Involvement of membrane sulphydryl groups in phagocyte activation

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INVOLVEMENT OF MEMBRANE SULPHYDRYL GROUPS IN PHAGOCYTE ACTIVATION.

Submitted by

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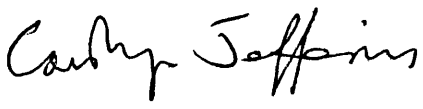

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"The Fabulous Maze was wove about with mysteries exciting;
The more you looked the more you saw enticing and inviting."

"The Fabulous Maze" by C. Mail.

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Contents

Abbreviations.

Aur	auranofin
cAMP	cyclic adenosine monophosphate
cyt B	cytochalasin B
DAG	diaycyl glycerol
DMSO	dimethylsulphoxide
Dpen	D-penicillamine
FMLP	formyl-methionyl-leucyl-phenylalanine
HAGG	heat-aggregated immunoglobulin G
IP_3	inositol trisphosphate
IAP	Islet Activating Protein from B. pertussis
LTB_4	leukotriene B_4
MNC	mononuclear cells
NADPH	reduced nicotinamide adenine dinucleotide
NSAID	non-steroidal antiinflammatory drugs
O_2^-	superoxide
PBS	phosphate buffered saline
PBSG	phosphate buffered saline with glucose
PHMPSA	parahydroxymercuriphenylsulphonic acid
PKC	protein kinase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocytes (neutrophils)
Psor Arth	psoriatic arthritis
RA	rheumatoid arthritis
SH	sulphydryl group (thiol)

SUMMARY

The project investigated any role of sulphydryl groups in the biochemical pathways which contribute to normal phagocyte function and which may be a point for therapeutic control of tissue damage in rheumatoid arthritis (RA). Neutrophils (PMN) and monocytes (MNC) were compared, and with each cell type comparison was made between the following groups: normal volunteers; patients with rheumatoid arthritis (RA) on non-steroidal antiinflammatory drugs (NSAID), on D-penicillamine (Dpen) or auranofin (aur), and patients with psoriatic arthritis (psor arth).

The choice of stimuli was designed to follow activation pathways stimulated at cell surface receptors, using FMLP and IgG, at a cytosolic receptor site, protein kinase C using PMA, and at a point between the two, namely G-proteins, using fluoride. The involvement of sulphydryl groups (SH) was investigated by external blockade with parahydroxymercuriphenylsulphonic acid (PHMPSA).

The effect of PHMPSA was examined in assays for the release of superoxide (O_2^-), hydrogen peroxide (H_2O_2), myeloperoxidase and for the elevation of internal calcium. Also, G-protein involvement in the pathways leading to O_2^- production was investigated using the islet-activating protein of Bordetella pertussis (IAP).

PMN and MNC from patients on second line therapy showed a significantly enhanced spontaneous peroxide response in the presence of exogenous calcium but not in its absence.

All groups showed an inhibition of both the peroxide and O_2^- responses to FMLP and heat aggregated IgG (HAGG) after SH blockade; the MNC peroxide response to PMA was inhibited, and the PMN O_2^- response was enhanced. PHMPSA inhibited the fluoride O_2^- response of MNC from all groups, but the PMN response was significantly enhanced in the patient groups but in the normal group in cells from male donors only.

PHMPSA inhibited basal and stimulated levels of internal calcium release from normal PMN but enhanced myeloperoxidase release.

IAP inhibited the FMLP O_2^- response in PMN and MNC from all groups. It had no effect on the PMA responses. Fluoride O_2^- response was inhibited in both PMN and MNC from the normal group and in MNC from the D-pen group.

The modulatory effects of PHMPSA demonstrated that there are surface thiols involved at the point of receptor-G-protein interaction in pathways to NADPH oxidase activation, which do not appear to be modulated by the inflammatory disease process in rheumatoid arthritis, and which may be different or differentially accessible in PMN and MNC.

IAP inhibited the fluoride response of normal PMN and MNC, and of MNC from the Dpen group but not those of the NSAID group. It did not affect the response of PMN from the patient groups.

Chapter One

INTRODUCTION

This introduction has been designed to give a broad picture of phagocytes and of RA, and to give a more detailed description of the elements of the phagocyte cell concerned with activation which have been used in experiments, and to indicate where SH groups may be important. Production of O_2^- and H_2O_2 have been used as the main model functions for investigating the role of SH in cell activation. Phagocytes were used for investigation for a number of reasons: they are easily obtained in sufficient quantity from peripheral blood; they produce reactive oxygen metabolites which can be measured unambiguously by simple biochemical assay; there is literature to document the receptors known to activate H_2O_2 and O_2^- production; they persist at sites of damage in RA and they form part of the immune system which may be defective in RA.

PMN were purified from other leukocytes to >95%. Experiments investigating azurophilic enzyme release were restricted to PMN only. Monocytes were not usually separated from lymphocytes mainly to minimalize time-induced deterioration of PMN and to reduce the loss of cells through extra manipulations. Monocytes were considered to be the overriding contributers to the H_2O_2 and O_2^- production in the mixed cell preparation. Thus, MNC refers to the mixed cell population whilst assuming that the monocyte population alone has contributed significantly to the result. Some preliminary experiments investigating internal calcium release were undertaken using purified monocytes.

The mobilization of internal calcium was monitored in PMN as was myeloperoxidase release to see if these pathways were altered by SH blockade in a manner which paralleled the effect on H_2O_2 and O_2^- production. Monocytes did not produce enough MPO to make the assay practical for them with the limitations dictated by the amount of blood obtainable. Similarly the calcium mobilization assay was mainly restricted to PMN both for reasons of

cell number and because in a MNC preparation lymphocytes would also contribute to the estimation of total calcium which is assessed by cell lysis.

In this project immediate use of PMN and MNC assumed that each cell type had been subjected to minimal activation, and that the MNC monocyte population was immature in terms of macrophage characteristics. The similarities in PMN and MNC functions were exploited and the implications of the results discussed in the light also of their differences.

A. SIGNAL TRANSDUCTION PATHWAYS.

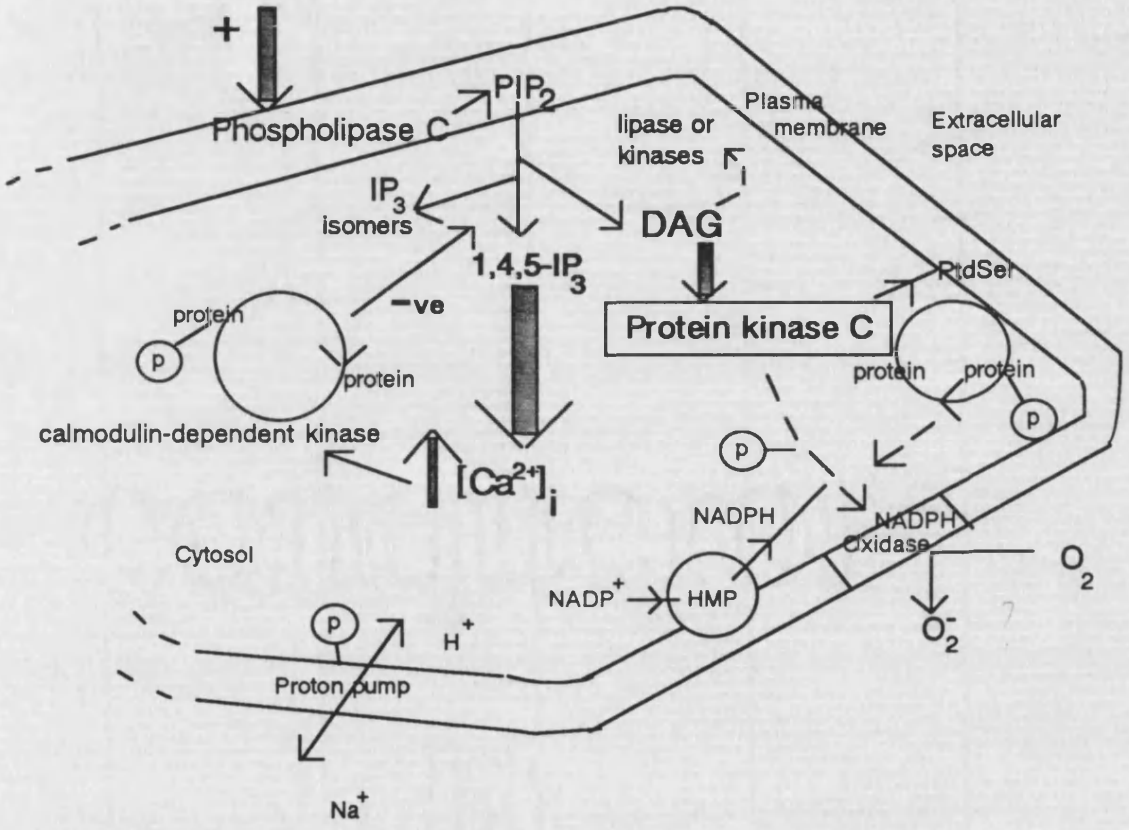
Cells can communicate with their surroundings including other cells by contact with specific receptors borne in their plasma membranes, and which are exposed at the external surface. On ligand binding, information is relayed to the inside of the cell in a manner dependent on the receptor and on the cell type. Understanding these signal transduction pathways may enable them to be used as sites for therapeutic modulation, or lead to a clearer picture of drug interactions which cause unwanted side-effects for instance and so allow better targetting.

Pathways involved in the control of granule release or of O_2^- production from phagocytes may be interrelated and each may have multifaceted control sites for the production of second messengers and other molecules (such as inflammatory mediators). Pathways include those for cyclic AMP production, for protein kinase C activation, for calcium release and for production of arachidonic acid metabolites. Diagram A gives an outline of separate PMN transduction pathways, outlined in isolation for clarity.

Diagram A.

Signal Transduction Pathways.

