent variables, leukocyte count was identified as having the best predictive potential of 63.8% with further additive contributions from

intracellular LECL (3.4%) and serum thiocyanate (1.9%). These variables accounted for 69.1% of the variation in extracellular LECL.

With FEF_{50}/FVC, FEV_1/FVC, FEF_{25}, FEF_{25-75}, and FEF_{75-85} individually as the dependent variable, and extracellular and intracellular LECL, pack-years, cigarettes per day, leukocyte counts, PMNL counts, age, and thiocyanate levels as independent variables, extracellular LECL responses (for the parameters FEV_1/FVC, FEF_{50}/FVC, and FEV_{25-75}) and pack-years (for the parameters FEF_{25} and FEF_{75-85}) were identified as having the most important predictive potential. In some cases, the predictive potential was increased by the inclusion of other variables, as shown in Table 8.

Using "Lung Function" as dependent variable and the same independent variables, the following in order of decreasing importance contributed significantly: extracellular LECL (26%), with further respective contributions of 5.1% and 4.5% from pack-years and PMNL count. These variables in combination explained 35.6% of the variation in lung function. The relative weights of lung function measurements in this composite variable "Lung Function" were 0.44, 0.40, 0.46, 0.46 and 0.46 for FEV_{50}/FVC, FEV_1/FVC, FEF_{25}, FEF_{25-75}, and FEF_{75-85}, respectively.

TABLE 8

*STEPWISE MULTIPLE REGRESSION ANALYSIS WITH SYSTEMATIC EVALUATION OF INDIVIDUAL SPIROMETRIC PARAMETERS AS DEPENDENT VARIABLES AND OF COMPOSITE ANALYSIS WITH "LUNG FUNCTION" TO ESTABLISH THE BEST PREDICTORS OF RESPIRATORY DYSFUNCTION **

Spirometric Parameter	Best Predictor	Additional Contributory Predictors
FEV_1/FVC	Extracellular LECL (18.8%)	PMNL count (5.8%) Pack-years (3.4%)
FEF_{50}/FVC	Extracellular LECL (20.9%)	-
FEF_{25}	Pack-years (21.5%)	Extracellular LECL (6.1%)
FEF_{25-75}	Extracellular LECL (22.1%)	-
FEV_{75-85}	Pack-years (22.6%)	Extracellular LECL (7.6%) PMNL count (6.2%)
Composite analysis ("Lung Function")	Extracellular LECL (26%)	Pack-years (5.1%) PMNL count (4.5%)

LECL = luminol-enhanced chemiluminescence;
MNL = mononuclear leukocytes;
PMNL = polymorphonuclear leukocytes

* The best predictors of variability (percent) of each spirometric parameter are shown in the second column, and further additive contributions derived from inclusion of other significant independent variables are shown in the third column. Results of the composite analysis "Lung Function", which unifies all five spirometric parameters, are also shown.

2.3.5 Comparison of Age, Smoking History, Laboratory Investigations, and Spirometry in Cigarette Smokers with Normal and Abnormal Lung Functions

The results for the three groups of smokers (Groups A, B, and C) are shown in Table 9. Relative to the smokers with no spirometric changes (Group A), increases in cigarettes smoked per day, pack-years, thiocyanate levels, circulating leukocyte counts, and peak intracellular and extracellular LECL responses were noted in Groups B and C, whereas decreases were noted in the individual spirometric parameters. The corresponding mean (± SEM) peak extracellular and intracellular LECL responses of nonsmokers were 975 ± 83 rlu and 1,413 ± 121 rlu, respectively. These did not differ significantly from the corresponding values for Group A smokers.

2.3.6 Comparison of Kinetics of LECL Responses in Smokers and Nonsmokers

The results are shown in Figure 9 for nonsmoking control subjects and cigarette smokers in Groups A (n=35), B (n=12), and C (n=13). The bimodal FMLP/CB-activated LECL responses are clearly evident. Relative to nonsmokers, those in Group A had slightly increased, but statistically insignificant, initial (within 1 minute) extracellular and late (8 to 10 minute) intracellular LECL responses. Those in Group B had greater extracellular (mean increase, 49%) and intracellular (mean increase, 19%) FMLP/CB-activated LECL responses relative to Group A. The most striking differences were observed in the

TABLE 9

COMPARISON OF AGE, LEUKOCYTE AND PMNL COUNTS, SMOKING HISTORIES, THIOCYANATE LEVELS, LECL RESPONSES, AND SPIROMETRY IN GROUPS A, B, and C *

	Group			p Values §	
	A (n = 35)	B (n = 12)	C (n = 13)	A versus B	A versus C
Age, yr	27.0 ± 0.6	28.1 ± 1.1	31.0 ± 1.8	< 0.12	<0.12
White blood cell count x 10^3/,ul	7.6 ± 0.3	8.9 ± 0.7	10.2 ± 0.9	< 0.13	<0.0007
PMNL count, x 10^3/,ul	5.10 ± 0.24	6.69 ± 0.65	6.59 ± 0.82	< 0.05	<0.12
Cigarettes per day	19.5 ± 1.7	27.9 ± 3.8	26.6 ± 1.1	< 0.02	<0.001
Pack-years	8.5 ± 1.0	18.0 ± 3.9	17.8 ± 2.5	< 0.02	<0.001
Blood thiocyanate, ,umol/L	87.9 ± 0.6	90.4 ± 1.3	92.7 ± 1.2	< 0.09	<0.002
Chemiluminescence, rlu					
Extracellular response	1,075 ± 69	1,601 ± 160	2,044 ± 225	< 0.0005	<0.0001
Intracellular response	1,417 ± 120	2,109 ± 218	2,223 ± 217	< 0.01	<0.003
Lung functions, % pred					
FEV_{50}/FVC	107.8 ± 3.9	76.7 ± 3.5	64.2 ± 3.0	< 0.0001	<0.0001
FEV_1/FVC	95.8 ± 1.1	88.4 ± 1.4	82.5 ± 2.1	< 0.001	<0.0001
FEF_{25}	118.9 ± 4.1	79.1 ± 2.1	65.0 ± 2.4	< 0.0001	<0.0001
FEF_{25-75}	121.9 ± 4.1	86.5 ± 2.4	71.3 ± 2.4	< 0.0001	<0.0001
FEF_{75-85}	131.4 ± 4.9	89.6 ± 3.4	70.4 ± 2.6	< 0.0001	<0.0001
FEV_1	105.5 ± 2.0	101.6 ± 1.9	94.9 ± 3.0	< 0.10	<0.002

LECL = luminol-enhanced chemiluminescence;

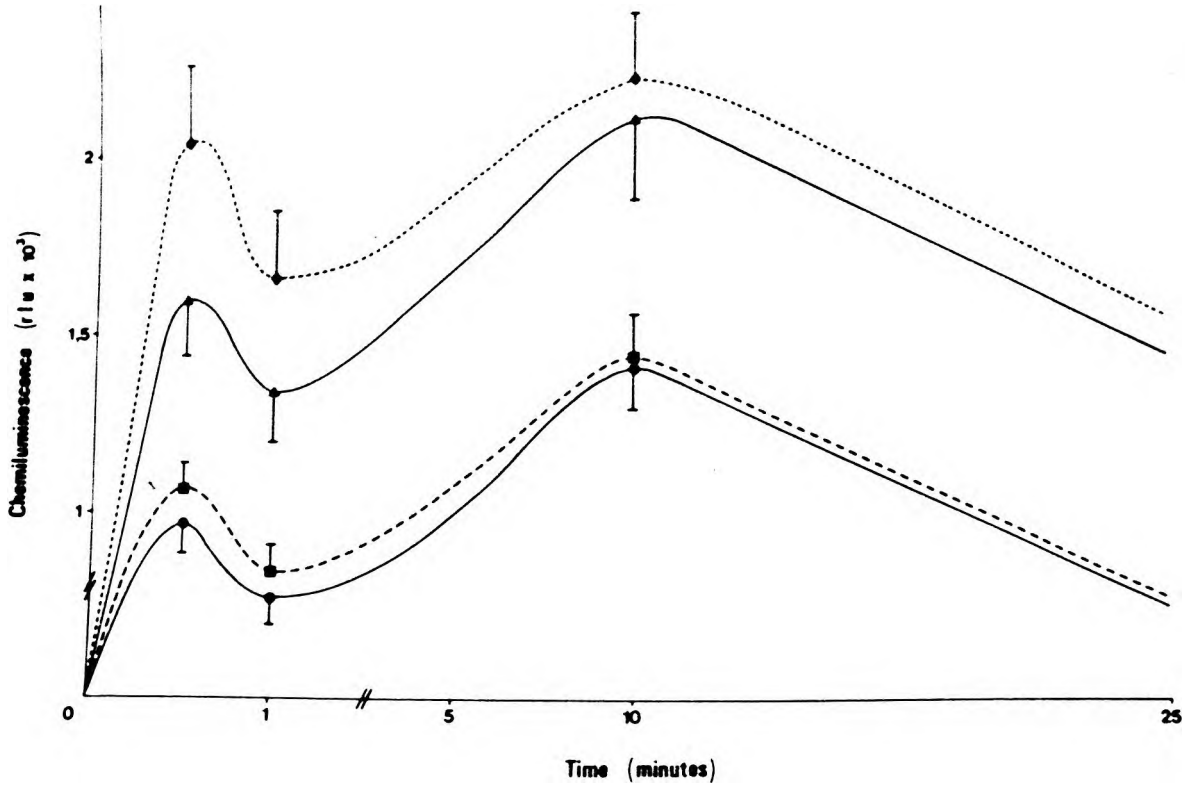
MNL = mononuclear leukocytes;

PMNL = polymorphonuclear leukocytes

* Results expressed as the mean value ± SEM for each investigation.

§ Kruskall-Wallis test; statistically significant differences between Groups B and C were observed for the spirometric parameters FEV_{50}/FVC ($p<0.01$). FEV_1/FVC ($p<0.05$), FEF_{25} ($p<0.001$), FEF_{25-75} ($p<0.0001$), and FEF_{75-85} ($p<0.001$).

FIGURE 9 Kinetics of FMLP/CB-activated luminol-enhanced chemiluminescence (LECL) of whole blood phagocytes from nonsmokers (closed circles) and cigarette smokers in Groups A (closed squares), B (closed triangles) and C (closed diamonds).



The results are expressed as the mean values for each group with standard error bars shown for the peak extracellular (within 1 min) and later occurring intracellular LECL responses. Results or statistical analyses for Groups A, B and C are shown in Table 9. No statistically significant differences were observed between nonsmokers and Group A smokers.

extracellular LECL responses of those in Group C. Relative to Group A the mean percentage increase in the extracellular LECL was 90% and, by comparison with Group B, was 28%. The intracellular LECL responses of FMLP/CB-activated blood phagocytes of those in Group C differed from those in Group A (mean increase, 57%) but were similar to those in Group B. The mean total leukocyte counts were 6.3 ± 9.3, 7.6 ± 0.3, 8.9 ± 0.7, and 10.2 ± 0.9 x 10^3/μl blood for the nonsmokers and Groups A, B and C, respectively. When the extracellular LECL responses were corrected for variations in leukocyte count, the observed trend, although reduced in magnitude, was still evident, with mean increases of 27 and 42% in extracellular LECL responses for Groups B and C, respectively, when compared with those in Group A. Importantly, the unstimulated (in the absence of FMLP/CB) LECL values did not differ between the various groups with values of 120 ± 12, 97 ± 7, 126 ± 13, and 130 ± 13 rlu for nonsmokers and Groups A, B, and C, respectively. These results demonstrate that circulating phagocytes from cigarette smokers with early small airways disease are primed to hyperreact with FMLP/CB stimulation of membrane-associated oxidative metabolism.

2.3.7 Influence of Cellular and Humoral Interactions on the Elevated FMLP/CB-Activated LECL Responses of Cigarette Smokers

These results are shown in Table 10. Relative to the responses observed with nonsmokers' unfractionated blood, the FMLP/CB-activated LECL responses of smokers' PMNL added to reconstituted nonsmokers' blood selectively depleted of autologous PMNL remained unchanged. Similarly, PMNL from nonsmokers were unaffected by mixing with PMNL-depleted blood from smokers. When PMNL were numerically adjusted and added back to autologous and nonsmokers' PMNL-depleted blood, at concentrations equivalent to those of matched nonsmoking control subjects, the FMLP/CB-activated LECL responses observed with smokers' blood were still evident despite correction for PMNL count. Additionally, the extracellular FMLP/CB-activated LECL responses of whole blood and purified PMNL from 18 different cigarette smokers in Groups A, B, and C were strongly correlated ($p<0.009$).

2.3.8 Analysis of all the Correlations After Correction of LECL for Leukocyte Count

The correlates between the extracellular LECL (pre- and post-correction for white cell count) age, WCC, PMNL count, pack-years and thiocyanate are seen in Table 11. It is noteworthy that the only significant correlate that is retained post-correction is with thiocyanate ($p<0.005$). There was no correlation however with pack-years ($p<0.25$) nor with age ($p<0.08$).

TABLE 10

INVESTIGATION OF CELLULAR AND HUMORAL INTERACTIONS IN THE ELEVATED FMLP/CB-ACTIVATED LECL RESPONSES OF PMNL FROM CIGARETTE SMOKERS *

Test System	FMLP/CB-activated LECL	
	Extracellular	Intracellular
Unfractionated blood from nonsmokers	660 ± 148	715 ± 140
Nonsmokers' PMNL + smokers' PMNL-depleted blood	632 ± 132	720 ± 105
Unfractionated blood from smokers	2,404 ± 258	2,516 ± 312
Smokers' PMNL + nonsmokers PMNL-depleted blood	2,256 ± 234	2,004 ± 223
Smokers' numerically standardised PMNL + autologous blood	1,198 ± 198	1,173 ± 174
Smokers' numerically standardised PMNL + nonsmokers' PMNL-depleted blood	1,060 ± 146	1,011 ± 129

LECL = luminol-enhanced chemiluminescence;
MNL = mononuclear leukocytes;
PMNL = polymorphonuclear leukocytes

* Results are expressed as the mean values ± SEM in rlu. Blood samples from seven different smokers in Groups B and C were paired with blood from age- and sex-matched nonsmokers. In the last two systems, correction was made for increased PMNL counts in smokers' blood with adjustment to the concentration (of PMNL) present in the blood of the matched nonsmoking control subject. Reaction systems were pre-incubated for 30 min at 37°C prior to activation with FMLP/CB.