Ai. Phosphatidylinositolbisphosphate Phospholipase C. (The PIP_2 -Specific PLC)

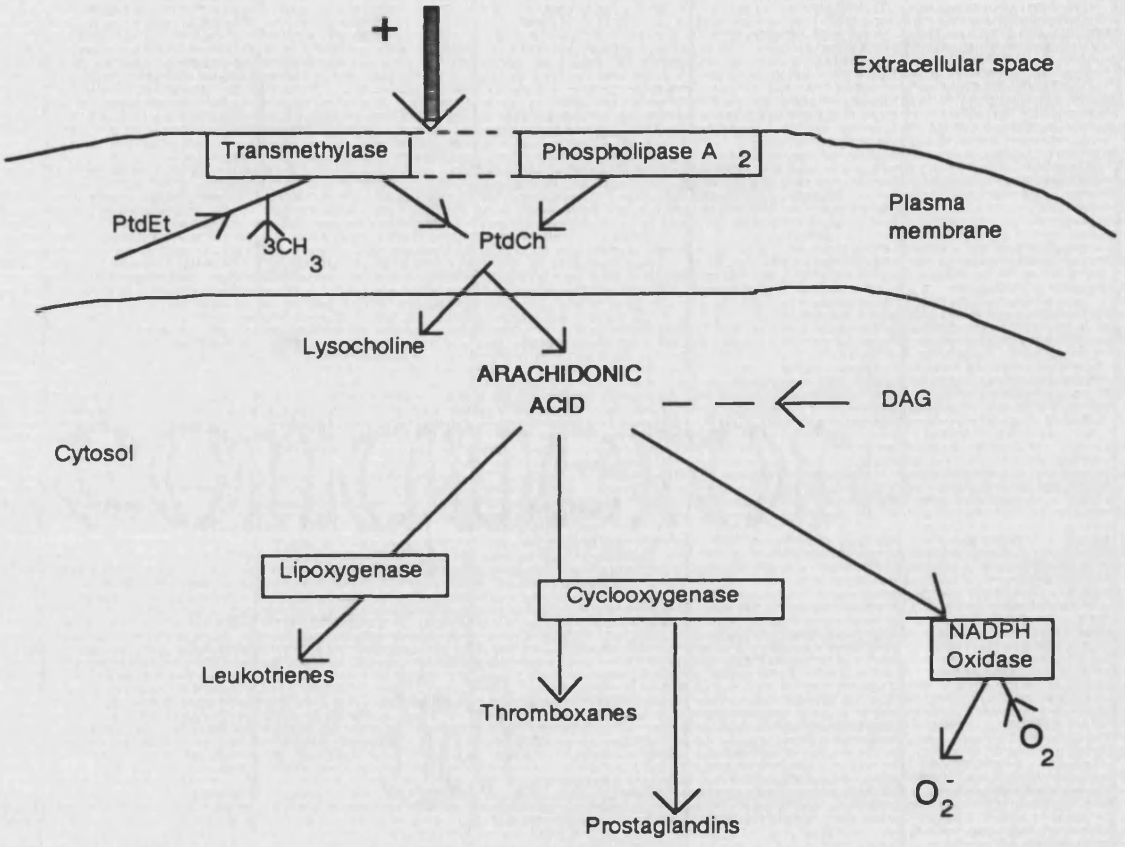


Key:

(P)	= phosphorylation
DAG	= diacylglycerol
IP_3	= inositol trisphosphate
HMP	= hexose monophosphate shunt

Aii. Phospholipase A_2

(PLA_2)



Refinements in control may include the interaction of different stimuli such as may be found in heterologous priming of responses such as those of TNF-alpha and PMA (Berkow et al 1987) or GM-CSF and FMLP (Corey and Rosoff 1989); a dual or biphasic dose response to an external ligand, such as the regulation of PAF receptor expression by PKC activation (O'Flaherty et al 1987), differential affinity states of a receptor depending on the state of the cell (Fletcher and Gallin 1980); subtypes of G-proteins, which control the positive or negative nature of the signal and which may interact with more than one receptor or effector mechanism (Burch et al 1986); isozymic effector mechanisms such as protein kinase C (Nishizuka 1988) and compartmentalized intracellular substrate availability for second messenger production. These elements in control may be cell type specific, such as protein kinase C isozymes (Nishizuka 1984;1986;1988) and phospholipases C which are both cell type- and species-specific and may be altered by cell activation or differentiation (Homma et al 1989).

Activity of adenylate cyclase and of PLC or PLA_2 are usually considered to be mutually exclusive events, adenylate cyclase activity requiring higher calcium levels which also activate calmodulin-specific protein kinases, the cAMP and calmodulin-dependent phosphorylating events forming down-regulation for those induced by an initial signal via one of the other two pathways. However all three pathways may be interrelated in a single stimulation event, Fc receptor-mediated stimulation involving both PLC and PLA_2 for instance (Sakata et al 1987) and FMLP activating adenylate cyclase as well (Bokoch and Gilman 1984).

A picture of the mechanisms activated by the receptors used in this project is necessary before the receptors (namely those for FMLP and the Fc receptors for IgG) can be described with clarity, and so the next two sections introduce effector enzymes and their modulation.

i. Adenylate cyclase.

Widespread finding of dual G-protein-mediated regulation of adenylate cyclase by hormonal and adrenergic stimuli in different cell types has made this a much studied effector mechanism (Ross and Gilman 1980) which will be outlined in the section on G proteins. It is regulated intracellularly by phosphorylation (Katada et al 1985) and by calcium concentrations (Lim et al 1983) and may regulate other phagocyte signal transduction pathways. Cyclic AMP is consistently reported to be transiently elevated in FMLP stimulation of PMN (Simchowitz et al 1980), an event to which no definite function has yet been ascribed. The relative speeds at which FMLP products arise suggest that cAMP is necessary for an early event. Its elevation is independent of O_2^- production and degranulation (Simchowitz et al 1983). It may be related to the need for exogenous calcium for maximal effect of FMLP stimulation and is independent of inhibition of Gi-like proteins by pertussis toxin (IAP) (Okajima and Ui 1984). However the increase in internal calcium is IAP inhibited (Verghese et al 1986). Lipocortin inactivation is dependent on phosphorylation by a cAMP-dependent protein kinase (Hirata et al 1981), and so cAMP may be required for activation of PLA_2. Adenylate cyclase has not been found to be activated by Fc receptor-mediated events.

ii. Phospholipase A_2

In PMN plasma membranes PLA_2 is a neutral, calmodulin-dependent, phosphatidylcholine-specific enzyme which cleaves arachidonic acid in particular from the sn-2 ester bond, phosphatidylcholine (PtCh) being supplied by transmethylation of phosphatidylethanolamine (PtEth) (Hirata 1979). Its active site has structural similarities with PKC (Maragore 1987). The PLA_2 inactivator lipocortin is, in turn, inactivated by phosphorylation by a tyrosine kinase, as seen following FMLP activation (Hirata 1981), although it may also

be phosphorylated by seryl-threonyl-specific kinases, and thereby its regulation may depend on the stimulus (Pepinsky and Sinclair 1986). PLA_2 activity can be stimulated for instance by FMLP receptor occupancy (Duque et al 1986) and by stimulation of complement receptors with opsonized zymosan (Maridonneau-Parini et al 1986). The products of this activity are arachidonic acid and lysocholine, both of which may be further metabolised or may be active in their own rights as second messengers. The important products of arachidonic acid metabolism are those of the cyclooxygenase (prostaglandins) and lipoxygenase (leukotrienes) pathways, which may be regulated by the interaction of protein kinase C or its products (McColl et al 1987). Lysocholine has no known second messenger activity as such, but may be further metabolised to DAG, and able to activate PKC. It may also be metabolized to platelet activating factor (PAF), which is a powerful chemotaxin for phagocytes and which is released by monocytes (Elstad et al 1988). PtEth is a normal cytoplasmically oriented constituent of the plasma membrane and PtCh resides in the outer side and is not available to membrane PLA_2. Thus before PLA_2 activity occurs transmethylation takes place. A second, acidic PLA_2 resides in azurophil granules, being released to the extracelluar space on degranulation (Balsinde et al 1988), an event which would promote local acidity. Control of this enzyme and its substrate specificity may be relevant to PLA_2 activity in disease and its modification by antimalarial drug therapy (Hurst et al 1986), especially as it has been found to show a substrate preference for arachidonic acid - bearing phospholipids in RA synovial fluid compared with OA synovial fluid (Loeser et al 1988).

Activation of (plasma membrane) PLA_2 by FMLP may be indirect through PLC (Matsumoto et al 1988). Substrate availability may be altered by peroxidative damage (van Kuijk et al 1987) which, in turn, could result in altered substrate availability to the lipoxygenase and cyclooxygenase pathways. PLA_2 has been

suggested to play a preventative role in peroxidative damage (van Kuijk et al 1987) which may divert it from its normal activity. Again in abnormal conditions, arachidonic acid may be released into the external environment where it may activate phagocytes directly (Naccache et al 1989). Arachidonic acid induces an initial rise in internal calcium similar to that seen with FMLP, but it also induces a slower more sustained rise. PLA_2 activity is also governed by extracellular viscosity, which may be lowered in disease states, promoting activity (Yedgar 1987). One would expect lowered extracellular viscosity to increase membrane fluidity and this increases transmethylation (Lenaz et al 1983) and thereby PLA_2 substrate availability. However, it may also alter substrate specificity (Gonzalez-Buritica et al 1989). An alteration in activity in this manner is unlikely to be restricted to only one activation system.

iii. Activation of protein kinase C - The PIP_2 Pathway.