TABLE 11

CORRELATION OF EXTRACELLULAR LECL WITH AGE, LEUKOCYCTE COUNT, PMNL COUNT, PACK-YEARS AND THIOCYANATE PRE- AND POST-CORRECTION FOR WHITE CELL COUNT

	Age	Leukocyte Count	PMNL Count	Pack-years	Thiocyanate
Extracellular	0.9	0.66	0.57	0.30	0.58
LECL Precorrection	0.48	0.0001	0.0001	0.02	0.0001
Extracellular LECL	0.02	0.16	0.1	0.15	0.3
Post Correction	0.08	0.21	0.29	0.2	0.005

Definition of abbreviations: See Table 6, page 68.

The results are expressed as the correlation coefficients (uppermost) with the paired corresponding p value below

The correlates between extracellular LECL and the spirometric parameters are recorded in Table 12. Significance is retained for all spirometric parameters, especially for FEV_1/FVC ($p<0.0006$). As with the pre-correction values there is no correlate between FEV_1 and LECL ($p<0.22$) which as mentioned previously is not surprising as all the smokers were young and asymptomatic and would not be expected to manifest changes in FEV_1 at this time.

The comparisons of LECL values for groups A, B and C as defined in the "Methods" sections are seen in Table 13. It can be noted that the change in LECL between group A and C is still highly significant ($p<0.0007$) and represents an increase of 35%. The corresponding mean (±SEM) peak extracellular and intracellular responses of non smokers were 975 ± 83 rlu and 1,413 ± 121 rlu respectively which did not differ significantly for the corresponding values for group A (after correction for white cell count).

Stepwise multiple regression analysis with FEF_{50}/FVC, FEV_1/FVC, FEF_{25}, FEF_{25-75} and FEF_{75-85} individually as the dependent variable, and extracellular and intracellular LECL (after correction for white cell count), pack-years, leukocyte counts, PMNL counts, age, and thiocyanate levels as independent variables (Table 14) demonstrates that LECL is still an important contributor to the variation of the spirometric parametrics individually and as a composite. Extracellular LECL contributed 18.98% to FEV_1/FVC, 4.52% to FEF_{50}/FVC, 7.61% to FEF_{25-75}, 7.62% to FEF_{75-85} and 8.74% to "lung function".

TABLE 12

CORRELATION OF SPIROMETRIC PARAMETERS WITH LECL PRE- AND POST-CORRECTION FOR WHITE CELL COUNT

	FEF_{50}/FVC	FEV_1/FVC	FEF_{25}	FEF_{25-75}	FEF_{75-85}	FEV_1
Leukocyte Count	-0.38 0.001	-0.27 0.03	-0.35 0.005	0.36 0.008	-0.34 0.008	0.1 0.4
Extracellular LECL Pre-correction	-0.48 0.0001	0.40 0.0002	-0.48 0.0001	-0.50 0.0001	0.48 0.0001	0.19 0.15
Extracellular LECL Post-correction	0.38 0.01	0.43 0.0006	0.36 0.004	0.42 0.001	0.43 0.00017	0.16 0.22

Definition of abbreviations: See Table 6, page 68.

The results are expressed as the correlation coefficients (uppermost) with the paired corresponding p values below.

TABLE 13

COMPARISON OF LECL RESPONSES IN GROUPS A, B and C AFTER CORRECTION FOR WHITE CELL COUNT

	A	B	C	P values §	
				A vs B	A vs C
Extra LECL	2,816.5 ± 148.80	3,634.58 ± 284.26	3,949.23 ± 221.01	0.0205	0.0007
Intra LECL	3,866.5 ± 288.67	4,707.17 ± 320.33	4,426.62 ± 390.84	*0.0338	*0.1536

Results expressed as mean value ±SEM for each investigation

* Not significant
§ Kruskall-Wallis Test

TABLE 14

STEPWISE MULTIPLE REGRESSION ANALYSIS WITH SYSTEMATIC EVALUATION OF INDIVIDUAL SPIROMETRIC PARAMETERS AS DEPENDENT VARIABLES TO ESTABLISH THE BEST PREDICTORS OF RESPIRATORY DYSFUNCTION AFTER CORRECTION FOR NEUTROPHIL COUNT

Spirometric Parameter	Best Predictor	Additional contributory Predictors
FEV_1/FVC	Extracellular LECL (18.98%)	Pack-years (5.18%) Intracellular LECL (4.54%)
FEF_{50}/FVC	PMNL count (15.01%)	Extracellular LECL (4.54%)
FEF_{25}	Pack-years (22.72%)	Thiocyanate (4.24%)
FEF_{25-75}	Pack-years (14.89%)	Extracellular LECL (7.61)
FEF_{75-75}	Pack-years (24.48%)	Extracellular LECL (7.62%)
Lung function	Pack-years (20.64%)	Extracellular LECL (8.74%)

For definition of abbreviations see Table 6, page 68

NOTE: Extracellular LECL presents the value after correction for white cell count

The best predictors of variability (percent) of each spirometric parameter are shown in the second column and further additive contributions are shown in this third: Results of the computer derived composite analysis "lung function" which unifies all five spirometric parameters are also shown.

2.4 DISCUSSION

Previous reports have indicated that cigarette smoking alters the metabolism of circulating PMNL and intrapulmonary macrophages to produce increased amounts of RO when these cells are purified, numerically standardised and activated (Hoidal et al. 1981; Hoidal and Niewoehner, 1982; Ludwig and Hoidal, 1982). The present study demonstrates similar metabolic alterations (as measured by LECL) in peripheral whole blood phagocytes of cigarette smokers. Although the whole blood LECL method used here may be influenced by interactions of the various cellular and humoral components with the assay system, potential interference by nonphagocytic elements is probably negligible as evidenced by the absence of FMLP/CB-activated LECL responses in blood from children with chronic granulomatous disease and the results of the fractionation/reconstitution experiments described here. In favour of the method are its rapidity and strong positive correlation with corresponding data obtained using purified PMNL. Relative to PMNL, the contribution of monocytes to LECL of FMLP/CB-activated whole blood is small.

Apart from leukocyte counts, cigarettes smoked per day and serum thiocyanate levels were most strongly correlated with LECL responses, especially with the extracellular component. The absolute leukocyte count, which rises significantly relative to cigarettes smoked per day ($p<0.0001$) and pack-years ($p<0.002$) obviously contributes directly to the magnitude of LECL responses. However, this is not the only factor involved, as

significantly elevated LECL responses were still observed in Group C after correction for neutrophil count. In addition, spirometric parameters correlated well with corrected LECL, and multiple regression analysis indicated that cellular RO production is important in its own right. Interestingly, it was also observed that four of 12 and six of 13 smokers in Groups B and C, respectively, had normal circulating white cell counts of $<9.6 \times 10^3/ul$, yet had markedly elevated LECL responses. In keeping with the other investigations it is clear that numerically standardised, purified PMNL and macrophages from cigarette smokers generate significantly greater amounts of reactive oxidants during exposure to phorbol myristate (PMA) acetate (Ludwig and Hoidal, 1982) and FMLP/CB (Klebanoff, 1980) relative to those from nonsmokers. Ludwig and Hoidal described these effects using both circulating and marginated PMNL and also observed that the enhanced oxidant release persisted for at least 14 hours after abstention from smoking (Ludwig and Hoidal, 1982), the only differences being that these investigators used PMA as the activator of PMNL and that enhanced oxidative metabolism was observed only with PMNL from smokers with leukocyte counts of $>9,000x/ul$. Irrespective of these differences, phagocytes from some smokers seem primed to hyperreact to both FMLP and PMA. Why this should be so and why this does not occur in all smokers to the same degree is as yet unknown. At present, the influence of smoking patterns, type of cigarettes, genetic predisposition, and anti-oxidant status individually or in combination has not been established.

Extracellular LECL responses and pack-years were most strongly correlated with spirometry. Multiple regression analysis with systematic evaluation of each spirometric parameter as the dependent variable revealed that these same parameters were also the best predictors of flow-volume abnormalities. Extracellular LECL was the best predictor for FEV_1/FVC, FEF_{50}/FVC, FEF_{25-75}, and pack-years for FEF_{25} and FEF_{75-85}. With "Lung Function", a composite of the five spirometric parameters, extracellular LECL, pack-years, and PMNL count accounted for 35.6% of the variation in pulmonary function with the major contribution from extracellular LECL (26%). This clearly indicates that extracellular LECL is strongly correlated with spirometric abnormalities in cigarette smokers and may be mechanistically involved. Taken in conjunction with pack-years, extracellular LECL which is determined by both PMNL count and production from individual cells is a good predictor of respiratory dysfunction in cigarette smokers, ie. it appears to be the product of the two which is more important, suggesting that pulmonary damage is associated with a chronically elevated extracellular LECL which does not occur in all smokers. It is possible therefore that elevated extracellular LECL responses in young cigarette smokers with low pack-years may identify a risk group for the development of smoking-related respiratory dysfunction. In support of this, 13 of the 35 smokers with normal spirometry and relatively low pack-years (8.6 ± 2.2) had raised extracellular LECL responses, ie. >1,200 rlu (1,497 ± 75 rlu) as opposed to 826 ± 51 rlu in the remaining 22 (8.5 ± 1.2

pack-years). The mean number of cigarettes smoked per day did not differ statistically between these subgroups, being 18 ± 2 and 22 ± 3, respectively.

The results obtained after correction for white cell count establish a strong correlate between pulmonary function as measured by spirometry and reactive oxidant production from individual PMNL, which extends these findings by confirming that total oxidant generation is determined both by production from individual cells as well as by the absolute PMNL count, and in addition indicates that neutrophil "priming" may be important in the aetiology of smoke-induced disease. This latter contention is graphically illustrated by the fact that strong correlates between extracellular LECL and all the spirometric parameters were retained even after correction for white cell count.

The reason why and the mechanisms whereby PMNL from only some smokers are "primed" to produce excessive oxidants have not been established, but postulates exist as to the offending chemical substance. Certainly this study does demonstrate the importance of acute smoke exposure where the number of cigarettes smoked per day is highly correlated with extracellular LECL ($r = 0.47$; p 0.0001) but less with pack-years ($r = 0.3$; p 0.02). In addition, the strong relationship between thiocyanate and extracellular LECL post-correction is confirmatory. The burning cigarette generates thousands of chemicals, many of which interact with and change human physiological functions. Included amongst these are carbon monoxide, carcinogenic aromatic hydrocarbons, oxygen free

radicals and nicotine, all of which are potentially harmful. Animal experiments have specifically implicated nicotine as the smoke-derived PMNL priming agent because enhanced PMA-activated superoxide generation by PMNL occurs within 15 weeks of chronic cigarette smoke exposure and similar changes also occur approximately one hour after intraperitoneal injection of nicotine (0.02 and 0.2mg/kg) (Gillespie et al. 1987). The mechanism is a matter of conjecture, but may be related to a recently described phenomenon whereby susceptible smokers demonstrate alterations of apparent FMLP receptors (Anderson et al. 1987; Codd and Bridges, 1986). In this regard T lymphocyte-derived, granulocyte-macrophage-colony stimulated factor (GM-CSF) has also been noted to prime PMNL FMLP-activated oxidative metabolism by altering the affinities and increasing the numbers of FMLP receptors on the cell membrane (Weisbart et al. 1986). Nicotine per se is also chemotactic for neutrophils and enhances neutrophil responsiveness to chemotactic peptides (Totti et al. 1983). It is possible therefore, that colony stimulating factors as well as pro-oxidative cytokines such as TNF released in response to cigarette smoke from activated pulmonary macrophages and T lymphocytes prime the PMNL in this manner.

Cytokines interact to initiate and maintain the inflammatory reaction and it is possible that PMNL priming involves the interaction NAP with the cytokines mentioned above. This is perhaps in a similar manner to lipopolysaccharide which induces production of both tumour necrosis factor and NAP by the

macrophage, the endothelium and fibroblasts. These substances, both enhance chemotaxis and adhesion and as mentioned above may interact with colony stimulating factors to cause the release of excessive RO from neutrophils (Leonard and Yoshimura, 1990).

In those smokers with normal PMNL function it is possible that hormonal factors inhibit their response to nicotine. However, this study and others failed to demonstrate hormonal factors present in smokers' plasma that have a negative influence on FMLP/CB-activated LECL responses of smokers' PMNL (Codd and Bridges, 1986; Ludwig and Hoidal, 1982).

Smoking related pulmonary disease is obviously dependent on a complex interaction between inherent susceptibility, PMNL, macrophages and hormonal factors. It should also be recognised that the process is multi-factorial, involving factors such as the stimulation of phagocyte migration into the lungs, the increased elastolytic activity resulting from the greater number of phagocytes, as well as the inaccessibility of inhibitors to proteolytic enzymes (Campbell et al. 1983). Other potential modifications, the role of which need to be clarified, are the influence of brand of cigarette, the presence or absence of a filter and the effect of additives such as menthol.

CHAPTER 3

INVESTIGATION OF THE ROLE OF PHAGOCYTES AND ANTI-OXIDANT NUTRIENTS IN OXIDANT STRESS MEDIATED BY CIGARETTE SMOKE

3.1 INTRODUCTION

By neutralising phagocyte-derived reactive oxidants and thereby protecting tissues against oxidative injury, innate biological anti-oxidant defence mechanisms such as superoxide dismutase, catalase, glutathione peroxidase, protein and non-protein sulphydryls, alpha tocopherol, ß-carotene (BC) and ascorbate may regulate the rate at which spirometric parameters deteriorate in cigarette smokers (Galdston et al. 1984; Nishikimi, 1975; Plonka and Metodiewa, 1979; Taylor and Oey, 1982; Theron and Anderson, 1985; Slade et al. 1985; Wayner et al. 1987). In support of this are observations that cigarette smoking is associated with decreased levels of ascorbate and BC in plasma (Chow and Bridges, 1984; Kallner et al. 1981; Smith and Hodges, 1987) and VE in the lungs (Pacht et al. 1986), and that decreased plasma and leukocyte concentrations of VC and VE may be found in other conditions with increased activity and number of PMNL (Barton et al. 1976; Kallner et al. 1981; Pacht et al. 1986). In fact a strong relationship has also been reported to exist between a deficiency in the anti-oxidant activity of plasma, the presence of a family history of lung disease and an abnormal FEV_1/FVC ratio (Taylor et al. 1986). This suggests that these nutrients are consumed during neutralisation of phagocyte derived oxidants (Hemila et al. 1984) and that baseline anti-oxidant nutrient status may be a determinant of inflammation-related tissue damage and carcinogenesis (Frei et al. 1988). In this regard, and once

again using cigarette smoking as a model of chronic inflammation, the previous study was extended by acquiring data on the relationship between (1) plasma concentrations of the anti-oxidants VC, BE and BC; (2) the generation of RO by activated PMNL; (3) smoking histories and (4) spirometric parameters in asymptomatic cigarette smokers.