Stimulation of G-protein-mediated activation of a phosphatidylinostol bisphosphate-specific phosphodiesterase, PLC, causes the hydrolysis of PIP_2 to diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG activates protein kinase C by lowering its calcium requirement to basal levels (Wolf et al 1985). Pathways which involve activation of protein kinase C can be investigated by its direct stimulation with phorbol myristate acetate (PMA) (Nishihara et al 1985) a non-metabolizable analogue of DAG; thus an activation process can be stimulated at a site distal to plasma membrane constituents thought to be involved in the physiological activation of PKC such as receptors and G-proteins, and effector enzymes such as PLA_2 or PLC. When investigating pathways to O_2^- production, if blockade does not inhibit the response to PMA it can be deduced that no blockade occurs from PKC activation onwards and that PKC is involved in the activation pathway. PKC is a tissue-specific family of kinases which are differentially activated by PMA and inactivated by calpain and which have different requirements for calcium (Nishizuka 1988). They have

variable regions in their structure which may indicate different substrate specificities. In phagocytes they do not need internal calcium above basal levels for activation by DAG but do require translocation to phosphatidylserine in the plasma membrane where the catalytic fragment is cleaved by calpain and released into the cytosol (Nishizuka 1988). A tissue has yet to be found which contains only one subtype of PKC (Carpenter et al 1987).

PMA is a potent stimulus of the respiratory burst (Ohta et al 1985) and of the release of specific but not azurophilic granules (Wright et al 1977). The phosphorylation profile of cells is altered by stimuli which activate NADPH oxidase, including PMA (Andrews and Babior 1983), and the pattern of this includes PKC phosphorylation of the alpha subunit of G-proteins (Matsumoto et al 1986), of inositol phospholipids (Cockroft et al 1985), of the proton pump (Moolenaar et al 1984), and of a subunit of the NADPH oxidase complex itself (Gennaro et al 1985). (Other substrates for PKC are tabulated in Nishisuka 1986). The exact function of all the phosphorylation events is unknown but in the case of the G-protein alpha subunit, phosphorylation may increase the affinity of the subunit for the other subunits and promote GTPase activity, forming a negative feedback signal as phosphorylation only occurs of the dissociated (that is, activated) alpha subunit (Matsumoto et al 1986). Phosphorylation is known to be a negative feedback mechanism for beta-adrenergic receptors so that they no longer interact with their G-proteins (Sibley et al 1987). PKC activation has been suggested to be a negative feedback mechanism for both PLC (Muldoon et al 1987), and for PLA_2 (Fuse and Tai 1987) although inhibition of PLA_2 has been found to inhibit PMA-induced O_2^- production (Duque et al 1986), and PMA stimulates release of labelled arachidonic acid from methylated phospholipids (Hirata 1979). As arachidonic acid itself can activate the release of O_2^-, the PLA_2 connection may be a feed forward event. PKC is an integral part of O_2^- production by at least one

pathway, but it is difficult to determine precisely how, probably because it is involved in a number of interrelated events which may regulate or occur at the same time as the process. Inhibitors of PKC such as H7 (Kawamoto et al 1984) are also used to delineate its effects, and a natural inhibitor of PKC has been found to be associated with specific granule membranes on their association with the plasma membrane (Balazovich et al 1986).

Physiological production of DAG involves concomitant release of inositol trisphosphates (IP3), of which mainly 1,4,5-IP3 releases calcium from intracellular stores (Spat et al 1986; Berridge and Irvine 1989). The use of PMA has established that calcium need not be elevated above basal levels for O_2^- production. Use of a PKC-blocking agent has also established that PKC activation is not essential for O_2^- production by FMLP or C5a for instance (Gerard et al 1986) and that there are therefore at least two pathways to this end. PKC activation occurs in FMLP stimulation (Ohta et al 1985) and FcR-mediated stimulation (Sakata et al 1987) and may function to regulate the metabolic products of arachidonic acid release, causing the preferential synthesis of lipoxygenase products by PMN (McColl et al 1987). PKC activation causes a drastic change in PMN morphology and this may be a function of the calcium- and pH-independent promotion of actin assembly (Sheterline et al 1986). PKC activation of actin assembly can be induced independently of its kinase activity by an otherwise inactive analogue of PMA (White et al 1984). Others have found actin assembly also with FMLP stimulation, but to no significant degree with phorbol esters (Bengtsson et al 1986).

iv.Calcium.

Activation of PKC by arachidonic acid is dependent on calcium but not on phosphatidylserine (McPhail et al 1984), suggesting that calcium may be necessary for accessibility of membrane components to each other, perhaps by

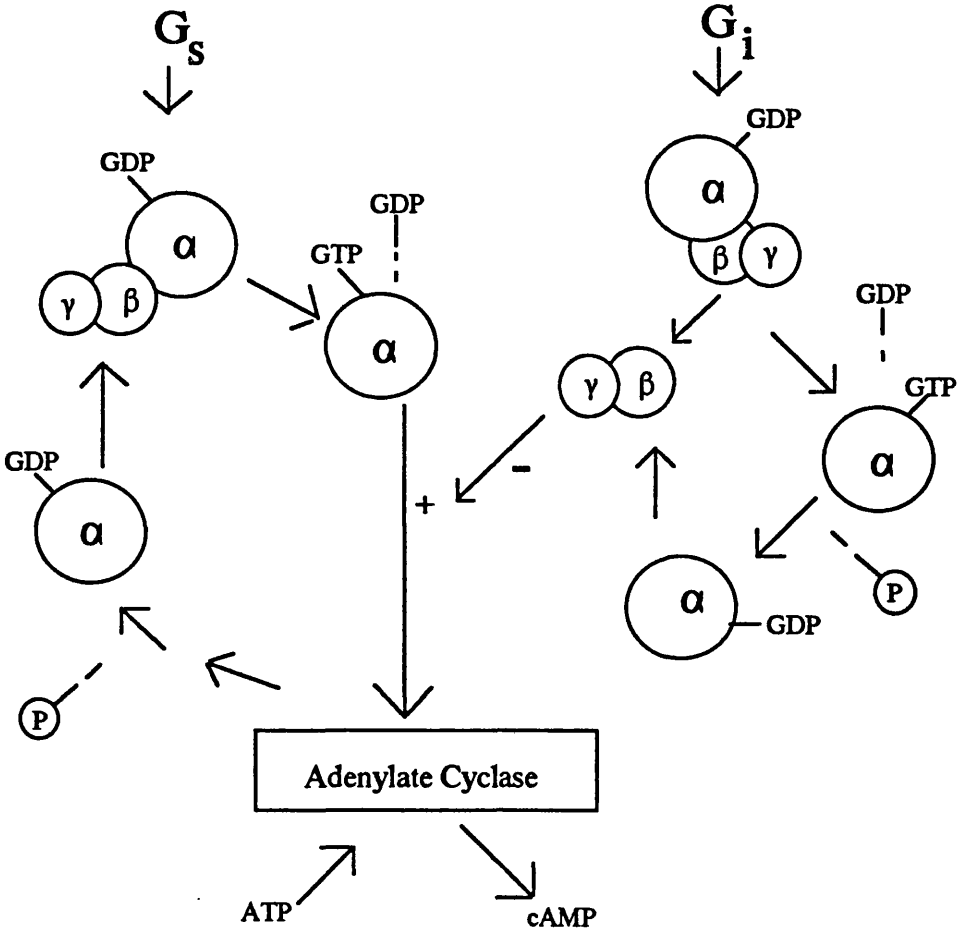
modifying the local charge distribution. The calcium ionophore A23187 induces low levels of O_2^- from PMN (Simchowitz 1983; Ohta et al 1985), arachidonic acid release and degranulation (Bokoch and Gilman 1984) and production of LTB_4 (Sellmayer et al 1987). A23187 does not mobilize PIP_2 but does induce an IAP-insensitive accumulation of inositol phosphates by an unidentified route (Ohta et al 1985) and acts synergistically with low concentrations of a diacylglycerol analogue, OAG, to promote a O_2^- response from PMN at doses at which neither stimulus alone would be active (Penfield and Dale 1984). Alone, A23187 activates PLA_2 to release arachidonic acid metabolites as can be seen using the PLA_2 inhibitor mepacrine (Bokoch and Gilman 1984), which suggests that FMLP-stimulated cells require activation of PLA_2 by exogenous calcium for full activity. External calcium is necessary for maximal O_2^- release by FMLP but in the presence of EGTA FMLP still stimulates PLC but the nature of the inositol phosphate isomers differs from those found in the presence of calcium (Dillon et al 1987). As the concomitant release of DAG activates PKC, the isomeric nature of the inositol phosphates may be concerned more with pathways other than O_2^- activation, such as degranulation. This could also be indicated by the fact that the O_2^- release is greater with calcium, FMLP receptors being upregulated by degranulation. Hexadecyl methylglycerol is a PKC inhibitor which prevents O_2^- release by FMLP without affecting IP_3 release or calcium mobilization (Kramer et al 1989), further reinforcing the view that calcium mobilization is not an activator of O_2^- release, either directly or by activation of another pathway independent of PKC activation. More evidence is supplied by the enhanced phosphorylating rate and activity seen in response to PMA in the presence of calcium (White 1984). The roles of calcium differ depending on the pathway concerned, and where activation appears to be of more than one by a single stimulus such as FMLP, with no clear picture of the order of events, identification of these is complex and isolation may be impossible.

v. G-Proteins.

An important family of GTP-binding proteins (G-proteins) mediate many receptor-activated pathways such as those involving adenylate cyclase (Sternweis and Gilman 1978), guanylate cyclase (Manning and Gilman 1983), phosphatidylinositol metabolism or PLA_2 activation (Fuse and Tai 1987), all of which control the release of second messengers within the cell. G-proteins are best explained taking adenylate cyclase regulation as an example (and can be seen diagrammatically in Diagram B). The role of G-proteins in activation by the specific stimuli used in this project will be described in appropriate sections.

DIAGRAM B

Adenylate Cyclase Regulation by G-Proteins.



key:

α
β
γ } G-protein subunits

p = phosphate group

G_s =Cholera toxin-inhibitable G interaction (+ stimuli)

G_i =Pertussis toxin-inhibitable G interaction (- stimuli)

cAMP = cyclic adenosinemonophosphate

GDP = guanosinediphosphate

GTP = gaunosinetriphosphate

Adenylate cyclase, as reviewed by Ross and Gilman (1980) and by Gilman (1987), is an almost ubiquitous enzyme in eukaryotes, which catalyses the formation of cyclic AMP, an important second messenger. Generally, binding of a specific ligand to its receptor promotes the exchange of GDP for GTP on a G-protein subunit, and this regulates adenylate cyclase activity. Whether this ligand-binding stimulates or represses adenylate cyclase activity depends on the receptor and on the G-protein it modifies, there being both stimulatory and inhibitory receptors which activate different G-proteins.

Detailed analysis of G-proteins has revealed a similar trimeric structure in both types which regulate adenylate cyclase, the most useful physiological difference being that the stimulatory type, or G_s, is ADP-ribosylated by Cholera spp. toxin (Schleifer et al 1982) and the inhibitory type, or G_i, is ADP-ribosylated by the islet-activating protein (IAP) of Bordetella pertussis (Bokoch et al 1983). The G_s and G_i nomenclature has been retained when referring to G-proteins identified elsewhere, and has been supplemented by other "G" subscripts for systems in which the two toxins are not active, such as G_p for a placental G-protein (Evans et al 1986) and G_o for that from bovine brain (Higashijima et al 1987). In brief, the toxins irreversibly ADP-ribosylate the G-alpha subunits which causes them to dissociate from their beta-gamma complexes, and renders them impervious to receptor-mediated control. In the case of adenylate cyclase regulation either toxin results in permanent activation of the enzyme. It is worth stressing that G_s and G_i G-proteins in systems other than adenylate cyclase are identified by their ADP-ribosylation whilst not presuming their mode of action; whereas this nomenclature is now generally accepted, other subscripts may vary depending on the reference.

The G-protein heterotrimer consists of three subunits, alpha, beta and gamma. The alpha and gamma subunits are each a single peptide but the beta subunit consists of two peptides. The approximate molecular weights are 41KD($alpha_i$) or 45KD($alpha_s$) (Bokoch et al 1983), 35KD and 36KD(beta) (Northup et al 1983), and 8KD(gamma) (Kuhn 1980). Ligand-activated receptor associates with the alpha subunit and promotes the exchange of bound GDP for GTP; this has been suggested to be by a phosphoenzyme transferring a phosphate to GDP (Ohtsuki 1987). The presence of magnesium supports this activation step, being found particularly as a cofactor for phosphate transfer reactions in many systems (Macdonald and Martin 1988). Association of all three G-subunits is necessary for efficient receptor-catalyzed nucleotide exchange (Gilman 1987). In membrane preparations this is also dependent on the presence of magnesium ions. This exchange lowers the affinity of the receptor for its ligand and also causes dissociation of the G-protein alpha subunit from the beta-gamma complex. The alpha subunit has intrinsic GTPase activity which slowly catalyses the GTP to GDP conversion, increasing the affinity of the alpha subunit for the beta-gamma complex and they reassociate. The GTPase reaction is a slow one, which is preceded by the regulation of adenylate cyclase by the GTP-alpha subunit. The association of the GTP-alpha subunit with the cyclase in its turn promotes the GTPase activity. In purified G_0 preparations from bovine brain, GTP binding causes a conformational change enhanced by the presence of magnesium ions, and which declines slowly as GTP hydrolyses to GDP (Higashijima et al 1987). The slow hydrolysis allows a build-up of active alpha subunits whilst the receptor is occupied, amplifying the single signal.

In a second mode of regulation which could be the pathway of inhibitory modulation of adenylate cyclase, rather than direct action of the alpha subunit on the adenylate cyclase, subunit dissociation could allow an excess of beta-gamma complexes to "mop up" alpha subunits, thereby preventing activation. It has not

been proved conclusively what the regulatory mechanism is in the case of inhibition of adenylate cyclase. The possible deactivation by the beta-gamma complex has not been ruled out and it has been suggested as the active participant for other G-protein systems, such as in the regulation of potassium gating (Logothetis et al 1987). In a short review, Bourne (1989) notes that PLA_2 figures largely in evidence for the beta-gamma complex in positive effector activation, and that yeasts found to be genetically deficient in the G-alpha genes demonstrate a complete reversal of the conventional G-protein model by the beta-gamma mediated activation of their pheromone response lacking negative control. This indicates a broad diversity in G-protein mediation, as yet only partly explored.

Human PMN contain G-proteins which are substrates for both cholera toxin and for IAP (Gierschik et al 1986); by using monoclonal antibodies, two different substrates for the latter have been identified. cDNA analysis identified two G_i-alpha subunit subtypes (Didsbury and Snyderman 1987); Suki and workers (1987) found three nonallelic genes by this method for subtypes of G_i-alpha subunits, with little difference between species of mammal, with expression possibly being cell type-specific. It was suggested that the different subtypes could be used to identify the effector enzyme they modulate in vivo, type 1, (41KD), activating PIP_2-specific phospholipase C, for instance. However, it has also been found that the different G_i-alpha subtypes can all activate potassium channels (Yatani et al 1988). Mattera et al (1989) identified three splice variants of the adenylate cyclase-regulating G_s-alpha. In this work it was demonstrated that three of their splice variants (produced by expressing cDNA in E. coli) could each activate both adenylate cyclase and calcium channels in cell-free systems and in membrane vesicles. It may be that if the splice variants exhibit no specificity in the effector activated, that the variability is concerned with the specificity of receptor-G-protein interaction. Beta-

subunits are expressed more uniformly than the alpha-subunits, but the two peptide chains are coded for by different genes (Fong et al 1986), those from different systems not only being functionally interchangeable between G-protein systems in vitro, but being in fact identical on protein analysis; this has been found for the beta-subunits of the bovine brain G-protein G_o and those of retinal transducin (Pines et al 1985). However, in a reconstituted system, G_s alpha discriminated between beta/gamma subunits from transducin and from bovine brain G_o but G_i-alpha did not (Hekman et al 1987). Diversity of G-protein subunits has been found to be due to different genes (Suki et al 1987), and to alternative splicing of mRNA (Northup et al 1983) and it may also be due to post-translational modification.

It could be important to see how expression of G-subunit genes varies with development and with cellular differentiation. It is reasonable to presume that this could relate to oncogenesis, in which cells are seen to revert to a less-differentiated phenotype and to have aberrant control mechanisms. The G-protein-like ras genes are commonly expressed in an constitutive fashion in tumour cells, changes have been found in G-proteins expressed in the 3T3-LI cell-line as it differentiates for instance (Gierschik et al 1986), and similarly in rat brain (Milligan et al 1987), and in a regionally-specific manner in the chicken heart (Luetje et al 1987).

The G-protein regulation of activation pathways involves specific receptors, one or more G-proteins with (tissue-specific) subunit subtypes, and a number of effector enzymes or gating mechanisms. So far, investigations have not determined whether binding of a single specific ligand activates only one G-protein subtype, nor whether the G-protein/s once activated normally regulate one or more effector mechanisms at the same time in whole cells - if they do not, then the interchangeable action of subunits from various sources in in vitro work suggests that spatial organization within the membrane must be very well

defined. The main focus of work has been on the receptor-alpha subunit interaction; the beta and more especially the gamma subunits have not been investigated in so much detail.

Two proteins considered to be related to the G-protein family are transducin, which is found abundantly in retinal rod outer segments and which mediates between light-activated rhodopsin and guanylate cyclase (Yamazaki et al 1987a;b), and the p21 ras oncogene products which are small molecular weight G-alpha-like proteins, thought to mediate growth factor promotion and which are commonly found as mutations in human tumours (Pai et al 1989). Transducin has the classic model heterotrimeric structure but with only a single peptide in its beta subunit. Transducin is especially useful because a large amount is available and is easily purified, and it has a high degree of sequence homology with G_s and G_i (Manning and Gilman 1983). Whereas transducin is sensitive to both cholera toxin and to IAP, p21 is sensitive to neither. The p21 ras protein mutants are valuable in G-protein functional analysis (e.g. Tong et al 1989). Bearing in mind the frequency of finding mutant p21 in tumours, perhaps a certain amount of caution should be used when including G-subunit variants in a "normal" list, when they have been identified from cDNA made from immortalized cell lines - such as those found in HL60 (Didsbury and Snyderman 1987).

Genetic analysis and peptide sequencing of the receptors found to be modulated by G-proteins (Dohlman et al 1987) allows extrapolation of information from work on different systems. The membrane-spanning regions of several G-protein-linked receptors contain a particularly high degree of sequence homology, across four species (three mammalian, one avian), and between receptors from different tissues; their structure as suggested by peptide analysis is very similar, and genetic analysis has shown none of them to contain introns. Reconstitution experiments have shown that each of these receptor proteins can

interact with more than one type of G-protein. The $Beta_2$-adrenergic receptor has the group structure for instance (Dohlman et al 1987) and it has been shown in unilamellar phospholipid vesicles to be able to activate G_i. In whole cells it is seen to activate G_s (Asano et al 1984). The homologous regions lie in the presumed membrane-spanning regions, with extramembranous loops formed by the regions in between which are not particularly homologous.

In PMN and MNC (both in monocytes and macrophages), G-proteins have been identified which mediate chemotactic receptor-activated pathways to the hydrolysis of PIP_2 by its specific PLC (Okajima et al 1985 and Verghese et al 1986 respectively). They have been identified as G_i-like because they are IAP-sensitive, but the effect they mediate is stimulatory, the hydrolysis yielding two second messengers, inositoltrisphosphate (IP_3) and diacylglycerol (DAG). They may also exert control over calcium gating, but whether or not this effect is by the G-proteins themselves or by second messengers which they cause to be released is uncertain. Work with FMLP receptors on both PMN (Snyderman and Pike 1984) and MNC (Koo et al 1983) has demonstrated that whilst ligand binding promotes the GDP/GTP exchange reaction on G-alpha subunits, receptor binding to the G-alpha subunit decreases the receptor affinity for ligand. Thus the G-proteins have a two directional controlling role in cell activation, in the interior of the cell on effector mechanisms, and at the cell surface on receptor affinity. The association of PIP_2-specific PLC activity which is inhibited by IAP, seems to be unique to phagocytic cells.