3.2 SUBJECTS AND METHODS

Smoking Subjects:

A total of 72 asymptomatic young cigarette smokers were recruited to the study after informed consent and after clearance by the Committee for Research into Human Subjects of the University of the Witwatersrand had been obtained. Thirty-six were male and 36 were female. All were current smokers with pack-years ranging from 0.6 to 54 pack-years. All were resident in Johannesburg in similar environmental circumstances and none were involved in occupations potentially damaging to the lungs.

3.2.1 Reactive Oxidant Estimation

The previously described whole blood luminol-enhanced chemiluminescence (LECL) method was used (see Section 2.2.5, page 63).

3.2.2 Measurement of VC, VE and BC

Plasma levels of VC and total tocopherols were measured using modified colorimetric methods (Attwood et al. 1974); Leland et al. 1978) and the results expressed as micrograms per millilitre plasma, whilst BC was assayed using high pressure liquid chromatography (Vuilleumier et al. 1983) and the results expressed as nanograms per millilitre.

3.2.3 Other Investigations

Circulating total leukocyte counts and plasma levels of C-reactive protein (CRP) were also measured in the cigarette smokers. Those with positive CRPs were excluded from the study.

3.2.4 Lung Functions

These were performed as per Section 2.2.5 (page 63).

3.2.5 Statistical Analysis

Correlations of anti-oxidant nutrient levels with smoking histories, circulating leukocyte counts, extracellular and intracellular LECL responses and spirometry were calculated using Spearman's correlation coefficient.

3.3. RESULTS

This study confirmed previously described correlations (Table 6, page 68) between extracellular LECL responses and circulating leukocyte counts ($p<0.0001$), pack-years ($p<0.003$),

and cigarettes smoked per day ($p<0.0006$). The corresponding values for intracellular LECL were $p<0.0001$, $p<0.04$ and $p<0.02$. Likewise extracellular LECL was strongly correlated with abnormalities of the various spirometric parameters ($p<0.03$-$p<0.0001$) with the exception of FEV_1. Lesser correlations were observed with intracellular LECL.

3.3.1 Correlations between Anti-Oxidant Nutrient Levels, Smoking Histories, Leukocyte Counts and LECL Responses

These are shown in Table 15. No statistically significant correlations were observed between plasma levels of VC, VE and BC and any of the other measured parameters.

3.3.2 Correlations Between Anti-Oxidant Nutrient Levels and Spirometry

These are shown in Table 16. No statistically significant correlations were observed between levels of VC, VE and BC and any of the spirometric parameters tested.

3.4 DISCUSSION

Cigarette smoking is associated with decreased levels of VC, VE and BC in the circulation and in the lungs (Chow and Bridges, 1984; Pacht et al. 1986; Smith and Hodges, 1987). Since depletion of these anti-oxidant nutrients is not related to altered dietary intake (Bridges et al. 1990) it is probable that they are consumed during neutralisation of ROs in inhaled tobacco smoke or by oxidants released by smoke-activated

TABLE 15 CORRELATION OF PLASMA LEVELS OF VITAMIN C, VITAMIN E AND BETA-CAROTENE WITH LEUKOCYTE COUNT, LECL RESPONSES AND SMOKING HISTORY IN 72 ASYMPTOMATIC CIGARETTE SMOKERS

	Vitamin C	Vitamin E	Beta-carotene
Leukocyte count	-0.17*	0.07	-0.10
	0.18	0.60	0.46
Extracellular LECL	0.08	-0.004	0.09
	0.53	0.98	0.50
Intracellular LECL	-0.02	0.02	-0.11
	0.89	0.87	0.37
Cigarettes/day	-0.05	-0.01	0.14
	0.69	0.92	0.09
Pack-years	0.08	0.06	0.16
	0.53	0.67	0.23

* These are expressed as the correlation coefficients (uppermost) with the corresponding p values below.

LECL = Luminol enhanced chemiluminescence

TABLE 16 CORRELATION OF PLASMA LEVELS OF VITAMIN C, VITAMIN E AND BETA-CAROTENE WITH SPIROMETRIC PARAMETERS IN 72 ASYMPTOMATIC CIGARETTE SMOKERS

	Vitamin C	Vitamin E	Beta-carotene
FEF_{50}/FVC	0.10*	0.13	-0.14
	0.48	0.39	0.35
FEV_1/FVC	-0.13	0.07	-0.10
	0.37	0.64	0.50
FEF_{25}	0.04	0.09	-0.16
	0.76	0.56	0.28
FEF_{25-75}	0.15	0.06	-0.10
	0.32	0.70	0.52
FEF_{75-85}	0.10	0.20	-0.09
	0.49	0.20	0.54

* These are expressed as the correlation coefficients (uppermost) with the corresponding p values below.

phagocytes (Hoidal et al. 1981; Ludwig and Hoidal, 1982). It is conceivable that the status in plasma of the anti-oxidant nutrients VC, VE and BC is a determinant of susceptibility to oxidant-mediated pulmonary dysfunction and carcinogenesis in cigarette smokers.

This study attempted to correlate plasma levels of VC, VE and BC with the frequency of spirometric abnormalities in, and with the generation of ROs by circulating phagocytes from asymptomatic cigarette smokers. This confirmed the previous findings that the release of ROs is significantly correlated with pulmonary dysfunction. However there was no meaningful correlation between the plasma levels of the three anti-oxidants with smoking histories, ROs or spirometry, which is probably due to variations in smoking habits and daily intakes of the anti-oxidant nutrients by the smokers. Identical daily consumption of VC, VE and VC over an extended period would have been ideal, but logistically difficult. These negative observations may also be attributed to a number of other factors. Firstly, circulating levels of VC, VE and BC may not be representative of the local situation in the lungs of cigarette smokers (Pacht et al. 1986) or alternatively, leukocyte levels may, in the case of cigarette smoking, be more relevant than plasma levels. In support of this Bridges and co-workers (1990) have recently described a study similar to that reported here, in which plasma and leukocyte levels of vitamins A, C and E and total carotenes from 160 male smokers and an equal number of

matched non-smokers were correlated with smoking histories and spirometry (Bridges et al. 1990). Levels of vitamins A, C and carotenes, although significantly depressed in the group of cigarette smokers, did not correlate with smoking histories or spirometry (Bridges et al. 1990). Both plasma and leukocyte VC levels were depressed in smokers, while dietary intakes did not differ between the groups (Bridges et al. 1990). Plasma, but not leukocyte, VC correlated significantly with dietary intake, while leukocyte, but not plasma, VC was negative associated with the number of cigarettes smoked in the past 24 hours (Bridges et al. 1990). These data indicate that leukocyte levels of nutritional anti-oxidants, rather than plasma levels, may be a better indicator of oxidant stress in cigarette smokers. Yet another possibility is that physiological levels of VC, VE and BC are inadequate to deal with the smoking-related increase in oxidant stress, and that adequate protection is attainable only with supra-physiological levels of these agents. This is supported by data from recent short-term intervention studies with VC, VE or BC in cigarette smokers (see later, Chapter 4).

In conclusion, it is clear that while the nutritional anti-oxidants VC, VE and BC are important in protection against oxidant-mediated damage (Frei et al. 1988; Steinberg et al. 1989), the plasma levels of these agents are apparently not predictive of predisposition to oxidant-mediated spirometric abnormalities in cigarette smokers. Future research should target on the relevance of leukocyte and tissue levels of VC, VE and BC as possible determinants of pulmonary dysfunction.

CHAPTER 4

THE INVESTIGATION OF THE EFFECTS OF ORAL ADMINISTRATION OF VITAMIN E AND β-CAROTENE ON THE CHEMILUMINESCENCE RESPONSES OF CIRCULATING PMNL FROM CIGARETTE SMOKERS

4.1 INTRODUCTION

Anti-oxidant therapeutic strategies are of potential value in acute and chronic inflammatory conditions associated with excessive oxidant generation and overburdened physiological anti-oxidant defences. The intravenous administration of liposome-encapsulated anti-oxidant enzymes has been successfully applied in animal models, but this form of therapy is expensive and impractical at present (Yusa et al. 1984). VE and BC however, are easily administered, relatively non-toxic and inexpensive and represent a more practical form of therapy if proved efficacious.

In the present study cigarette smoking has been used as a model of chronic inflammation to investigate the effects of oral administration of nutritional anti-oxidants on stimulus-activated luminol-enhanced chemiluminescence responses of circulating phagocytes.

4.2 SUBJECTS AND METHODS

4.2.1 Cigarette Smokers

Sixty asymptomatic young subjects, 41 females and 19 males with a mean age of 33 ± 4 years, were recruited to the study after informed consent and clearance from the Committee for Research Into Human Subjects of the University of the Witwatersrand, Johannesburg had been obtained. All participants were current smokers with mean pack-years and urinary cotinine

levels of 13 ± 2 and 71 ± 7 uM respectively. None of the participants had evidence of any respiratory, cardiac or systemic disease and all denied taking any medication or vitamin supplements for one week preceding, and throughout the six week period of supplementation with the anti-oxidants. Likewise, their smoking habits remained unchanged throughout the trial.

4.2.2 Study Design

The trial was double-blind and placebo controlled. Sixty cigarette smokers were randomly assigned to 3 main treatment groups designated Groups 1, 2 and 3 with 20 individuals in each group. Individuals in Group 1 received a total of 900 international units (IU) (IU; 1 IU of VE = 1mg) of vitamin E (VE) daily for 6 weeks, administered orally as 1 x 300 IU tablet every 8 hours. Individuals in treatment Group 2 received 40mg of ß-carotene (BC) daily (2 x 20mg tablets) which was administered orally for 6 weeks. Smokers in Group 3 received matched VE/BC placebo tablets for 6 weeks. All treatment material was supplied by F. Hoffmann-La Roche, Basel, Switzerland. The laboratory investigations described below were performed before anti-oxidant/placebo supplementation and after 4 and 6 weeks of supplementation with VE or BC or matched placebo and finally, 12 weeks after the cessation of treatment. The daily dosages of VE and BC were selected on the basis of safety and tolerance considerations (Hillman, 1957; Mathews-Roth et al. 1974).

4.2.3 Chemicals and Reagents

Unless indicated these were obtained from the Sigma Chemical Co.

4.2.4 Reactive Oxidant Estimation

A previously described luminol-enhanced chemiluminescence (LECL) method (Section 2.1.1) was used to investigate the effects of administration of VE, BC or placebo on reactive oxidant generation by blood phagocytes. This method has the following advantages: (a) large numbers of specimens can be processed in a single batch; (b) artefacts due to cell processing procedures are eliminated as are false negative results due to washing away of orally administered anti-oxidants during preparative procedures, and (c) the leukocytes are retained in an essentially physiological environment.

The technique was similar to that previously described (Section 2.1.1) except that blood phagocytes were also activated by the addition of 100 µl of the tumour promoter phorbol 12-myristate 13-acetate (PMA; 100ng/ml final concentration). Spontaneous and stimulus-activated LECL were measured using an LKB Wallac (Turkey, Finland) luminometer (model 1251). LECL readings were integrated for 5 sec intervals and recorded as millivolts per second (m.volts.sec^{-1}). As previously described, activation of blood with FMLP/CB resulted in a bimodal LECL response which corresponds to an early-occurring extracellular response and a later-occurring intracellular

response, which peak within 1 minute and 5-10 minutes respectively. PMA-activation of blood however generated a single peak of LECL which was maximal within 5-12 minutes. The results have been corrected for leukocyte counts and are expressed as peak LECL responses in m.volts.$sec^{-1}/10^6$ leukocytes. As determined previously, LECL generated during activation of whole blood with FMLP/CB originates almost exclusively from phagocytes, especially neutrophils.

4.2.5 Plasma Anti-Oxidant Activity

In these investigations the oxidant scavenging activity of plasma taken before, during and after the administration of the anti-oxidants and placebo was measured. Briefly, almost pure neutrophil suspensions were prepared from heparinised blood taken from adult human volunteers. To measure superoxide scavenging activity a sensitive lucigenin-enhanced chemiluminescence (CL) assay was used (Bender and Van Epps, 1983). Five hundred microlitres of time-matched plasma, from each individual, taken before, during (4 and 6 weeks) and 12 weeks after the administration of the anti-oxidants/placebo were co-incubated with 10^6 neutrophils and 200 μM lucigenin (bis-N-methyl-acridinium nitrate) in 900 μl of HBSS for 30 minutes at 37°C, after which the neutrophils were activated with 10ng/ml (final concentration) PMA and lucigenin-enhanced CL recorded as above (Bender and Van Epps, 1983). To measure the scavenging by plasma of oxidants generated by the

MPO/H_2O_2/halide system an almost identical assay was used, the only differences being the numbers of neutrophils (2×10^5) and substitution of lucigenin with 0.1mM luminol.

Similar experiments were performed to investigate the anti-oxidant activity of the same plasma specimens using a cell-free, xanthine oxidase/hypoxanthine superoxide-generating system with and without added horseradish peroxidase (HRP). Final reaction volumes of 1ml contained 500 µl plasma, 200 µM lucigenin, 100 milliunits xanthine oxidase and 100 µM hypoxanthine. Lucigenin was substituted with luminol when horseradish peroxidase (50m.units) was included in the assay system.

4.2.6 Measurement of Plasma Concentrations of VE and BC

Total tocopherols were assayed by a modified colourimetric method (Quaife et al. 1949) and the results expressed as micrograms per millilitre plasma, while BC was assayed using high pressure liquid chromatography (Vuilleumier et al. 1983) and the results expressed as nanograms per millilitre.

4.2.7 Other Investigations

Leukocyte counts were performed using standard haematological methods. Cotinine in urine was assayed by a spectrophotometric method and the results expressed as nanograms per millilitre urine (Barlow et al. 1987).

4.2.8 Expression and Statistical Analyses of Results

The results of each series of investigations are expressed as the mean value ± the standard error of the mean (SEM). The significance levels of changes in the various laboratory parameters which occurred during the administration of the anti-oxidants were calculated using Wilcoxon's matched pairs signed ranks test for comparison of measured parameters after 4 and 6 weeks of administration of VE, BC or placebo with the corresponding pre-treatment values.

4.3 RESULTS

4.3.1 Side Effects and Compliance

There were no side-effects necessitating withdrawal from the trial in any of the treatment groups. Fifty individuals completed the first stage of the trial (4 weeks) and 49 completed the first and second stages (6 weeks). Three individuals (one in each treatment group) absconded during the first 4 weeks of the trial, and a fourth (in Group 2) during weeks 5 and 6. Four were non-compliant (2 in Group 1 and one each in Groups 2 and 3) as determined by interview, tablet count and/or plasma levels. One individual (Group 2) suffered injuries in a motor vehicle accident, while 2 (one each in Groups 2 and 3) developed bacterial infections which required antimicrobial chemotherapy.