Receptor-mediated activation of these pathways has been effected in this project by use of FMLP and HAGG, and fluoride has been used to activate G-proteins directly. These ligands and their receptors are described after an introduction to phagocytes.

B. PHAGOCYTES.

This overview of PMN and monocytes is focused on the respiratory burst and phagocytic activities which they have in common and which have been exploited in this project, and the receptors which have been used to stimulate their activity.

i. Generally.

Neutrophils (PMN) and monocytes originate from the myeloid branch of the haemopoietic stem cells in bone marrow.

Monocytes are large cells with an oval or kidney-shaped nucleus, endoplasmic reticulum (ER), many cytoplasmic granules, a golgi apparatus and obvious mitochondria (Roos and Balm 1979). Monocytes leave the marrow as precursors of tissue macrophages, still capable of division and differentiation, and at the same time operating in their own right in antigen presentation and the release of immunomodulatory secretory molecules and in locating, ingesting and digesting particles such as tissue debris, including PMN containing foreign matter (Johnston 1988). Monocytes spend 3-4 days in the circulation before migrating to the tissues and differentiating further into one of a number of cell types depending on the tissue in which they take up residence. There they may survive for many months (Hurst and Nuki 1981).

PMN are also large cells (10-15uM), have a very distinct lobed nucleus with marginated heterochromatin, highly granulated cytoplasm with little ER, and few mitochondria (Scott and Horn 1970). Normally PMN enter the circulation as mature end cells, designed to locate and ingest foreign matter such as cell debris or bacteria, and to release chemical mediators concerned with inflammation and immune regulation. PMN have a half life of only a few hours which is normally spent in the circulation either freely or as marginated cells

adhering to blood vessel walls, eventually being removed by kupfer cells in the liver.

Table 1 indicates the proportion of phagocytes found in the cell population of normal human blood.

Table 1. Range of Normal Cell Numbers in The Blood.

Red cells	$3.9\text{-}6.5 \times 10^6/mm^3$
Neutrophils	$2.5\text{-}7.5 \times 10^3/mm^3$
Lymphocytes	$1.5\text{-}3.5 \times 10^3/mm^3$
Monocytes	$2\text{-}8 \times 10^2/mm^3$
Eosinophils	$0.4\text{-}4.4 \times 10^2/mm^3$
Basophils	$0.15\text{-}1 \times 10^2/mm^3$

ii. Activation:

On stimulation by inflammatory mediators, PMN and monocytes adhere to blood vessel endothelial cells, move out of the vasculature and migrate towards a site of damage or inflammation, where they may release factors which contribute to the inflammatory condition such as free radicals or the products of degranulation. Whereas PMN are able to migrate in direct response to chemical signals, monocytes may require a signal from PMN which are at the inflammatory site (Doherty et al 1988).

iii. The Respiratory Burst.

The process activated in phagocytes in which reactive oxygen metabolites are produced is termed the respiratory burst. It includes an increase in oxygen consumption, changes in cell shape, volume and density, an increase in internal pH, activation of a proton pump and the hexose monophosphate shunt, and activation of the NADPH oxidase complex which generates superoxide (O_2^-) and results in H_2O_2 formation. It is absolutely dependent on extracellular oxygen (Curnutte and Babior 1975). (NADPH oxidase will be described fully later). Alterations which may be seen at the same time include changes in the phosphorylation profile of cell proteins and lipids, changes in internal calcium levels, cyclic nucleotides and of lipids and phospholipids. These events can be activated by a number of different stimuli, some of which are dependent on exogenous calcium ions, are sometimes dose-dependent and which may also involve the differential regulation of degranulation (Richter et al 1989). The activators of the respiratory burst are diverse and despite it being reported as being independent of degranulation, degranulation has not been reported to occur without the production of reactive oxygen metabolites. Investigation of the respiratory burst includes the plasma membrane and cytoplasmic events as well as the NADPH oxidase enzyme complex itself. In this project the pathways to activation which may have been involved are described fully in the sections on the various stimuli used. These pathways include activation of phospholipase A_2, of PIP_2-specific phospholipase C, of protein kinase C, of internal calcium release and of primary granule release.

PMN release an array of reactive oxygen metabolites which may modify the function of plasma constituents such as alpha-1 antitrypsin, and granule enzymes (such as elastase and collagenase) which may destroy extracellular tissue. Upon activation, monocytes may undergo further maturation and

include accessory cell functions such as antigen presentation aswell as those similar to PMN, and may also release factors such as TNF-alpha (Renz et al 1988), IL-1 (Johnston 1988), and LTB_4 (Elstad 1988), which modify PMN function. As activated PMN have a net destructive role, their regulation by monocytes may be critical in the resolution of an inflammatory lesion. Monocytes release TNF-alpha which may control the manner of PMN death, containing their toxic contents until phagocytosis can remove the cells (Laster et al 1988).

PMN and monocytes have receptors in common, such as those for FMLP (Snyderman and Pike 1984; Niedel et al 1979), IL-1 (Parker et al 1989), IgG (Huizinga 1989) and complement factors (Wright and Silverstein 1982; Henson 1977). These cells may be able to respond also to more general stimuli which bind to carbohydrate subunits on the cell surface - an event mimicked in vitro by plant lectins and which may be effected in vivo by bacterial cell walls or aged glycoproteins and glycolipids (Vlassara et al 1987).

PMN have limited protein synthetic ability, so expression of receptors at the cell surface is restricted to those already present and those which can be released from intracellular stores, namely those on granule membranes (Gardner et al 1986), although synthesis of new mRNA has been reported (Lindemann 1988). Given time however, activated monocytes can also alter gene transcription and synthesise new molecules (Navarro et al 1989). Common receptors between cell types do not preclude uncommon functions which may depend on the transduction mechanism within the cells; for instance, stimulation of the receptors for the Fc portion of IgG molecules, Fc-gamma R (FcR in this thesis), is inhibited in PMN by pertussis toxin (IAP) and by cholera toxin (CT) whereas that of monocytes is not affected by CT (Brown et al 1987), although FcR stimulation induces a respiratory burst in both cell types, as will be described later. Monocytes and PMN may respond to the same stimulus

differently; IFN-gamma induces FcR1 expression on PMN (Petroni et al 1988) whilst it also regulates IL-6 synthesis in monocytes (Navarro et al 1989) demonstrating that the IFN-gamma signal transduction pathways differ. Both also have receptors for complement factors which can act in concert with binding of IgG to FcR (Malbran et al 1987). Either FMLP or IgG may activate cells on their own but there are a number of agents which do not appear to do so on PMN or monocytes but which 'prime' the cell to effects of other stimuli. This suggests sophisticated internal regulation of signalling to coordinate a response with either dose or combinations of factors. For instance TNF-alpha, which is slightly chemotactic for both cell types, inhibits the chemotactic response of both to FMLP, but enhances the O_2^- response of PMN whilst not affecting that of monocytes (Kharazmi et al 1988). The priming mechanism may suggest a degree of synergy between the cell types. For instance, TNF-alpha from monocytes upregulates complement receptor expression on PMN (Berger et al 1988), and monocyte IL-1 primes PMN for an enhanced reponse to FMLP (Sullivan et al 1989); similarly PMN-released oxygen metabolites control prostaglandin production by monocytes (Flescher et al 1989).

The contents of phagocyte granules is diverse (Kane and Peters 1975; Segal and Peters 1977; Johnston 1988; Rice et al 1987). The marker enzymes assayed for PMN azurophilic, specific and tertiary granules usually being myeloperoxidase, vitamin B_{12}-binding protein and beta-galactosidase respectively (Kane and Peters 1975). Release of granule contents may be independent of free radical generation and may be controlled by regulation of internal calcium concentration (Perez et al 1987). Monocytes do not have specific granules; also, as they differentiate into the macrophage phenotype their granule constituents alter and they lose myeloperoxidase in particular.

Investigations of respiratory activity (Roos and Balm 1978) determined that the relative dependence of each cell type on a particular energy supply delineated the

priority of PMN activity to be bactericidal and that of monocytes to be digestive, and the energy for phagocytosis of mitochondrial origin in monocytes but from the Embden-Meyerhof pathway in PMN (Reis and Roos 1978). Monocytes and PMN both have the respiratory burst enzyme complex NADPH oxidase but in monocytes it is not found to have any activity in granule membranes as they have no specific granules. O_2^- production in response to concanavalin A is dependent on activity of serine (acid) proteases in PMN but less so in monocytes (Kitagawa et al 1980). Some of the differences between the cell types are typified by different receptors, such as FcR for monomeric IgG (FcR1) on monocytes and not usually on PMN, and also in the contents of granules, their membranes and their release. The serine protease requirement of O_2^- production reflects this, and thus the degranulation induced by activation is an integral part of the activation process. Experiments suggesting that degranulation does not occur may reflect an absence of the particular product being assayed, not a total absence of degranulation, granules being released in a highly specific manner (Richter et al 1989). Similarities in part of the activation process, such as the production of O_2^- in the two cell types, need not necessarily include degranulation. In both cell types a transient increase in calcium appears necessary for stimulation of O_2^- by FMLP but the release of O_2^- does not correlate with this transient rise (Kemmerich et al 1988; Ohta et al 1985). This suggests that the internal mechanism for activating NADPH oxidase is probably the same, but concurrent degranulation events need not be so, especially as granules differ between the cells, and stimulation by fluoride, which inhibits degranulation, occurs in both cell types. The respiratory burst may be an independently activated process, depending on the nature and strength of the stimulus. Concurrent degranulation may depend also on the cell type. Thus the findings which hold true in this thesis for the production of O_2^- or H_2O_2 may not include regulation of degranulation. This is particularly pertinent when considering the effect of free radical generation both on enzymes

type. Thus the findings which hold true in this thesis for the production of O_2^- or H_2O_2 may not include regulation of degranulation. This is particularly pertinent when considering the effect of free radical generation both on enzymes released during degranulation and on controlling factors of these in the extracellular fluid, as will be described later in the section on free radicals.

The production of adhesion molecules has different significance on PMN and on monocytes; PMN may increase expression of these molecules after adhesion from intracellular stores (granule membranes), but monocytes adhere poorly without increasing their expression first, a process taking several hours and requiring new protein synthesis (Tonnesen 1989). Thus expression of the same surface protein has a different mechanism and priority in activation of the two cell types. The availability of these receptors at the time of activation may be significant because they include cysteine-rich subunits which could be vulnerable to free radicals; modification of these molecules may not be relevant in either cell type in the initial phase of activation - in PMN because they are not essential for adhesion, and in MNC because they are not present - but may be so in more chronic conditions.

C. ACTIVATING LIGANDS.

i. Fluoride

The fluoride ion was discovered to stimulate the respiratory burst (Sbarra and Karnovsky 1959). Uniquely among the halides, fluoride ion stimulates O_2^- production in PMN (Curnutte et al 1975); an effect reversed by washing and repeated on restimulation of the cells. It acts indiscriminately on all G-proteins, but, importantly, means that the response of a system can be further defined by specifically leaving out the receptor-ligand interaction. Fluoride does not induce phagocytosis or the release of azurophil granules (Curnutte et al 1987).

Inhibition of the effects of fluoride by ADP-ribosylation of G-proteins indicate that they are the site of fluoride activation. For instance, in whole guinea pig PMN IAP inhibited fluoride-induced translocation of PKC to the membrane (Toper at al 1987), and in human platelets IAP inhibited thromboxane but not IP_3 release by fluoride (Fuse and Tai 1987).

Fluoride's mode of action has been suggested to be a substitution for the third phosphate group to GDP bound to alpha subunits of G-proteins, causing a GTP-like subunit dissociation (Cockcroft 1987). The atomic sizes of aluminium and phosphorus are similar, the van der Vaals radii of fluoride and oxygen are the same and the bond lengths of Al-F and P-O are almost the same (Bigay et al 1985). Its general action on all G-proteins means that the activity seen is the sum of a collection of stimulatory and inhibitory signals to all G-protein-linked second messenger systems, so the lack of degranulation or phagocytosis does not necessarily mean that these processes are not G-protein linked, but that the net effect of the fluoride stimulation is inhibitory on these processes. Similarly, aluminium fluoride was not seen to induce inositol phospholipid-specific PLC activation in mouse B lymphocytes (Harnett and Klaus 1988), which could be because of overriding stimulation of other G-protein-linked inhibitory paths, nor did it induce the phosphorylation profile found by other stimuli which activate the respiratory burst (Andrews and Babior 1983). It has been suggested

however that at concentrations over 10mM fluoride preferentially stimulates Gi-like rather than Gs proteins (Katada et al 1984).

It has not been proved conclusively whether or not fluoride requires the presence of aluminium to exert its effect, but the "pseudophosphate group" model suggests aluminium fluoride as the active species. Work in which there has been no added aluminium is unlikely to be totally aluminium-free, as in the presence of fluoride at least it can be leached from glassware (Strnad et al 1986), and in work using reconstituted systems it may form a contaminant of commercially prepared reagents (Sternweis and Gilman 1982). Another theory suggests that fluoride is required to negate the charge on aluminium for transportation across the plasma membrane, after which aluminium ion acts as a magnesium analogue (Macdonald and Martin 1988). There is a lag time for activation of the O_2^- response to fluoride of several minutes which could be accounted for by the time taken for it to cross the membrane. Neither theory approaches the question of why fluoride should be required in such large quantities - 18-20mM being the usual concentration range used in work with whole cells, a sharp peak of reactivity at these concentrations being seen in O_2^- production (Curnutte at al 1979;Strnad et al 1986; Hurst et al 1988) and in the release of calcium from intracellular stores (Strnad and Wong 1985). In cell free systems the effect of fluoride on the activation of NADPH oxidase has been found to be the same as when GTP-gamma-S was added (a non-hydrolysable GTP analogue) and the two together had no cumulative effect, suggesting that fluoride and GTP act at the same site (Gabig et al 1987). This activation was totally and specifically dependent on magnesium, suggesting that aluminium does not act as its analogue in this reaction.

As a general G-protein activator therefore, fluoride may be used to stimulate a reaction to investigate possible G-protein involvement, in conjunction with toxin-induced inhibition. But it has to be born in mind that effects may accumulate due to G-protein mediated pathways being stimulated which have

indirect effects on the process under investigation, and that a lack of stimulation or inhibition by fluoride may again be an effect of indirect interaction from unrelated pathways. Also, G-protein links are likely to be cell-type specific and so effects seen in one cell type may not be reproduced in another. Fluoride also inhibits ATP synthesis by the Emden-Meyerhof pathway (Roos and Balm 1978), is used as an enolase inhibitor (Curnutte and Babior 1975), and has been found to suppress protein phosphorylation (Palomares et al 1987), events not proven to be directly related to its G-protein-mediated effects.

ii. N-Formyl-Methionyl-Leucyl-Phenylalanine.

FMLP is an analogue of the principal chemotaxin of E. coli(Goetzl and Pickett 1980). It is also a product of mammalian mitochondria, and may get released by damaged cells (Carp 1982). It is a formylated tripeptide which activates both PMN and MNC. The nature and degree of response obtained on stimulation with FMLP may depend on the dose, the availability and affinity state of the receptors, and also on the contents of the surrounding environment. The specificity of FMLP resides in the formyl end, non-formylated tripeptides being ineffective as stimuli (Lehmeyer 1979). FMLP activates both PLA_2 and PLC (Duque et al 1986; Cockcroft et al 1985b), G-proteins are involved (Okajima and Ui 1984), it increases filamentous actin concentrations in an IAP-dependent manner (Bengtsson et al 1986) and entry of exogenous calcium ($calcium_e$) is necessary for a maximal response (Lehmeyer et al 1979). SH dependence of FMLP-stimulated production of peroxide by PMN has been found (Maslen 1985). The complexity of the response to FMLP lies in the receptor-bearing cell and in particular in the associations the ligand-bound receptor can make in the membrane and with the cytoskeleton. These events dictate the type of second messenger signal which then follows and the response obtained.

FMLP responses studied include:

Protein synthesis (Hughes et al 1987);

Phosphorylation (Huang et al 1984,1987; Hirata et al 1984);

Expression of new antigens (Emery et al 1988);

Aggregation (Boxer et al 1979; Ingraham et al 1987; Kowanatzki et al 1987);

Adhesion (King et al 1986; Boxer et al 1979; Kowanatzki et al 1987);

Association with the cytoskeleton (Jessaitis et al 1988);

Actin polymerization (Yassin et al 1985);

Arachidonic acid release (Bokoch and Gilman 1984); Phosphoinositide turnover (Bradford and Rubin 1985; Cockcroft et al 1985);

H_2O_2 production (Maslen 1985);

Azurophilic- and specific-granule enzyme release (Dahlgren 1988);

Change in oxygen consumption (Bass et al 1987);

Chemotaxis (Boxer et al 1979);

O_2^- production and calcium influx (Okajima and Ui 1984);

Internal calcium (Palmblad et al 1988).

The FMLP receptor is a 60-70KD monomeric glycoprotein (Painter et al 1982) which is thought to have a large enough stokes radius (38 to 40A°- Allen et al 1986; 47A°-Huang et al 1984) to allow it to span the plasma membrane (about 60A°- Stryer 1981). This latter view is supported by the finding that if cells are stimulated before extraction, the receptors co-isolate with the cytoskeleton (Jesaitis et al 1988). The extraction procedure of Allan et al (1986) suggested that the receptor was closely associated with lipids, and Huang et al found it closely associated with tyrosine kinase activity. There is a degree of variation in the number of FMLP receptors thought to reside in PMN membranes, with reports of $15x10^3$ per cell (Tennenberg et al 1988); $5\text{-}8x10^5$ per cell (Painter et al 1982); and $1.2x10^5$ per cell (Niedel et al 1979). The discrepancies may well depend on the method of isolation and extraction, because a pool of FMLP receptors resides in the membrane of specific granules (Fletcher et al 1983; Gardner et al 1986). Either contamination with granules or the promotion of a fusion event would alter receptor number. The transient nature of the effect of FMLP stimulation may be due to rapid receptor-ligand ingestion (Boxer et al

1979), with the FMLP ligand being rapidly digested (Niedel et al 1979); although internalization would in theory leave the receptor free to be returned to the plasma membrane, these workers did not find recycling of receptors or exposure of new ones. The brevity of response has been noted with release of IP_3 and lysozyme (Bradford et al 1985a); with microtubule assembly and chemotaxis (Boxer et al 1979); and with internal calcium release (Palmblad et al 1988).