4.3.2 Effects of Administration of VE, BC or Placebo on LECL Responses, Leukocyte Counts and Plasma Levels of the Anti-Oxidants

These results are shown in Tables 17, 18 and 19 for VE, BC and placebo administration respectively. Administration of VE for 4 weeks was accompanied by decreases of 37% ($p<0.005$), 11% (NS), and 29% ($p<0.005$) in the FMLP/CB-activated peak extracellular and intracellular, and peak PMA-activated LECL responses respectively. The background values remained unaltered. After 6 weeks of administration of VE there was an apparent rebound in stimulus-activated LECL responses, although decreased PMA-activated (12%, NS) and FMLP/CB-activated extracellular LECL (25%, $p<0.01$) were still evident, but less impressive, especially the former. The LECL responses returned to pre-treatment levels on cessation of administration of VE.

Administration of BC for 4 weeks was associated with decreases of 19% ($p<0.025$), 19% ($p<0.025$) and 10% (NS) in the FMLP/CB-activated peak extracellular and intracellular, and peak PMA-activated responses respectively. The background values were unchanged. After 6 weeks of administration of BC the corresponding respective decreases in peak LECL responses were 30% ($p<0.01$), 6% (NS) and 21% ($p<0.025$). Cessation of administration of BC was accompanied by a return of LECL responses to pretreatment levels.

The LECL responses of FMLP/CB- and PMA-activated blood did not change significantly during the administration of the VE/BC placebo (Table 19).

TABLE 17 THE EFFECT OF VITAMIN E ON LECL RESPONSES

Time of Testing	LECL (mV/s)			VE (μg/ml)	BC (ng/ml)	Leukocytes (10^6/ml)
	Extra*	Intra*	PMA			
Pre-administration of VE (n=17)§	333±31	309±30	480±37	14.5±1.1	228±34	8.14±0.5
After 4 weeks (n=17)	210±26 (p<0.005)	275±35	345±31 (p<0.005)	24.7±2.3 (p<0.005)	209±38	7.7±0.4
After 6 weeks (n=17)	249±23 (p<0.01)	375±29	429±38	22.2±2.2 (p<0.005)	209±31	8.2±0.5
Post-administration (n=14)	316±29	347±35	494±37	19.9±2 (n = 11)	261±43	7.6±0.6

Measurement of *FMLP/CB-activated extracellular and intracellular, as well as PMA-activated peak LECL responses, total blood leukocyte counts and plasma levels of VE and BC in 17 cigarette smokers before, during (4 and 6 weeks) and 12 weeks after administration of VE (900 IU daily). The results are shown as the mean values ± SEM for the individuals who completed the trial (n) and are expressed as m.volts.sec $^{-1}$, leukocytes x 10^6/ml plasma, μg VE/ml plasma and ng BC/ml plasma respectively. The LECL values for unstimulated blood were 53, 47, 27 and 42 m.volts.sec^{-1} for pre-administration, during the 4th and 6th weeks and post-administration respectively.

TABLE 18 THE EFFECT OF β-CAROTENE ON LECL RESPONSES

Time of testing	LECL nV/s			BC (ng/ml)	VE (μg/ml)	Leukocytes (10^6/ml)
	Extra	Intra	PMA			
Pre-administration of BC (n=16)§	333 ± 31	319 ± 38	492 ± 59	266 ± 38	12.9 ± 0.8	8.7 ± 0.5
After 4 weeks (n=16)	269 ± 24 (p<0.05)	257 ± 29 (p<0.025)	445 ± 42	865 ± 215 (p<0.01)	14.2 ± 1.3	8.0 ± 0.5
After 6 weeks (n=15)	232 ± 19 (p<0.01)	300 ± 34	388 ± 50 (p<0.025)	1107 ± 209 (p<0.005)	14.6 ± 1.2	8.5 ± 0.6
Post-administration (n=14)	303 ± 31	345 ± 31	476 ± 50	337 ± 62		8.3 ± 0.5

Measurement of FMLP/CB-activated extracellular and intracellular as well as PMA-activated peak LECL responses, total blood leukocyte counts, and plasma levels of VE and BC in 16 cigarette smokers, before, during and 12 weeks after administration of BC (40mg daily). The results are expressed as the mean values ± SEMs for each investigation. The respective LECL values for unstimulated blood were 35, 44, 27 and 44m.volts.sec^{-1}.

TABLE 19 THE EFFECT OF PLACEBO ON LECL RESPONSES

Time of testing	LECL nV/s			VE (μg/ml)	BC	Leukocytes (10^6/ml)
	Extra	Intra	PMA			
Pre-administration of placebo (n=17)§	267 ± 23	266 ± 29	427 ± 39	15.6 ± 1.3	171 ± 22	7.8 ± 0.5
After 4 weeks (n=17)	253 ± 28	326 ± 35	495 ± 46	14.8 ± 1.1	218 ± 30	7.7 ± 0.4
After 6 weeks (n=17)	231 ± 17	303 ± 41	391 ± 37	13.3 ± 1.3	162 ± 18	7.3 ± 0.4
Post administration	216 ± 15	274 ± 51	455 ± 53		168 ± 30	6.7 ± 0.4

Measurement of FMLP/CB-activated extracellular and intracellular as well as PMA-activated peak LECL responses, total blood leukocyte counts, and plasma levels of VE and BC in 17 cigarette smokers, before, during and after the administration of matched-placebo. The results are expressed as the mean values ± SEMs for each investigation. The respective LECL values for unstimulated blood were 27, 31, 39 and 32m.volts.sec^{-1}.

To identify possible relationships between the intensity of LECL responses and sensitivities to VE/BC-mediated inhibition, the smokers in Groups 1 and 2 were subdivided according to the magnitudes of their pre-treatment PMA and FMLP/CB-activated LECL responses into high-, mid- and low-range responders and analysed for sensitivity to VE (4 weeks) and BC (6 weeks). These results are shown in Figures 10 and 11 for VE and BC respectively. In the VE group an association between the magnitude of pre-administration stimulus-activated LECL and susceptibility to VE-mediated inhibition was evident, especially with PMA. However the differences between low, medium and high responders were not statistically significant. The sensitivity to BC-mediated inhibition was directly proportional to the magnitude of the pre-administration PMA and FMLP/CB-activated LECL responses. This differential sensitivity was not due to differences in the plasma levels of BC, which were similar for each subgroup prior to and during administration of this agent (shown in the legend to Figure 11).

Mean leukocyte counts were unchanged in all groups throughout the trial, whilst VE and BC levels increased significantly in Groups 1 and 2 respectively during administration of these agents.

4.3.3 Plasma Anti-oxidant Activity

These results are shown in Tables 20 and 21 for the neutrophil-containing and cell-free oxidant generating systems respectively. The spontaneous and PMA-activated lucigenin-

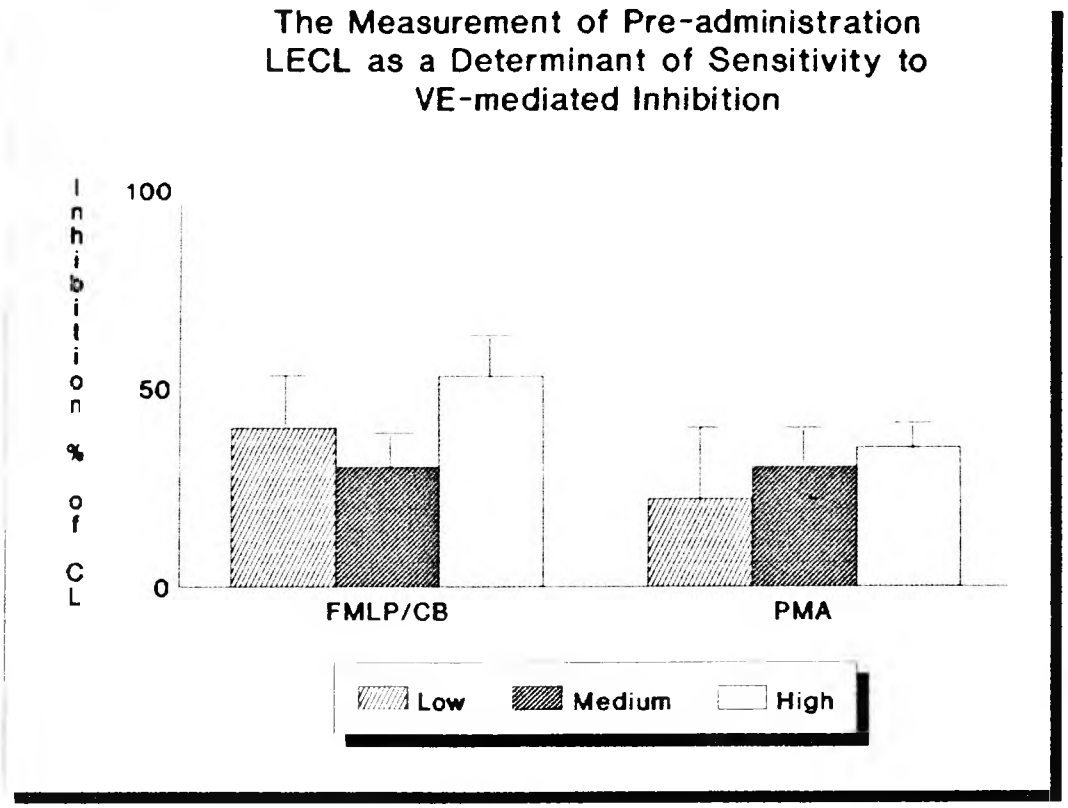


FIGURE 10

Smokers in Group 1 were subdivided according to the magnitudes of their pre-treatment FMLP/CB- and PMA-activated LECL responses into low (L, n = 6), medium (M, n = 8) and high (H, n = 3) responders and analysed for the sensitivity of their LECL responses to VE-mediated inhibition after 4 weeks of administration. The results are expressed as the mean percentages inhibition ± SEMs. The mean pre-administration plasma VE concentrations for the L, M and H subgroups were 13 ± 2, 15 ± 2 and 17 ± 2 ug/ml respectively. The corresponding values after 4 weeks of administration of 900 IU of VE/day were 23 ± 3, 25 ± 4 and 27 ± 7 ug/ml.

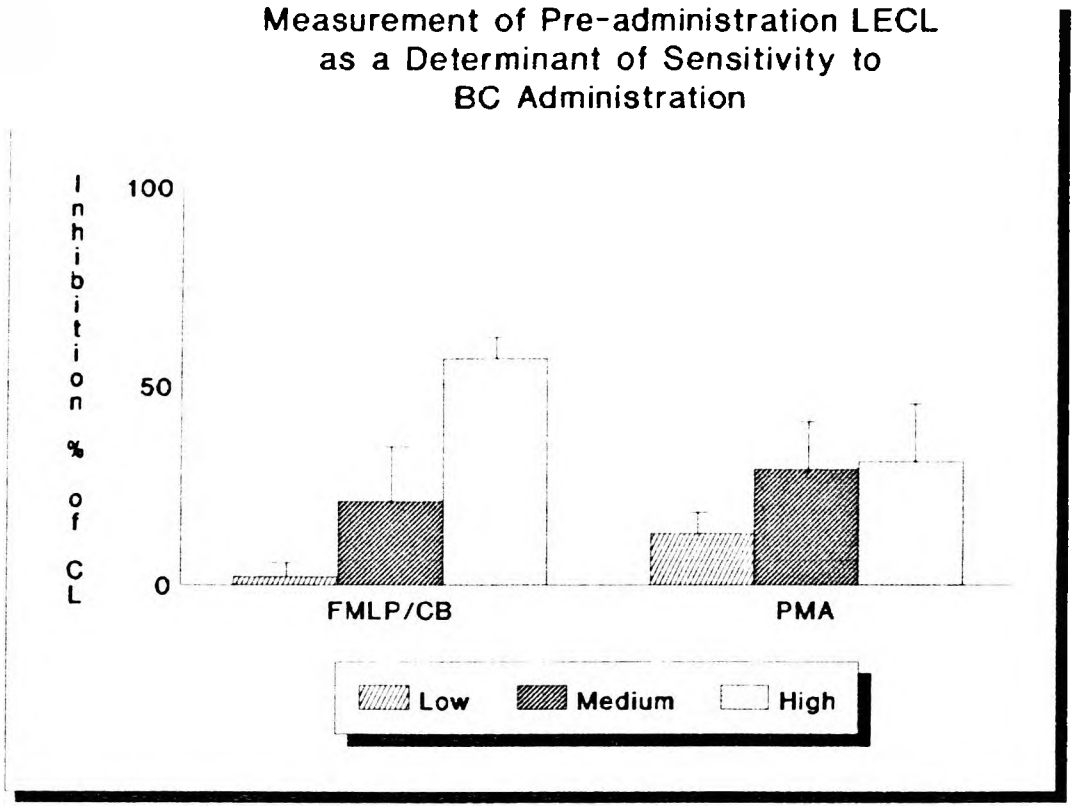


FIGURE 11
Smokers in Group 2 were also subdivided according to the magnitudes of their pre-treatment FMLP/CB- and PMA-activated LECL responses into low (L, n = 4), medium (M, n = 6) and high (H, n = 5) responders and analysed for the sensitivity of their LECL responses to BC-mediated inhibition after 6 weeks of administration. The results are expressed as the mean percentages inhibition ± SEMs. The mean pre-administration plasma BC concentrations for the L, M and H subgroups were 285 ± 76, 299 ± 76 and 252 ± 67ng/ml respectively. The corresponding values after 6 weeks of administration of 40mg of BC daily were 1,159 ± 409, 1,425 ± 442 and 694 ± 143ng/ml.

TABLE 20 Measurement of the effects of matched plasma, taken before and during the administration of VE (4 weeks), BC (6 weeks) or placebo on the peak lucigenin- and luminol-enhanced CL responses of control PMA-activated neutrophils.

Source of Plasma	Lucigenin CL		Luminol CL	
	Pre-	During	Pre-	During
Group 1 smokers (VE) (n = 10)	5,435 ± 963	5,643 ± 1152	315 ± 45	330 ± 50
Group 2 smokers (BC) (n = 10)	4,590 ± 466	4,559 ± 560	325 ± 55	245 ± 25
Group 3 smokers (placebo) (n = 8)	4,711 ± 690	4,741 ± 1733	250 ± 25	230 ± 35

The results are expressed as the mean values ± SEMs in m.volts.sec^{-1}. The background values for unstimulated neutrophils have been subtracted. For Groups 1, 2 and 3 the mean pre-administration plasma levels of VE were 15 ± 1, 13 ± 0.9 and 16 ± 1,ug/ml respectively and the corresponding values during supplementation were 25 ± 3, 15 ± 1 and 14 ± 0.9,ug/ml. The respective pre-administration values for BC were 242 ± 43, 304 ± 43 and 171 ± 29ng/ml and the corresponding values for plasma taken during administration were 200 ± 43, 1,245 ± 242 and 190 ± 29ng/ml.

TABLE 21 Measurement of the effects of matched plasma, taken before and during the administration of VE, BC or placebo on the peak lucigenin- and luminol-enhanced CL responses generated by a xanthine oxidase/ hypoxanthine/horseradish peroxidase system.

Source of Plasma	Lucigenin CL		Luminol CL	
	Pre-	During	Pre-	During
Group 1 smokers (VE) (n = 14)	1,008 ± 390	800 ± 235	424 ± 110	407 ± 110
Group 2 smokers (BC) (n = 12)	1,134 ± 207	1,091 ± 215	411 ± 149	232 ± 93 ($p<0.05$)
Group 3 smokers (placebo) (n = 15)	1,051 ± 179	954 ± 135	801 ± 125	740 ± 115

The results are expressed as the mean values ± SEMs in $m.volts.sec^{-1}$. For Groups 1, 2 and 3 the mean pre-administration plasma levels of VE were 15 ± 2, 12 ± 0.9 and 15 ± 2,ug/ml respectively and the corresponding values during administration were 26 ± 3, 14 ± 2 and 14 ± 2,ug/ml. The pre-administration respective values for BC were 238 ± 52, 280 ± 57 and 157 ± 14ng/ml and the corresponding values for plasma taken during administration were 228 ± 62, 850 ± 323 and 182 ± 38ng/ml.

enhanced CL responses of neutrophils co-incubated with matched plasma taken after 4 and 6 weeks of administration of VE, BC or placebo did not differ from the corresponding values obtained in the presence of matched pre-administration plasma (Table 20). Likewise the luminol-enhanced CL responses of neutrophils co-incubated with plasma taken during administration of VE or placebo were not significantly altered. However the PMA-activated luminol-enhanced CL responses of neutrophils co-incubated with plasma taken during the administration of BC were significantly decreased (Table 20). With the cell-free hypoxanthine/xanthine oxidase lucigenin-enhanced CL system no significant superoxide scavenging activity was detected with plasma taken during the administration of VE, BC or placebo (Table 21). Likewise the CL responses generated by the hypoxanthine/xanthine oxidase/HRP luminol-enhanced system in the presence of plasma taken during the administration of VE or placebo did not differ from the responses observed with pre-administration plasma. However the corresponding responses with plasma taken during BC supplementation were significantly ($p<0.05$) decreased (Table 21).

4.4 DISCUSSION

Cigarette smoking, since it is uncomplicated by the administration of chemotherapeutic agents, represents a useful human model for the study of chronic inflammatory processes such as the interaction between hyperactive phagocytes and

anti-oxidant defenses. The inflammatory response in the lungs leads to the release of elevated levels of phagocyte-derived reactive oxidants (Hoidal and Niewoehner, 1982) which are the possible mediators of smoking-related spirometric abnormalities (Janoff et al. 1983; Johnson and Travis, 1979), localised immune dysfunction (Holt, 1987; McSharry and Wilkinson, 1986) and an increased frequency of chromosomal aberrations (Lundberg and Livingstone, 1983) and carcinogenesis (US Department of Health and Human Services, 1982). These changes are accompanied by decreased plasma and tissue levels of the nutrients VE, BC and ascorbate which indicates that the smoking-related chronic inflammatory response leads to an imbalance of anti-oxidant/oxidant homeostasis (Nishikimi, 1975; Plonka and Metodiewa, 1979; Slade et al. 1985; Wayner et al. 1987). Individuals with such an imbalance may be particularly susceptible to oxidant-inflicted tissue damage and disease.

In this study the administration of VE and BC to cigarette smokers was associated with decreased PMA- and FMLP/CB-activated LECL responses of blood phagocytes. The administration of VE (900 IU/day) to smokers was accompanied by decreased FMLP/CB- and PMA-activated LECL responses of blood phagocytes, representing a decline in the production and/or reactivity of oxidants originating from the MPO/H_2O_2/halide system (De Chatelet et al. 1982). After 4 weeks of intake of VE, statistically significant decreases in the FMLP/CB-activated extracellular (37%, $p<0.005$) and PMA-activated (29%; $p<0.005$) LECL responses of blood phagocytes were observed, which is in

agreement with the findings of Baehner and colleagues that VE inhibits RO production (Baehner et al. 1977). After 6 weeks of administration of VE however, the inhibition of the FMLP/CB LECL responses although still evident, was of lesser magnitude (25%; $p<0.01$), while the inhibition of PMA-activated LECL (12%) was not statistically significant. This apparent rebound in the stimulus-activated LECL responses during the sixth week of administration of VE could not be attributed to alterations in the plasma levels of the anti-oxidant and may therefore represent physiological adaptation to sustained intake of high levels of VE. It has recently been reported that the activity of a hepatic membrane-associated VE binding protein is inversely related to VE intake (Verdon and Blumberg, 1988). If such a mechanism of VE transport exists in human neutrophils it may explain the rebound of LECL responses during prolonged administration of VE. The apparent transient nature of the VE-mediated inhibition of stimulus-activated LECL responses of human neutrophils ex vivo has not previously been described and may have important therapeutic implications. When the data were analysed to assess if the magnitude of pre-administration LECL was a determinant of sensitivity to VE-mediated inhibition, it was found that although tendencies were evident, the pre-administration LECL response did not significantly influence sensitivity to VE.

The administration of BC to cigarette smokers was also accompanied by inhibition of both FMLP/CB- and PMA-activated LECL responses of blood phagocytes. After 4 weeks of intake of

this anti-oxidant, statistically significant decreases in both extracellular (19%; $p<0.05$) and intracellular (19%; $p<0.025$) FMLP/CB-activated LECL responses were observed, with a slight (10%) decline in the PMA-activated responses. The inhibitory effects of BC administration on the FMLP/CB-activated extracellular and PMA-activated LECL increased in magnitude after 6 weeks of administration (30%; $p<0.01$ and 21%; $p<0.025$ respectively), while the inhibitory effects on FMLP/CB-activated intracellular LECL were of lesser magnitude. BC intake is therefore associated with a gradual, cumulative, inhibitory effect on the generation of reactive oxidants by stimulus-activated neutrophils from cigarette smokers which is consistent with its role as a scavenger rather than an agent affecting cellular function. Interestingly, sensitivity to BC-mediated inhibition of LECL was in direct proportion to the pre-administration oxidant-generating capacities of the phagocytes. This differential sensitivity of LECL responses to administration of BC has also been described for ascorbate (Theron and Anderson, 1988). In the placebo-treated group the LECL responses remained unchanged throughout the study period. Cessation of administration of VE and BC was accompanied by a return to pre-administration LECL values.

It has previously been reported that co-incubation of VE acetate at concentrations of up to 40 μg/ml with cell-free and neutrophil oxidant-generating systems <u>in vitro</u> did not inhibit either the generation or reactivity of superoxide, or of oxidants generated by the peroxidase/H_2O_2/halide system

(Anderson and Theron, in press). BC did not scavenge superoxide, nor did it influence the production of this oxidant by phagocytes or cell-free oxidant-generating systems. The failure of VE to influence phagocyte membrane-associated oxidative metabolism in these studies was attributed to the inability of this agent to interact optimally with phagocytes in vitro (Anderson and Theron, in press). Alternatively the observed effects of VE may be due to a transient membrane stabilising effect of this agent (Diplock et al. 1977). Since a whole blood method was used for the investigations described in the present ex vivo study, additional experiments were performed to identify the sites of anti-oxidant activity of VE and BC ie. cell-associated, or extracellular in plasma, or both, as well as the mechanisms of anti-oxidant activity. Plasma taken during the administration of VE did not influence the production of superoxide or the generation of oxidants by the MPO/H_2O_2/ halide system in control PMA-activated neutrophils or by cell-free oxidant-generating systems, which excludes an extracellular scavenging activity of VE. These findings are compatible with a cell-associated inhibitory effect of VE on H_2O_2 generation and/or reactivity by phagocytes as described by Baehner and co-workers (Baehner et al. 1982; Butterick et al. 1983). Furthermore, Mahoney and Azzi have described an inhibitory effect of VE (α-tocopherol) on brain protein kinase C and it is this property which could explain its activity in this setting (Mahoney and Azzi, 1988). Similar investigations were performed with plasma from blood taken during the administration

of BC to the cigarette smokers showed that this anti-oxidant apparently does not scavenge superoxide nor does it interfere with neutrophil membrane-associated oxidative metabolism, but rather acts predominantly as an extracellular scavenger of oxidants generated by the MPO/H_2O_2/halide system.

There has been concern that interfering with reactive oxidant generation by neutrophils may have deleterious effects on host defence. In vitro, BC at concentrations as great as 600 ug/ml does not decrease neutrophil microbicidal activity (Seifter et al. 1988), and in addition BC has been used in higher doses and for longer periods without adverse clinical effects (Mathews-Roth et al. 1974). Oral administration of VE (1,600 IU/day for 2 weeks) caused only moderate impairment of microbicidal activity (Baehner et al. 1982), and daily intake of 4,000 IU for several years did not cause any untoward effects (Burton and Ingold, 1984; Fahn, 1990). As mentioned previously, the effects of BC on FMLP/CB- and PMA-induced responses was greater in those patients with higher baseline RO status. In other words, this anti-oxidant decreased the elevated LECL responses toward normal, but not below.

The results of this study have demonstrated that the anti-oxidants VE and BC, when administered to cigarette smokers, regulate the generation (VE) or reactivity (BC) of phagocyte derived oxidants. However the investigations should be extended to identify the time of onset and duration of the anti-oxidant activities, as well as the optimal dosages and administration schedules, particularly for VE, which could sustain these

effects and minimise possible rebound phenomena. The prophylactic and therapeutic potential of these agents, individually and in combination, could then be established in certain high-risk target groups.

4.5 CONCLUDING COMMENTS

This thesis has reviewed the role of reactive oxidants in the aetiology of emphysema due to cigarette smoking and the role of anti-oxidants in the prevention of such disease.

The strong relationships between spirometry and reactive oxidant production are important because they extend to clinical relevance the findings of Hoidal in 1981. This is the first study that directly links cigarette smoking and reactive oxidant production to the deterioration of lung function parameters, and emphasises their probable causative role. Of no less importance is the fact that excessive generation of reactive oxidants can be modified, at least in the short term, by the administration of nutrients. While further investigation as to mode of delivery of these agents and a longitudinal study confirming benefit in larger cohorts of volunteers are required, it should be emphasised that prophylactic intervention cannot substitute for complete abstinence. However, a considerable number of smokers are unable or unwilling to stop, and it is this group that could benefit most. The manner of administration of these nutrients is obviously critical. Previously described negative effects of VE have probably been due to the rebound

phenomenon described in this thesis. VE would probably be more effective than BC if this problem could be overcome by an altered dosage schedule as it appears to inhibit the mechanism of activation of NADPH oxidase and is not merely a scavenger (Mahoney and Azzi, 1988).

Finally, this report lends optimism to the view that anti-oxidant strategies may be of value in other diseases of oxidant excess and, in the future, it may be routine to employ them in such diverse diseases as the adult respiratory distress syndrome and rheumatoid arthritis.

REFERENCES

Allen RC. Lucigenin chemiluminescence: a new approach to the study of polymorphonuclear leukocyte redox activity. In: De Luca MA, McElrosy WD (eds). Bioluminescence and Chemiluminescence. Academic Press Inc.: New York, 1987: 63-73.

Allen RC, Loose LD. Phagocyte activation of a luminol-dependant chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochem Biophys Res Commun 1976; 69: 245-252.

Allen RC, Stjennholm RL, Steel RH. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem Biophys Res Commun 1972; 47: 679-685.

Anderson R, Theron AJ. Physiological potential of ascorbate, ß-carotene and α tocopherol individually and in combination in the prevention of tissue damage, carcinogenesis and immune dysfunction mediated by phagocyte derived oxidants. World Rev Nutr Diet 1990; 62: 27-58.

Anderson R, Theron AJ, Ras GJ. Regulation by the anti-oxidants ascorbate, cysteine and dapsone of the increased extra-cellular and intracellular generation of reactive oxidants by activated phagocytes from cigarette smokers. Am Rev Respir Dis 1987; 79: 1665-1673.

Anderson R, Smit MJ, Joone GK, van Staden AM. Vitamin C and cellular immune functions - protection against hypochlorous-acid mediated inactivation of glyceraldehyde-3-phosphate dehydrogenase and ATP generation in human leukocytes as a possible mechanism of ascorbate-mediated immunostimulation. Ann NY Acad Sci 1990; 587: 34-48.

Attwood EC, Robey ED, Ross J, Francis B, Kramer JJ. Determination of platelet and leukocyte vitamin C and the levels found in normal subjects. Clin Chim Acta 1974; 54: 95-105.

Babior BM. Oxygen-dependant microbial killing by phagocytes. N Engl J Med 1978; 298 (12): 659-668.

Babior BM. The respiratory burst of phagocytes. J Clin Invest 1984a; 73: 599-561.

Babior BM. Oxidants from phagocytes: agents of defense and destruction. Blood 1984b; 64 (5): 989-966.

Babior BM. The respiratory burst oxidase. Trends Biochem Sci 1987; 12: 241-243.

Baehner RL, Boxer L, Allen JM, Davis J. Autooxidation as a basis for altered function by polymorphonuclear leukocytes. Blood 1977; 50: 327-335.

Baehner RL, Boxer LA, Ingraham L, Butterick C, Haak R. The influence of vitamin E on juman polymorphonuclear cell metabolism and function. Ann NY Acad Sci 1982; 393: 237-250.

Baggiolini M, Wymann M. Turning on the respiratory burst. Trends in Biochemical Science 1990; 69-72.

Bainton D, August J. Multivesicular bodies of human neutrophils, not granules, immunolabelled with the 2 major lysozymal glycoproteins. (Abstr) J Histochem 1988; 36: 953.

Baker MS, Feigan J, Lowther DA. The mechanism of chondrocyte hydrogen peroxide damage. Depletion of intracellular ATP due to suppression of glycolysis caused by oxidation of glyceraldehyde-3-phosphate dehydrogenase. J Rheumatol 1989; 16: 7-14.