As a chemotaxin in vivo, FMLP must induce directional motility, followed by a secondary response in the appropriate area. That is, the cell must move towards the site of FMLP production (namely an invasion of bacteria) and only then release bactericidal agents and indulge in phagocytosis. Thus is explained the reason for various responses to FMLP being dose-dependent. The responses to FMLP are obtained in the nanomolar range; Lehmeyer et al (1979) found that whereas only 10-20% of receptors needed to be occupied for chemotaxis to occur in human PMN, a 50-fold concentration was required to elicit O_2^- release ($E.D._{50}$ 10nM). Very low concentrations of FMLP induce chemotaxis, ligand binding itself being the directional signal, and as the concentration of FMLP increases with the approach towards the site of its production, the threshold of binding concentration for, say, degranulation is reached - a response which would be inappropriate at the time when chemotaxis was initiated. The finding of Nieldel et al that there was no renewed exposure of FMLP receptors is not consistent with the finding of receptors on granule membranes, and it would be essential that the number of receptors be maintained for the threshold to be reached at the appropriate site. This makes a recycling mechanism logical, because it would mean that no secondary response (i.e. degranulation) need be induced at the same time. Concentrations required for different responses depend on the presence of divalent cations and, in vitro, on whether or not cytochalasin B is used as a prelude to FMLP stimulation. For instance, in the work of Lehmeyer et al (1979), the responses of O_2^- production, specific-

granule enzyme release and chemiluminescence, were significantly enhanced by either the addition of calcium to the reaction mixture or by preincubation with cytochalasin B. Measurement of oxygen consumption with and without these additions showed that the calcium addition could cause an actual increase in cell activity, as there was an increase in oxygen consumption, whereas with cytochalasin B there was not. The PKC activated by FMLP is one which predominantly phosphorylates cytosolic proteins (Pontremoli 1986), and for which membrane proteins are poor substrates. But cytochalasin B enhances the translocation of PKC to the membrane (Pike et al 1986) - an effect of which is to increase the phosphorylation of 46KD membrane protein thus enhancing the FMLP O_2^- response. The effect of exogenous calcium may also be to enhance translocation of PKC (Burnham et al 1989).

A functional cell-type specific interaction of PLC and actin has been described (van Haelst et al 1988). These findings are significant because a) in the physiological environment, calcium would be present and b) because cytochalasin B is routinely used in vitro to enhance the FMLP response.

An enhancing effect on the FMLP response has been noted with agents which in themselves do not elicit the response seen. These include the effect of prior stimulation, or 'priming', with platelet activating factor (PAF) (Ingrahm et al 1987) or with tumour necrosis factor (TNF-alpha) (Berkow et al 1987). This suggests secondary control of the response stimulated by the main activating ligand, namely FMLP. It was noted in work with guinea pig macrophages that those elicited from the peritoneum had a lower O_2^- response to FMLP than those from the model inflammatory site, which could not be explained by any difference in receptor number (Snyderman and Pike 1980). This effect could indicate that priming has occurred in macrophages from inflamed tissue. FMLP does not usually induce significant LTB_4 synthesis in PMN (Sellmayer et al 1987), however, after GM-CSF or PAF priming it does (Wirthmueller et al 1989). This important finding demonstrates altered responsiveness to FMLP.

In the first few seconds after FMLP stimulation, there is a transient rise in the level of cyclic AMP (Bokoch and Gilman 1984; Okajima and Ui 1984) which is thought could be due to an FMLP-induced inhibition of cAMP phosphodiesterase (Verghese et al 1985) but this may not be so (Iannone et al 1989). There are G-protein regulatory mechanisms in FMLP-activated pathways and those controlling adenylate cyclase which may have mutually exclusive activation depending solely on the distance between the two mechanisms, proximity allowing the brief interaction seen. In purified plasma membrane preparations FMLP can be used to inhibit adenylate cyclase (Lad et al 1985) an effect which is abolished by IAP, suggesting a close relationship between the regulatory mediators in the two systems, but not necessarily any cooperative use in whole cells. However it could be that an increase in cyclic AMP (cAMP) allows activation of a cAMP-mediated event, such as phosphorylation of lipocortin by a cAMP-dependent tyrosine kinase as previously mentioned (Hirata et al 1981). The cAMP rise is independent of degranulation and O_2^- production, its inhibition not affecting these events (Simchowitz et al 1983). The FMLP- induced rise in cAMP is not inhibited by IAP (Okajima and Ui 1984). The increase in cAMP could come from a) inhibition of cAMP-specific phosphodiesterase b) activation of adenylate cyclase by the Gi-like G-protein of the FMLP receptor, or c) by prevention of adenosine inhibition of adenylate cyclase via the 'P' site (Bushfield et al 1989). FMLP stimulation of PIP_2 hydrolysis in human plasma membrane preparations is GTP-dependent and is inhibited by IAP. Smith et al (1985) found no formation of lysophospholipids, and therefore no PLA_2 activity. This work demonstrated FMLP activation of PIP_2 hydrolysis linked by G-proteins. In isolating the membrane preparation, it is possible that PLA_2 activity was lost, and so the negative result from these experiments is not conclusive. The inhibitory effect of IAP on FMLP-induced PIP_2 hydrolysis has been found also in whole PMN (Ohta 1985; Bradford et al 1986). In whole PMN also, FMLP-

induced arachidonic acid release has been found to be via the PLA_2-mediated hydrolysis of phosphatidylcholine synthesized by the transmethylation pathway (Hirata et al 1979) and this arachidonate synthesis was found to be IAP sensitive (Ohta et al 1985). Both PLA_2 activation and PLC activation by FMLP are IAP sensitive. This does not help to elucidate which pathway is activated at any one time or whether or not they are mutually exclusive in this respect. The dose-dependent activation of two different pathways for alternative second messenger release has been shown for glucagon, which at lower doses activates a PLC pathway and at higher doses activates adenylate cyclase (Wakelam et al 1986). And to indicate another possibility, PLA_2 is activated in retinal rod outer segments by the beta-gamma subunit of transducin (Jelsema et al 1987), the alpha subunit of which activates guanylate cyclase.

The PLA_2-specific inhibitor p-bromophenacylbromide (pBPB) inhibited FMLP-induced activation and it inhibited O_2^- production induced by PMA (Duque et al 1986). This may suggest that PLA_2 is indeed activated by FMLP but indirectly, by PKC phosphorylation. This effect, too, may be indirect - such as phosphorylation of the kinase which phosphorylates lipocortin! This theory is suggested because arachidonic acid release is dependent on internal calcium above basal levels and is inhibited by TMB8, an internal calcium antagonist (Ohta et al 1985) and because PKC itself is not dependent on a rise in cytosolic calcium.

The fine regulation of the internal effects of FMLP stimulation may depend on calcium levels within the cell as controlled by a combination of release of calcium from intracellular stores and of passage of exogenous calcium, both of which are inhibited by IAP (Ohta et al 1985; Okajima and Ui 1984). These workers found that inhibition of calcium mobilization, whilst not affecting PIP_2 hydrolysis, did inhibit arachidonic acid release, and that in the absence of exogenous calcium arachidonic acid release was only about 25% of that in its presence.

The number of receptors occupied is a control mechanism for the response elicited by FMLP and cAMP elevation may regulate the initial pathway activated. Further control of the effects of FMLP can be seen in changes in binding affinity and in cytoskeletal involvement. A number of events have been seen to alter FMLP binding affinity, such as an increase in plasma membrane fluidity which can be induced, in vitro, by increasing the saturated or monounsaturated fatty acid content of the membrane (Tanaka et al 1988) or by addition of alcohols (Yuli et al 1982). An increase in fluidity not only increases the affinity of FMLP binding but also has divergent effects on the response - chemotaxis being enhanced and O_2^- production and lysosomal enzyme release being inhibited without altering receptor number (Yuli et al 1982), coincident with a lack of PKC stimulation (Pike et al 1986). These two papers combine to make a case for chemotaxis and secretion being modulated by different effector enzymes. Similarly, in PMN membrane preparations G-protein interaction with the ligand-bound receptor lowers the ligand-receptor binding affinity (Koo et al 1983). The changes observed in receptor affinity are a combination of an increase in numbers of low affinity receptors exposed at the cell surface by degranulation, and a change in the affinity of receptors already at the cell surface. Products of the signal transduction event could feed back to induce the changes in fluidity (assuming no competing metabolic pathway to be one hundred per cent efficient). Free arachidonic acid is a product of both PLA_2 and PLC activity, and the alcohol inositol triphosphate of the latter. Thus a cycle could be envisaged in which on FMLP binding to a few, high affinity receptors, chemotaxis could be induced; the increase in the affinity induced by cytoskeletal interaction could simultaneously repress the secretory functions until the concentration of FMLP was high enough to ensure a large degree of ligand binding to low affinity receptors to override this; following PLA_2 activation, released arachidonic acid could raise membrane fluidity and again induce conditions more conducive to chemotaxis. If the concentration of FMLP was

high enough to induce PLC activation, both arachidonic acid and inositol triphosphate would also contribute to an increase in fluidity. As well as feeding back to stop the O_2^- and secretory responses, this latter event could be a means of ensuring that the chemotactic response was not lost after a high concentration of FMLP had been encountered. This may be more significant in the case of MNC activation than of PMN, the latter being less able to sustain prolonged involvement in stimulatory conditions.

FMLP binding induces an increase in cytoskeleton-associated actin and rapid and transient association of the receptor with the cytoskeleton at chemotactic doses (1nM), in a manner inhibitable by IAP (Sha'afi et al 1987). It has been suggested that the cytoskeletal association segregates the FMLP-bound receptor from its interactive G-proteins (Jesaitis et al 1988). Inhibiting actin polymerization with cytochalasin B to enhance degranulation and O_2^- production could also prevent internalization of receptors and prolong possible interaction with G-proteins. Cytochalasin B reduces phosphatidylinositol phosphate kinase activity, so microfilaments probably facilitate PLC activity by controlling the site of substrate production (Pike et al 1988). This suggests that the PLC pathway may be concerned with negative feedback in the O_2^- response, and the disruption of this could be responsible for the enhancement seen in the FMLP response with cytochalasin B.

iii. <u>The IgG Fc Receptors.</u>

(Fc-gamma-R :- FcR in this thesis).

There are currently three classes of human IgG Fc receptor; these are summarized in Table 2. Polymorphisms have been found within each class of FcR. In the case of FcR111 for instance, two polymorphic types have been attributed to differences in primary amino acid structure (Ory et al 1989) and Ravetch et al (1989) identified two distinct FcR111 genes on human chromosome 1; also the location of the three genes for FcR11.