Barlow RD, Stone RB, Wald NJ, Puhakainen EVJ. The direct barbituric acid assay for nicotine metabolites in urine: a simple colorimetric test for the routine assessment of smoking status and cigarette smoke intake. Clin Chim Acta 1987; 165: 45-52.

Barton GMG, Roath OS. Leucocyte ascorbic acid in abnormal leucocyte states. Int J Vit Nutr Res 1976; 46: 271-274.

Becker EL. Leukocyte stimulation: receptor, membrane, and metabolic events. Fed Proc 1985; 45: 2148-2161.

Bender JG, van Epps DE. Analysis of the bimodal chemiluminescence pattern stimulated in human neutrophils by chemotactic factors. Infect Immun 1983; 41: 1062-1070.

Bennett JP, Cockcroft S, Gomperts BD Use of cytochalasin B to distinguish between early and late events in neutrophil activation. Biochim Biophys Acta 1980; 601: 584-591.

Berend N, Woolcock AJ, Marlin GE. Correlation between the function and structure of the lung in smokers. Am Rev Respir Dis 1979; 119: 695-705.

Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr. Recombinant tumor necrosis factor induced procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci USA 1986; 83: 4533-4537.

Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA Jr. Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J Clin Invest 1985; 76: 2003-2011.

Bieri JG, Corash L, Hubbard VC. Medical uses of vitamin E. N Engl J Med 1983; 308: 1063-1070.

Blow AM, Barrett AJ. Action of the human cathepsin G on the oxidized B chain of insulin. Biochem J 1977; 161: 17-19.

Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase. Translation during activation. J Cell Biol 1983; 97: 57-61.

Boudier C, Holle C, Bieth JG. Stimulation of the elastolytic activity of leukocyte elastase by leukocyte cathepsin G. J Biol Chem 1981; 256: 10256-10258.

Boudier C, Pelletier A, Pauli G, Bieth JG. The functional activity of alpha-1-protease inhibitor in bronchoalveolar lavage fluid from healthy human smokers and non-smokers. Clin Chim Acta 1983; 132: 309-315.

Bowler RG. Determination of the thiocyanate in blood serum. Biochem J 1944; 38: 385-388.

Boxer GJ, Curnutte JT, Boxer LA. Polymorphonuclear leukocyte function. Hospital Practice 1985; 20: 69-90.

Brawn K, Fridovich J. DNA strand scission by enzymatically generated oxygen radicals. Arch Biochem Biophys 1981; 206: 414-419.

Bridges RB, Chow CK, Rehm S. Micronutrient status and immune function in smokers. Ann NY Acad Sci 1990; 587: 218-231.

Briheim G, Stendahl O, Dahlgren C. Intra- and extracellular events in luminol-dependent chemiluminescence of polymorphonuclear leukocytes. Infect Immun 1984; 45: 1-5.

Britigan BE, Cohen MS, Rosen GM. Hydroxyl radical formation in neutrophils. N Engl J Med 1988; 318: 858-859.

Britigan BE, Rosen GM, Chap Y, Chen MS. Do human neutrophils make hydroxyl radicals? Determination of free radicals generated by human neutrophils activated with soluble or particulate stimulus using electron paramagnetic resonance spectrometry. J Biol Chem 1986; 261: 4426-4431.

Buckley BJ, Tanswell AK, Freeman BA. Liposome mediated augmentation of catalase in alveolar type II cells protects against H_2O_2 injury. J Appl Physiol 1987; 63: 359-367.

Burton GW, Ingold KU. ß-carotene: an unusual type of lipid anti-oxidant. Science 1984; 224: 569-573.

Butterick CJ, Baehner RL, Bozer LA, Jersild RA. Vitamin E: a selective inhibitor of the NADPH oxidoreductase enzyme system in human granulocytes. Am J Pathol 1983; 112: 287-293.

Campbell EJ, Senior RM, McDonald JA, Cox DL. Proteolysis by neutrophils. Relative importance of cell substrate contact and oxidative inactivation of proteinase inhibitors in vitro. J Clin Invest 1983; 70: 845-852.

Cantin AM, Hubbard RC, Crystal RG. Gluthathione deficiency in the epithelial lining fluid of the lower respiratory tract in idiopathic pulmonary fibrosis. Am Rev Respir Dis 1989; 139: 370-372.

Caplowitz N, Aw T, Ookhten SM. The regulation of hepatic glutathione. Annu Rev Pharmacol Toxicol 1985; 25: 715-744.

Carp H, Janoff A. Possible mechanisms of emphysema in smokers: In vitro suppression of serum elastase-inhibitory activity by fresh cigarette smoke and its prevention by anti-oxidants. Am Rev Respir Dis 1978; 118: 617-621.

Carp H, Janoff A. The in vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. J Clin Invest 1979; 63:793-797.

Carp H, Janoff A. Potential mediator of inflammation. Phagocyte derived oxidants suppress the elastase-inhibitory capacity of alpha-1-proteinase inhibitor in vitro. J Clin Invest 1980; 66: 987-995.

Chan-Yeung M, Abboud R, Dy Buncio A, Vedal S. Peripheral leucocyte count and longitudinal decline in lung function. Thorax 1988; 43: 462-466.

Chan-Yeung M, Dy Buncio A. Leucocyte count, smoking and lung function. Am J Med 1984; 76: 31-37.

Charo IF, Yuen C, Perez HD, Goldstein IM. Chemotactic peptides modulate adherence of human polymorphonuclear leukocytes to monolayers of cultured endothelial cells. J Immunol 1986; 136: 3412-3419.

Chow CK, Bridges RB. Chronic cigarette smoking and plasma micronutrients (abstr). Fed Proc 1984; 43: 861.

Clark RA. Chemotactic factors trigger their own oxidative inactivation by human neutrophils. J Immunol 1982; 129 (6): 2725-2728.

Clark RA. The human neutrophil respiratory burst oxidase. J Infect Dis 1990; 161: 1140-1147.

Clark RA, Malech HL, Gallin JI, Nunoi H, Volpp BD, Pearson DW, Nauseef WM, Curnutte JT. Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. N Engl J Med 1989; 321: 647-652.

Clark RA, Stone PJ, El-Hag A, Calore JD, Franzblau C. Myeloperoxidase catalysed inactivation of alpha-1-proteinase inhibitor by human neutrophils. J Biol Chem 1981; 256: 3348-3353.

Clark SC, Kamen R. The human hematopoietic colony-stimulating factors. Science 1987; 236: 1229-1237.

Codd EE, Bridges RB. Altered chemotactic peptide binding in tobacco smokers' neutrophils. Regul Pept 1986; 14: 243-251.

Cohen AB, Chenoweth DE, Hugli TE. The release of elastase, myeloperoxidase and lysozyme from human alveolar macrophages. Am Rev Respir Dis 1982; 126: 241-247.

Cohen G. Hochstein P. Glucose-6-phosphate dehydrogenase and detoxification of hydrogen peroxide in human erythrocytes. Science 1961; 134: 1756-1757.

Cone F, Lellouch J, Schwartz D. Smoking and leukocyte counts: results of an epidemiological study. Lancet 1971; ii: 632-634.

Cosgreave IA, Moldeus P. Lung protection by thiol containing antioxidants. Bull Eur Physiopathol Respir 1987; 23: 275-277.

Cradock PR, White JG, Jacobs HS. Potentiation of complement (C5a)-induced granulocyte aggregation by cotochalasin B. J Lab Clin Med 1978; 91: 490-499.

Crawford DR, Schneider DL. Identification of ubiquinone-50 in human neutrophils and its role in microbicidal events. J Biol Chem 1982; 257: 6662-6668.

Cross AR, Parkinson JF, Jones OTG. Mechanism of the superoxide-producing oxidase of neutrophils: O_2 is necessary for the fast reduction of cytochrome b-245 by NADPH. Biochem J 1985; 226: 881-884.

Dahlgren C, Anlansson H, Magnusson KE. Pattern of formyl-methionyl-leucyl-phenylalanine-induced luminol and lucigenin dependent chemiluminescence in human neutrophils. Infect Immun 1985; 47: 326-328.

Dallegri F, Gorletti R, Ballestrero A, Ottonello L, Patrone F. Neutrophil induced depletion of adenosine triphosphate in target cells: evidence for a hypochlorous acid mediated process. J Lab Clin Med 1988; 112: 765-772.

De Chatelet LR, Long GD, Shirley PS, Bass DA, Thomas MJ, Henderson FW, Cohen MS. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. J Immunol 1982; 129: 1589-1593.

De Chatelet LR, Shirley PS. Evaluation of chronic granulomatous disease by a chemiluminescence assay of microlitre quantities of whole blood. Clin Chem 1981; 27: 1739-1744.

De Chatelet LR, Shirley PS, Johnson B. The effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. Blood 1976; 47 (4): 545-554.

Desroches PE, Weiss SJ. Proteolytic inactivation of alpha-one-proteinase by a neutrophil metalloprotein. J Clin Invest 1988; 81: 1646-1650.

Dewald B, Bretz U, Baggiolini M. Release of gelatinase from a novel secretory compartment of human neutrophils. J Clin Invest 1982; 70: 518-525.

Dinarello CA. Biology of interleukin 1. FASEB J 1988; 2: 108-115.

Diplock AT, Lucy JA, Verrinder M, Zieleniewski A. α-Tocopherol and the permeability to glucose and chromate of unsaturated liposomes. FEBS Lett 1977; 82: 341-344.

Dogra SC, Khanduja KL, Sharma RR. Effect of Vitamin A deficiency on the levels of glutathione and glutathione-S-transferase activity in rat lung and liver. Experientia 1982; 38: 903-904.

Doll R. The smoking induced epidemic. Can J Public Health 1981; 72: 372-381.

Doll R, Hill AB. A study of the aetiology of carcinoma of the lung. Br Med J 1952; 2: 1271-1286.

Doll R, Peto R. Cigarette smoking and bronchial carcinoma: dose and time relationships among regular smokers and lifelong non smokers. J Epidemiol Community Health 1978; 32: 303-313.

Dosman J, Bode F, Urbanetti J, Martin R, Macklem PT. the use of a helium-oxygen mixture during maximum expiratory flow to demonstrate obstruction in small airways in smokers. J Clin Invest 1975; 55: 1090-1099.

Estensen RD, Plageman PGW. Cytochalasin B: Inhibition of glucose and glucosamine transport. Proc Natl Acad Sci USA 1972; 69 (6): 1430-1434.

Faden H, Maciejewski N. Whole blood luminol-dependent chemiluminescence. J Reticuloendothel Soc 1981; 30: 219-224.

Fahn S. Anti-oxidant vitamin and beta-carotene in disease prevention: Parkinson's disease. Am J Clin Nutr 1990 (in press).

Fantone JC, Feltner DE, Brieland JK, Wand PA. Phagocytic cell-derived inflammatory mediators and lung disease. Chest 1987; 91 (3): 428-435.

Flenley DC. What should an ideal antioxidant do (and not do): Bull Eur Physiopathol Respir 1987; 23: 279-285.

Fletcher C, Peto R. The natural history of chronic airflow obstruction. Br Med J 1977; 1: 1645-1648.

Fletcher MP, Gallin JI. Human neutrophils contain an intracellular pool of putative receptors for the chemoattractant N-FMLP. Blood 1983; 62 (4): 792-799.

Freeman BA, Young SL, Crapo JD. Liposome-mediated augmentation of superoxide dismutase in endothelial cells prevents oxygen injury. J Biol Chem 1983; 258: 12534-12542.

Frei B, Stocker R, Ames BN. Antioxidant defences and lipid peroxidation in human blood plasma. Proc Natl Acad Sci 1988; 85: 9748-9752.

Fridovich I. Superoxide radical: an endogenous toxicant. Annu Rev Pharmacol Toxicol 1983; 23: 239-257.

Gabig TG, English D, Akard LP, Schell MJ. Regulation of neutrophil NADPH oxidase activation in a cell-free system by guanine nucleotides and fluoride. J Biol Chem 1987; 262: 1685-1690

Gadek JE, Fells GS, Crystal RG. Cigarette smoking induces functional anti-protease deficiency in the lower respiratory tract of humans. Science 1979; 206: 1315-1316.

Galdston M, Levytska V, Schwartz MS, Magnusson B. Ceruloplasmin. Increased serum concentration and impaired antioxidant activity in cigarette smokers, and ability to prevent suppression of elastase inhibitory capacity of alpha-1-proteinase inhibitor. Am Rev Respir Dis 1984; 129: 258-263.

Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci USA 1985; 82: 8667-8671.

Gillespie MN, Owasoyo JO, Kojima S, Jay M. Enhanced chemotaxis and superoxide anion production by polymorphonuclear leukocytes from nicotine treated and smoke-exposed rats. Toxicology 1987; 45: 45-52.

Gilman AG. G proteins and dual control of adenylate cyclase. Cell 1984; 36: 577-579.

Goldhaber JI, Ji S, Lamps T, Weiss JN. Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle. J Clin Invest 1989; 83: 1800-1809.

Goldman DW, Goetzl EJ. Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B_4. J Exp Med 1984; 159: 1027-1041.

Goldstein IM, Horn JK, Kaplan HB, Weissman G. Calcium-induced lysozyme secretion from human polymorphonuclear leukocytes. Biochem Biophys Res Common 1974; 60: 807-812.

Halliwell B. Oxidants and human disease. Some new concepts. FASEB J 1987 (1): 358-364.

Halliwell B, Gutteridge JMC. Free radicals in biology and Medicine. Oxford: Clarendon Press, 1985.

Hawkins D. Neutrophilic leukocytes in immunologic reactions *in vitro*: effect of cytochalasin B. J Immunology 1973; 110 (1): 294-296.

Heffner JE, Repine JE. Pulmonary strategies of antioxidant defences. Am Rev Respir Dis 1989; 140: 531-554.

Helman N, Rubenstein LS. The effects of age, sex, and smoking on erythrocytes and leukocytes. Am J Clin Pathol 1975; 63: 35-44.

Hemilä H, Roberts P, Wikström M. Activated polymorphonuclear leukocytes consume vitamin C. Febs Letters 1984; 178: 25-30.

Henson PM. The immunologic release of constituents from human neutrophils. II. Mechanisms of release during phagocytosis and adherence to non-phagocytosable surfaces. J Immunol 1971; 107: 1547-1557.

Henson PM, Henson JE, Fittschen C, Kimani G, Bratten DL, Riches DW. Phagocytic cells: Degranulation and secretion. In: Gallin JJ, Goldstein EM, Snyderman R. (eds). Inflammation: Basic principles and clinical correlates. Raven Press, New York: 1988.

Hibbs M, Bainton D. Human neutrophil gelatinase is a component of specific granules. J Clin Invest 1989; 84: 1395-1402.

Hillman RW. Tocopherol excess in man. Am J Clin Nutr 1957; 5: 597-600.

Hinshaw DB, Armstrong BC, Beals RF, Hyslop PA. A cellular model of endothelial cell ischaemia. J Surg Res 1988; 44: 527-537.

Hinshaw DB, Sklar LA, Bohl B, Schraufstatter IU, Hyslop PA, Rossi MW, Spragg RG, Cochrane CG. Cytoskeletal and morphological impact of cellular oxidant injury. Am J Pathol 1986; 123: 454-464.

Hjorth R, Jonsson A-K, Vretblad P. A rapid method for purification of human granulocytes using percoll. A comparison with dextran sedimentation. J Immunol Methods 1981; 43: 95-101.

Hoidal JR, Fox RB, Lemarbe PA, Perri R, Repine JE. Altered oxidative metabolic responses in vitro of alveolar macrophages from asymptomatic cigarette smokers. Am Rev Respir Dis 1981; 123: 85-87.

Hoidal JR, Niewoehner DE. Lung phagocyte recruitment and metabolic deterioration induced by cigarette smoke in humans and hamsters. Am Rev Respir Dis 1982; 126: 548-552.

Holman RG, Maier RV. Oxidant-induced endothelial leak correlates with decreased cellular energy levels. Am Rev Respir Dis 1990; 142: 134-140.

Holt PG. Immune and inflammatory function in cigarette smokers. Thorax 1987; 42: 241-249.

Honeycutt PJ, Niedel JE. Cytochalasin B augments diacylglycerol levels in stimulated neutrophils. Fed Proc 1986a; 45: 1135.

Honeycutt PJ, Niedel JE. Cytochalasin B enhancement of the diacylglycerol response in formyl-stimulated neutrophils. J Biol Chem 1986b; 261: 15900-15905.

Hugli TE. Structure and function of anaphylatoxins. Springer Semin Immunopathol 1984; 7: 193-219.

Hunninghake GW, Crystal RG. Cigarette smoking and lung destruction: accumulation of neutrophils in the lungs of cigarette smokers. Am Rev Respir Dis 1983; 128: 833-838.

Hurley JV. An electron microscopic study of leucocytic emigration and vascular permeability in rat skin. Aust J Exp Biol Med Sci 1963; 41: 171-186.

Hyslop PA, Hinshaw DB, Halsey WA, Schraufstatter IU, Sauerheber RD, Spragg RG, Jackson JH, Cochrane CG. Mechanisms of oxidant mediated cell injury: the glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. J Biol Chem 1988; 263: 1665-1675.

Idell S, Cohen AB. The protease-inhibitor hypothesis of emphysema. Recent Advances in Respiratory Medicine 1983; 2: 159-171.

Jackson JH, Gajewski E, Schraufstatter IU, Hyslop PA, Fuciarelli AF, Cochrane CG. Dizdaroglu M. Damage to the bases in DNA induced by stimulated neutrophils J Clin Invest 1989; 84: 1644-1649.

Jallat S, Carvallo D, Tessier LH, Ruecklin D, Roitsch C, Ogushi F, Crystal RG, Courtney M. Altered specification of genetically engineered α1-antitrypsin variants. Prot Eng 1986; 1: 29-35.

Janoff A. Elastase in tissue injury. Annu Rev Med 1985; 36: 207-216.

Janoff A, Carp H, Laurent P, Raju I. The role of oxidative processes in emphysema. Am Rev Respir Dis 1983; 127: 531-538.

Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, Kimbel P. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. Am Rev Respir Dis 1977; 115: 461-478.

Johnson D, Travis J. The oxidative inactivation of human alpha-1-proteinase inhibitor. J Biol Chem 1979; 254: 4022-4026.

Junod AF. Effects of oxygen intermediates on cellular functions. Am Rev Respir Dis 1987; 35: 532-534.

Kaiser H, Greenwald RA, Feinstein G, Janoff A. Degradation of cartilage proteoglycan by human leukocyte granule neutral protease; a model of joint injury. II. Degradation of isolated bovine nasal cartilage proteoglycan. J Clin Invest 1976; 57: 625-632.

Kallner AB, Hartmann D, Hornig DH. On the requirements of ascorbic acid in man: steady state turnover and body pool in smokers. Am J Clin Nutr 1981; 34: 1347-1355.

Kanofsky JR, Wright J, Miles-Richardson GE, Tauber AI. Biochemical requirements for singlet oxygen production by purified human myeloperoxidase. J Clin Invest 1984; 74: 1489-1495.

Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR. A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. J Clin Invest 1988; 82: 1963-1973.

Klebanoff SJ. Oxygen metabolism and the toxic properties of phagocytes. Ann Intern Med 1980; 93: 480-489.

Klebanoff SJ. Oxygen dependent cytotoxic mechanisms of phagocytes. Advances in Host Defence Mechanisms 1982; 1: 111-162.

Koo C, Lefkowitz J, Snyderman R. The oligopeptide chemotactic factor receptor on human polymorphonuclear leukocyte membranes exists in two affinity states. Biochem Biophys Res Commun 1982; 106: 442-449.

Koo C, Lefkowitz J, Snyderman R. Guanine nucleotides modulate the binding affinity of the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. J Clin Invest 1983; 72: 748-753.

Kreisle RA, Parker DW. Specific binding of leukotriene B to a receptor on human polymorphonuclear leukocytes. J Exp Med 1983; 157: 628-634.

Krinsky NI, Deneke SM. Interaction of oxygen and oxy-radicals with carotenoids. JNCI 1982; 69: 205-209.

Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium deficient rat liver. Biochem Biophys Res Commun 1976; 71: 952-958.

Lehmann EL. Non-parametrics: statistical methods based on ranks. New York: McGraw-Hill, 1975; 204-210.

Lehmeyer JE, Snyderman R, Johnston RB. Stimulation of neutrophil oxidative metabolism by chemotactic peptides: influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristate acetate. Blood 1979; 54: 35-45.

Leland G, Hansen MPH, Warwick WJ. An improved assay method for serum vitamins A and E using fluorometry. Am J Clin Pathol 1978; 70: 922-923.

Leonard EJ, Yoshimura T. Neutrophil attractant/activation protein-1 (NAP-I [interleukin 8]). Am J Respir Cell Mol Biol 1990; 2: 679-486.

Leoni P, Dean RT. Mechanisms of lysozymal enzyme secretion by human monocytes. Biochem Biophys Acta 1983; 762: 378-389.

Levine M. New concepts in the biology and biochemistry of ascorbic acid. N Engl J Med 1986; 314: 892-902.

Lim TPK. Airway obstruction among high school students. Am Rev Respir Dis 1973; 108: 985-988.

Litosch I, Fain JN. Regulation of phosphoinositide breakdown by guanine nucleotides. Life Sci 1986; 39: 187-194.

Logan IMK, Davies RE. Lipid oxidation: biological effects and antioxidant review. Lipids 1980; 15: 485-495.

Lord GP, Gazioglu K, Kaltreider N. The maximum expiratory flow volume in the evaluation of patients with lung disease. Am J Med 1969; 46: 72-79.

Lucy JA. Functional and structural aspects of biological membranes. A suggested structural note for vitamin E in the control of membrane permeability and stability. Ann NY Acad Sci 1972; 203: 4-11.

Ludwig RW, Hoidal JR. Alterations in leukocyte oxidative metabolism in cigarette smokers. Am Rev Respir Dis 1982; 126: 977-980.

Lundberg MS, Livingstone GK. Sister-chromatid exchange frequency in lymphocytes of smoking and non-smoking mothers and their newborn infants. Mutation Res 1983; 121: 241-246.

Machlin LJ, Bendich A. Free radical tissue damage: Protective role of anti-oxidant nutrients. FASEB J 1987; 1: 441-445.

Mahoney CW, Azzi A. Vitamin E inhibits protein kinase C activity. Biochem Biophys Res Comm 1988; 154: 694-697.

Mainardi C, Dixit S, Kang A. Degradation of type IV (basement membrane) collagen by a proteinase isolated from human polymorphonuclear leukocyte granules. J Biol Chem 1980; 255: 5435-5441.

Mainardi CL, Hasty DL, Sayer JM, Kang AH. Specific cleavage of human type III collagen by polymorphonuclear leukocyte elastase. J Biol Chem 1980; 255: 12006-12010.

Majerus PW, Connolly TM, Deckmyn H, Ross TS, Bross TE, Ishii H, Bansal VS, Wilson DB. The metabolism of phosphoinositide-derived messenger molecules. Science 1986; 234: 1519-1526.

Malech HL, Gallin JI. Neutrophils in human defenses. N Engl J Med 1987; 317: 687-694.

Malech HL, Root RK, Gallin JL. Structural analysis of human neutrophil migration: centriole, microtubule and microfilament orientation and function during chemotaxis. J Cell Biol 1977; 75: 666-693.

Mansour H, Brun-Pascaud M, Marquetty C, Gougerot-Pocidalo M, Hakim J, Pocidalo J. Protection from oxygen toxicity by inducers of the cytochrome P450 system. Am Rev Respir Dis 1988; 137: 688-694.

Mantovani A, Dejana E. Cytokines as communication signals between leukocytes and endothelial cells. Immunology Today 1989; 11: 370-375.

Marchesi VT, Florey HW. Electron micrographic observations on the emigration of leucocytes. Q J Exp Physiol 1960: 45: 343-348.

Marklund SL. Human copper-containing superoxide dismutase of high molecular weight. Proc Natl Acad Sci 1982; 79: 7634-7638.

Marrero O, Beck GJ, Schachter EN. Discriminating power of measurement from maximum expiratory flow volume curves. Respiration 1986; 49: 263-273.

Matheson NR, Wong PS, Schyler M, Travis J. Interaction of human α-1 proteinase inhibitor with neutrophil myeloperoxidase. Biochemistry 1981; 20: 331-336.

Mathews-Roth MM, Pathak MA, Fitzpatrick JB, Harber LC, Kass EH. B Carotene as an oral photoprotective agent in erythropoietic protoporphyria JAMA 1974; 228 (8): 1004-1008:

McCarthy DS, Spencer R, Greene R, Millic-Emili J. Measurement of 'closing volume' as a simple and sensitive test for early detection of small airways disease. Am J Med 1972; 52: 747-753.

McCay PB. Vitamin E: interactions with free radicals and ascorbate. Annu Rev Nutr 1985; 5: 323-340.

McDonald RJ, Berger EM, White CW, White JG, Freeman BA, Repine JE. Effect of superoxide dismutase encapsulated in liposomes or conjugated with polyethylene glycol on neutrophil bactericidal activity in vitro and bacterial clearance in vivo. Am Rev Respir Dis 1985; 131: 633-637.

McEver RP, Beckstead JH, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein is also synthesized by vascular endothelial cells and is localized in Weibel-palade bodies. J Clin Invest 1989; 84: 92-99.

McPhail LC, Snyderman R. Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other stimuli. Evidence that the same oxidase is activated by different transductional mechanisms. J Clin Invest 1983; 72: 192-200.

McSharry C, Wilkinson PC. Cigarette smoking and the antibody response to inhaled antigens. Immunol Today 1986; 7: 98.

Minkenberg I, Ferber E. Lucigenin-dependent chemiluminescence as a new assay for NAD(P)H-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. J Immunol Methods 1984; 71: 61-67.

Moldeus P, Cosgreave I, Berggren M. Lung protection by thiol containing antioxidant - N acetyl cysteine. Respiration 1986; 50: 31-43.

Morris JF, Koski A, Breese JD. Normal values and evaluation of forced end expiratory flow. Am Rev Respir Dis 1975; 111: 755-762.

Nakagawara A, Nathan CF, Cohn ZA. Hydrogen peroxide metabolism in human monocytes during differentiation in vitro. J Clin Invest 1981; 68: 1243-1247.

Nathan CF. Neutrophil activation on biological surfaces: massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J Clin Invest 1987; 80: 1550-1560.

Necotera P, Moore M, Bellomo G, Mirabelli F, Orrenius S. Demonstration and partial characterisation of glutathione disulfide-stimulated ATPase activity in the plasma membrane fraction from rat hepatocytes. J Biol Chem 1985; 260: 1999-2002.

Nishikimi M. Oxidation of ascorbic acid with superoxide anion generated with the xanthine-xanthine oxidase system. Biochem Biophys Res Commun 1975; 63: 463-468.

Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 1984; 398: 693-698.

Oppenheim JJ, Kovacs EJ, Matsushima K, Durum DK. There is more than one Interleukin I. Immunol Today 1986; 7: 45-56.

Pacht ER, Kasek H, Mohammed JR, Cromwell DG, David WB. Deficiency of vitamin E in the alveolar fluid of cigarette smokers: influence on alveolar macrophage cytotoxicity. J Clin Invest 1986; 77: 789-796.

Packer JE, Slater TF, Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 1979; 278: 737-738.

Padmanabhan R, Guadapaty Z, Liener I, Schwartz BA, Hoidal J. Protection against pulmonary oxygen toxicity in rats by the intratracehal administration of liposome-encapsulated superoxide dismutase or catalase. Am Rev Respir Dis 1985; 132: 164-167.

Parkos CA, Dinauer MC, Jesaitis AJ, Orkin SH, Curnutte JT. Absence of both the 91 kD and 22 kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease. Blood 1989; 73: 1416-1420

Peppin GJ, Weiss SJ. Activation of the endogenous metalloproteinase, gelatinase by triggered human neutrophils. Proc Natl Acad Sci USA 1986; 83: 4322-4326.

Perry P, Wolff S. New Giemsa method for the differential staining of sister chromatids. Nature 1974; 2351: 156-158.

Plonka A, Metodiewa D. ESR evidence of superoxide radical dismutation by human ceruloplasmin. Biochem Biophys Res Commun 1979; 95: 978-984.

Pober JS, Lapierre LA, Stolpen AH, Brock TA, Springer TA, Fiers W, Bevilacqua MP, Mendrick DL, Gimbrone MA Jr. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. J Immunol 1987; 138: 3319-3324.

Prentki M, Wollheim CG, Lew PD. Ca^{2+} homeostasis in permeabilized human neutrophils: characterization of CA^{2+}-sequestering pools and the action of inositol 1,4,5-triphosphate. J Biol Chem 1984; 259: 13777-13782.

Quaife ML, Scrimshaw NS, Lowry OH. Micromethod for the assay of total tocopherol in blood and serum. J Biol Chem 1949; 180: 7229-7230.

Reidt J, Murthy M, Sicignano A. Tanaka N, Musick W, Rossman M. Structure and heme environment of beef liver catalase at 2,5 A resolution. Proc Natl Acad Sci USA 1987; 78: 4767-4771.

Reilly CJ, Fukunaga Y, Powers JC, Travis J. Effect of neutrophil cathepsin G on elastin degradation by neutrophil elastase. Hoppe-Seyler's Z Physiol Chem 1984; 365: 1131-1135.

Reilly JT, Chapman HA. Association between alveolar macrophage plasminogen activator activity and indices of lung function. Am Rev Respir Dis 1988; 348: 1422-1428.

Ross D, Norbeck K, Moldéus P. The generation and subsequent fate of glutathionyl radicals in biological systems. J Biol Chem 1985; 260: 15028-15032.

Rowbotham B, Roeser HP. Iron-overload associated with congenital pyruvate kinase deficiency and high dose ascorbic acid ingestion. Aust NZ J Med 1984; 14: 667-669.

Sayers TJ, Wiltrout TA, Bull CA, Denn AC III, Pilaro AM, Lokesh B. Effect of cytokines on polymorphonuclear neutrophil infiltration in the mouse. Prostaglandin- and leukotriene-independent induction of infiltration by IL-1 and tumor necrosis factor. J Immunol 1988; 141: 1670-1677.

Schiffman E, Corcoras BA, Wahl SM. N-Formyl-methionyl-peptides as chemoattractant for leukocytes. Proc Natl Acad Sci 1975; 72 (3): 1059-1062.

Schleimer RP, Rutledge BK. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. J Immunol 1986; 136: 649-654.

Schreiber RD. The chemistry and biology of complement receptors. Springer Semin Immunopathol 1984; 7: 221-249.

Scott MD, Meshnick SR, Eaton JW. Superoxide dismutase-rich bacteria: Paradoxical increase in oxidant activity. J Biol Chem 1987; 262; 3640-3645.

Seelentag WK, Mermod JJ, Montesano R, Vassalli P. Additive effects of interleukin 1 and tumour necrosis factor-alpha on the accumulation of the three granulocyte and macrophage colony-stimulating factor mRNAs in human endothelial cells. EMBO J 1987; 6: 2261-2265.

Seely JE, Zuskin E, Bouhuys A. Cigarette smoking: Objective evidence of lung damage in teenagers. Science 1971; 172: 741-743.

Segal AW. Absence of both cytochrome $b-_{245}$ subunits from neutrophils in X-linked chronic granulomatous disease. Nature 1987; 326: 88-91.

Segal AW. The electron transport chain of the microbicidal oxidase of phagocytic cells and its involvement in the molecular pathology of chronic granulomatous disease. J Clin Invest 1989; 83: 1785-1793.

Seifter E, Mendecki J, Holtzman S, Kanefsky JD, Friedenthal E, Davis L, Weinzweig J. Role of Vitamin A and beta-carotene in radiation protection: relation to anti-oxidant properties. Pharmacol Ther 1988; 39: 357-365.

Shah SV, Baricos WH, Basci A. Degradation of human glomerular basement membrane by stimulated neutrophils: Activation of a metalloproteinase(s) by reactive oxygen metarocites. J Clin Invest 1987; 79: 25-31.

Sibille Y, Llwebuga-Mukasa JS, Polomski L, Merrill WW, Ingbar DH,Gee GBL. An in vitro model for polymorphonuclear-leukocyte-induced injury to an extracellular matrix: relative contribution of oxidants and elastase to fibronectin release from amnionic membranes. Am Rev Respir Dis 1986; 134: 134-140.

Sies H. Anti-oxidant activity in cells and organs. Am Rev Respir Dis 1987; 136: 478-480.

Slade R, Stead AG, Graham JA, Hatch GE. A comparison of lung antioxidant levels in humans and laboratory animals. Am Rev Respir Dis 1985; 131: 742-746.

Smith CD, Cox CC, Snyderman R. Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. Science 1986; 232: 97-100.

Smith CW. Molecular determinants of neutrophil adhesion. Am J Respir Cell Mol Biol 1990; 2: 487-489.

Smith JL, Hodges RE. Serum levels of vitamin C in smokers and nonsmokers. Ann NY Acad Sci 1987; 498: 144-152.

Snyderman R, Pike MC. Regulation of leukocyte function. Contemp Top Immunobiol 1984; 14: 1-28.

Spirometric Tables. Bull Eur Physiopathol Respir 1983 19 (Suppl 5): 45-51.

Spragg RG, Hinshaw DB, Hyslop PA, Schraufstatter IU, Cochrane CG. Alterations in adenosine triphosphate and energy charge in cultured endothelial and P388DI cells after oxidant injury. J Clin Invest 1985; 76: 1471-1476.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: Modifications of low density lipoproteins that increase its atherogenicity. N Engl J Med 1989; 320: 915-924.

Stockley RA, Shechter Y, Bonstein Y, Patchornik A. Selective oxidation of methionine residues in proteins. Biochemistry 1975; 14: 4497-4502.

Stone PJ, Calore JD, McGowan SE, Bernardo J, Snider GL, Franzblau C. Functional alpha-1-protease inhibitor in the lower respiratory tract of smokers is not decreased. Science 1983; 221: 1187-1189.

Stossel PT. Phagocytosis. 1st of 3 parts. N Engl J Med 1974; 290: 717-722.

Suttorp N, Toepper W, Roka L. Antioxidant defence mechanisms of endothelial cells: glutathione redox cycle versus catalase. Am J Physiol 1986; 251: 671-680.

Tabachnick BJ, Fidell LS. Using multivariate statistics. New York: Harper and Row, 1983; 372-44.

Tanswell AK, Tzaki MG, Byrne PJ. Hormonal and local factors influence antioxidant enzyme activity of rat fetal lung cells. Exp Lung Res 1986; 11: 49-59.

Taylor JC, Madison R, Kosinska D. Is anti-oxidant deficiency related to chronic obstructive pulmonary disease? Am Rev Respir Dis 1986; 134: 285-289.

Taylor JC, Oey L. Ceruloplasmin: plasma inhibitor of the oxidative inactivation of alphal-1-protease inhibitor. Am Rev Respir Dis 1982; 126: 476-482.

Test ST, Lampert MB, Osanna PJ, Theone JG, Weiss SJ. Generation of nitrogen-chlorine oxidants by human phagocytes. J Clin Invest 1984; 74: 1341-1349.

Test ST, Weiss SJ. The generation and utilization of chlorinated oxidants by human neutrophils. Adv Free Radical Biol Med 1986; 2: 91-116.

Theron A, Anderson R. Investigation of the protective effects of the antioxidants ascorbate, cysteine, and dapsone on the phagocyte-mediated oxidative inactivation of human alpha-1-protease inhibitor in vitro. Am Rev Respir Dis 1985; 132: 1049-1054.

Theron AJ, Anderson R. Investigation of the effects of oral administration of ascorbate on the functional activity of the serum α-1-protease inhibitor and oxidant release by blood phagocytes from cigarette smokers in a placebo-controlled double-blind, crossover trial. Int J Vitam Nutr Res 1988; 58: 218-224.

Totti N, McCusker KT, Campbell EJ, Griffin GL, Senior RM. Nicotine is chemotactic for neutrophils and enhances neutrophil responsiveness to chemotactic peptides. Science 1983; 223: 169-171.

Travis J, Solnesen GS. Human plasma protease inhibitors. Annu Rev Biochem 1983; 52: 655-709.

Truett AP, Verghese MW, Dillon SB, Snyderman R. Leukocyte activation by chemoattractants: A two phase sequential pathway mediates the respiratory burst. Clin Res 1987 (Abstr).

Turrens JF, Crapo JD, Freeman BA. Protection against oxygen toxicity by intravenous injection of liposome-entrapped catalase and superoxide dismutase. J Clin Invest 1984; 73: 87-95.

Ursini F, Maiorino M, Gregoli NC. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochem Biophys Acta 1985; 839: 62-70.

US Department of Health and Human Services. The health consequences of smoking: the changing cigarette. A report of the Surgeon General Public Health Service, Office of the Assistant Secretary for Health, Office on Smoking and Health. Rockville, MD: National Center for Health Statistics (DHHS publication no. [PHS]81-50179). 1982; 200-207.

US Department of Health and Human Services, Public Health Service. The health consequences of smoking - chronic obstructive lung disease - A report of the Surgeon General. Washington DC: US Government Printing Office. (DHEW Publication No (PHS) 84-50205. 1984.

Van Rensburg CEJ, Theron A, Richards GA, van der Merwe CA, Anderson RA. Investigations of the relationships between plasma levels of ascorbate vitamin E and B-carotene and the frequency of sister chromatoid exchanges and release of reactive oxidants by blood leucocytes from cigarette smokers. Mut Res 1989; 215: 167-172.

Verdon CP, Blumberg JB. Influence of dietary vitamin E on the intermembrane transfer of α-tocopherol as mediated by an α-tocopherol binding protein. J Nutr 1988; 189: 52-60.

Volpp BD, Nauseef WM, Clark RA. Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. Science 1988; 242: 1295-1297.

Vuilleumier JP, Keller HE, Gysel D, Hunzikar F. Clinical chemical methods for the routine assessment of the vitamin status in human populations. Part I: the fat soluble vitamins A and E and beta-carotene. Int J Vitam Nutr Res 1982; 53: 265-272.

Walter S, Nancy NR, Collier CR. Changes in the forced expiratory spirogram in young smokers. Am Rev Respir Dis 1979; 119: 717-724.

Walz A, Peveri P, Aschauer H, Thelen M, Kernan P, Dewald B, Barriolin M. Structure and properties of a novel neutrophil activating factor (NAF) produced by human monocytes. Agents and Actions 1989; 26: 148-155.

Wasil M, Halliwell B, Hutchison DC, Baum H. The anti-oxidant action of human extracellular fluids: effects of human serum and its protein components on the inactivation of alpha-one anti-protease by hypochlorous acid and hydrogen peroxide. Biochem J 1987; 243: 219-223.

Wayner DDM, Burton GW, Ingold KW, Barclay LRC, Locke SJ. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochem Biophys Acta 1987; 924: 408-419.

Weisbart RH, Golde DW, Gasson JC. Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f-met-leu-phe receptors. J Immunol 1986; 137: 3584-3587.

Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989; 320 (6): 365-376.

Weiss SJ, Curnette TT, Regiari S. Neutrophil mediated solubilization of the subendothelial matrix; oxidative and non oxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. J Immunol 1986; 136: 636-641.

Weiss SJ, Lampert MB, Test ST. Long lined oxidants generated by human neutrophils. Characterization and bio-activity. Science 1983; 222: 625-628.

Weiss SJ, Peppin GJ. Collagenolytic metallo-enzymes of the human neutrophil. Biochem Pharmacol 1986; 35 (19): 3189-3197.

Weiss SJ, Peppin G, Ortiz X, Ragsdale C, Test ST. Oxidative autoactivation of latent collagenase by human neutrophils Science 1985; 227: 747-749.

Weiss SJ, Regiari S. Neutrophils degrade subendothelial matrices in the presence of alpha-one proteinase inhibition: cooperative use of lysozymal proteinases and oxygen metabolites. J Clin Invest 1984; 73: 1297-1303.

Weiss SJ, Speizer FE. The Surgeon General's report on the health consequences of voluntary smoking. A commentary. Am Rev Respir Dis 1987; 1225-1226.

Weitberg AB, Weitzman SA, Destrempes M, Latt SA, Stossel TP. Stimulated human phagocytes produce cytogenic changes in cultured mammalian cells. N Engl J Med 1983; 308: 26-30.

Weitberg AG, Weitzman SA, Clark EP, Stossel TP. Effects of antioxidants on oxidant-induced sister chromatid exchange formation. J Clin Invest 1985; 75: 1835-1841.

Weitzman SA, Stossel TP, Mutation caused by human phagocytes. Science 1981; 212: 546-547.

Weitzman SA, Stossel TP. Effects of oxygen radical scavengers and antioxidants on phagocyte-induced mutagenesis. J Immunol 1982; 128: 2770-2772.

Weitzman SA, Weitberg AB, Clark EP, Stossel TP. Phagocytes as carcinogens: malignant transformation produced by human neutrophils. Science 1985; 227: 1231-1233.

White CW, Jackson J, Freeman B, Mimmack R, Abuchowski A, Davis FF, Kazo G, McCord J, Repine J. Intravenous polyethylene glycol conjugated superoxide dismutase and catalase prolongs survival in rats exposed to hyperoxia. Am Rev Respir Dis 1989; 129: 349-354.

White CW, Repine JE. Pulmonary anti-oxidant defense mechanisms. Experimental Lung Research 1985; 8: 81-96.

White JR, Huang C-K, Hill JM Jr, Naccache PH, Becker EL, Sha'afi RI. Effect of phorbol 12-myristate 13-acetate and its analogue 4α-phorbol 12,13-didecanoate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils. J Biol Chem 1984; 259: 8605-8611.

White RE, Coon MJ. Oxygen activation by cytochrome P450. Annu Rev Biochem 1980; 49: 315-356.

Willet WC. Selenium and cancer: Br Med J 1988; 297: 573-574.

Williams LT, Snyderman R, Pike MC, Lefkowitz RJ. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc Natl Acad Sci USA 1977; 74: 1204-1208.

Woolcock AJ, Vincent NJ, Macklem PT. Frequency dependence of compliance as a test for obstruction in the small airways. J Clin Invest 1969; 48: 1907-1106.

Yoshikawa T, Furokawa Y, Morakami M, Watanabe K, Kondo M. Effect of vitamin E on endotoxin-induced disseminated intravascular coagulation in rats. Thromb Haemost 1982; 48: 235-237.

Yusa T, Crapo JD, Freeman BA. Lipsome-mediated augmentation of brain SOD and catalase inhibits CNS O_2 toxicity. J Appl Physiol 1984; 57: 1674-1681.

Zakhireh B, Malech HL. The effect of colchicine and vinblastine on the chemotactic response of human monocytes. J Immunol 1980; 125: 2143-2153.

Zakhireh B, Root RK. Development of oxidase activity by human bne marrow granulocytes. Blood 1979; 54: 429-439.

Zaslow MC, Clark RA, Stone PJ, Calore J, Snider GL, Franzblau C. Myeloperoxidase-induced inactivation of α1-anti proteinase in hamsters. J Lab Clin Med 1985; 105: 178-184.

Zigmond SH. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. J Cell Biol 1977; 75: 606-616.

Zigmond SH, Levitsky HI, Kreel BJ. Cell polarity: an examination of its behavioral expression and its consequences for polymorphonuclear leukocyte chemotaxis. J Cell Biol 1981; 89: 585-592.