The receptors have epitopes in common on lymphocytes, monocytes and on PMN, all labelling with monoclonal antibody (Mab) KuFc79 (Vaughan et al 1985), which precipitated molecules of molecular weights 42KD, 33KD and 70KD, with extensive microheterogeneity of molecular weight suggested to be due to variation in carbohydrate content. The carbohydrate side chains (CHO) may introduce further, functional, heterogeneity among the FcR. Concanavalin A (which is alpha-mannoside-specific) binds to FcR111 and not to FcR11, and alters the binding of FcR111-specific Mab 3G8 (Kimberly et al 1989). Any alteration of CHO may therefore affect receptor function. In RA the sialic acid content of many plasma proteins is seen to be altered, including that on IgG (van Eijk et al 1986). If this extends to FcR, which seems likely, this may have important consiquences for cell activation. Recently it has been found that IgG isotypes and not alterations in CHO affect rheumatoid factor binding (Newkirk et al 1990).

Of the three types of receptor, only FcR1 demonstrates any significant binding of monomeric IgG. FcR11 and FcR111 require cross-linking for binding and activation (Bijsterbosch and Klaus 1985). Activation of FcR1 can be mimicked in vitro by preincubating cells with monomeric IgG under conditions which prevent internalization of receptors (such as incubating at 4^0c) followed by addition of $F(ab)_2$ fragment of a second antibody to the first one. Alternatively monomeric IgG can be heat-aggregated and used as a one-step stimulant of all three receptor sub-types. The work using these two methods can be cross referenced for work on cell activation and used separately for studying specific Fc receptors. It has been found that heteromeric cross-linking is possible (Malbran et al 1987). The complement component C3 has a high affinity for IgG. If the complement cascade is activated, C3b binds to IgG covalently in the C_h3 region. This heterodimer can then bind to both FcR and CR1, mimicking homologous cross-linkage with subsequent cell activation and internalization of

the complex. A third method for cross-linking has been described in which IgG-coated beads were used (Fitzharris et al 1987).

Anchorage of Fc receptors in the plasma membrane differs, which has important implications for the response that they can induce. FcR111 on PMN is anchored by a phosphatidylinositol (PI) linkage, and has no transmembrane-spanning region, but it is not found to be PI-linked in cultured monocytes (Huizinga et al 1988) or in NK cells (Ravetch et al 1989). Ravetch and workers determined that the FcR111 in PMN and monocytes were each the product of a different gene. In patients with paroxysmal nocturnal haematuria (PNH) where there is known to be defective PI-linkage, PMN were deficient in FcR111 expression but cultured monocytes were not. Activation of PMN was unaffected by this deficiency. It was noted that normal PMN in serum-free medium released FcR111 on stimulation with FMLP. Investigation of receptor expression may depend on the invitro conditions. McGuire and Sandilands (1987) demonstrated that release of FcR from normal monocytes was an artifactual event in vitro and was completely absent in the presence of serum, which supplied essential protease inhibitors. However in PNH, as FcR111 are synthesised normally and simply not linked to the cell membrane, they probably are released into the extracellular space, with unknown consequences. It is also possible that in RA synovial fluid the unidentified protease is not active or is absent. Kurosaki and Ravetch (1989) found FcR111 PI-linkage to be determined as a post-translational event, dependent on simultaneous expression of another protein. Absence of the second protein results in cleavage of the carboxy terminal end of the FcR molecule and PI-linkage. There is evidence that differing receptor linkage may be a fairly common way of altering receptor function from one cell type to another (Cross 1987), and that alterations in linkage may occur in malignancy (Conzelmann et al 1986). Release of diacylglycerol on receptor hydrolysis is likely also to be functionally relevant.

Considering the constitutive expression of FcR on PMN and monocytes, in experiments using cells directly from whole blood FcR11 and FcR111 can be involved in activation events in PMN, and FcR1 and FcR11 can be with monocytes. Any differences in the responses seen in the two cell types may be attributed to FcR1 activity in monocytes or FcR111 in PMN. The expression of FcR111 on monocytes in culture is indicative of their attaining macrophage-like characteristics (Huizinga et al 1988).

a. FcR Activation.

The ability of FcR1 to bind monomeric IgG does not preclude its activation by cross-linked IgG such as immune complexes. FcR1 may transmit a negative signal; in mouse B lymphocytes it inhibits proliferation induced by cross-linked anti-surface-bound IgG (Bijsterbosch and Klaus 1985). In keeping with the idea of a negative signal, Hammerling and Hoffmann (1987) suggest that Fc on antigen-presenting cells down-regulate T helper cell function. FcR have been found to be functional in T cell locomotion (Wilkinson and Higgins 1987) and proliferation (Boot et al 1989); in NK cell ADCC (Selveraj 1989), specifically on an NK T cell subset (Lanier et al 1985;1989); in B lymphocyte PLA_2 activity (Suzuki et al 1980) and surface Ig function (Bijsterbosch and Klaus 1985); in platelet clearance (Clarkson et al 1986) and are found to be expressed on endothelial cells after viral infection (Cines et al 1982).

b. FcR on PMN and Monocytes.

Human PMN produce O_2^- in response to heat-aggregated IgG (HAGG) and undergo degranulation and phagoytosis (Roos et al 1976). Preincubation with cytochalasin B (inhibiting phagocytosis) results in enhanced O_2^- production and granule enzyme release, indicative of cytoskeletal involvement. Willis et al (1988) used Mab to demonstrate that cross-linking FcR on PMN induced lysosomal enzyme release and O_2^- production. IgA_1 or IgG_1 induced significantly more beta-glucuronidase from PMN than other Ig subclasses, and IgG_1 did so from monocytes; effects doubled after cytochalasin B treatment

(Ferreri et al 1987). The release could be blocked by monomeric IgG, demonstrating that cross-linking, and not just receptor occupancy, is required for FcR activation. Cross-linking of FcR-FcR or of FcR-CR1 results in transduction of an activation signal and internalization of the receptor-ligand complex, in a down-regulation mechanism in common with other receptors. This is not seen to happen with monomeric binding, and in these circumstances the fate of ligand-bound FcR1 remains unclear (Jones et al 1985). It may be that FcR1 is involved with normal clearance of circulating IgG. In monocytes, adhesion of bound IgG to an antigenic surface can mimick cross-linking under conditions precluding internalization (Jungi et al 1987). This modified both receptor expression on the rest of the cell surface and cell function. In vivo this could be envisaged to occur with IgG bound to damaged cells (Hansson et al 1987) or with tissue-bound autoantibody.

Identification of the relative roles of FcR11 and FcR111 ascertained that there were 15-16x10^3FcR11/PMN and 110-120x10^3FcR111/PMN (Tosi and Berger 1988). Stimulating with HAGG and blocking with Mabs IV.3 and 3G8 respectively, these workers concluded that the role of FcR11 was to transduce the activation signal and that of the more numerous FcR111 was to bind ligand, thus ensuring a plentiful supply for FcR11. This model is reinforced by the finding that Mab CRB-FcR/Gran1 to FcR111 blocked all IgG binding to PMN whereas Mab C1KM5 to FcR11 only reduced it (Huizinga et al 1989a). These workers also found on blocking FcR11 that they were unable to activate the respiratory burst or phagocytosis with small immune complexes bound to FcR111, and vice versa, but that large complexes could activate FcR11 when FcR111 was blocked (Huizinga et al 1989b), suggesting that the lower numbers of FcR11 are spatially distributed in such a way that cross-linking is not accomplished by small complexes, a deficiency which may usually be made good by the relative abundance of FcR111. If this dual binding is mediated by only one of the two receptor types having a signal transduction mechanism, it

must strongly suggest that in the other instances of heterologous cross-linking (FCR-CR1 for instance) the necessity is again a spatial one. It also suggests that direct interaction between the transducing receptors (e.g. FcR11) is not necessary because they will be separated by the many FcR111 molecules. The capping seen by fluorescence experiments indicates that bound receptors do polarise. Capping could just be for the removal of the receptor-ligand complex with the loss of as little plasma membrane, and associated molecules, as possible. If, however, this is indeed to bring signal transducing receptors into apposition, then release of FcR111 would promote this. Its hydrolase could also provide a means of regulation. In other cell types FcR111 may have a different function. Current work by Huizinga and workers is investigating a possible signal transducing mechanism from FcR111 on PMN (verbal communication).

In work with Mab to FcR11, Feister et al (1988) found that the O_2^- response of human PMN was abolished by preincubation of cells with IAP. However the degranulation response was only partially inhibited, indicating that there are at least two pathways activated by FcR cross-linking, of which only that to O_2^- was totally regulated by an IAP-sensitive G-protein. The degranulation response was not completely immune to G-protein regulation. This could suggest that both the nature of the response and the degree may be mediated by G-proteins.

Fitzharris et al (1987) demonstrated that FMLP-primed PMN could be induced to release LTB_4 both extra- and intracellularly by cross-linking Fc receptors with IgG-coated beads. The fact that it is synthesised but not released in response to higher doses of FMLP alone (Sellmayer 1987) may suggest that FcR activation induces an essential membrane perturbation which does not occur with FMLP stimulation.

Monocytes synthesise arachidonic acid metabolites on FcR stimulation and this could be derived directly from FcR if they, too, bear the intrinsic PLA_2 activity

which has been found in FcR11 of B lymphocytes (Suzuki et al 1980). PLA_2 activity has been found to inhibit adenylate cyclase activity, and adenylate cyclase to inhibit binding to Fc-gamma 2bR (human FcR11 homologue) in the mouse macrophage cell line p388Di (Hirata et al 1987), and, in the same cell line, this could be blocked by inhibiting prostaglandin production and involved G-protein regulation (Nitta et al 1982). In work with guinea pig elicited peritoneal macrophages, FcR-mediated stimulation of O_2^- release was inhibitable by a PLA_2 inhibitor and partially by a PKC inhibitor (Sakata et al 1987), direct stimulation with arachidonic acid not being inhibited by PKC inhibitor; suggesting that FcR-mediated activation may be by more than one pathway perhaps depending on the receptor subtype, those activated in the guinea pig macrophage not being distinguished here. To determine if there is activation of more than one pathway by the same receptor subtype requires activation by cross-linking subtype-specific monoclonal antibodies. Using an IgG1 Mab to FcR11 (Mab C1KM5) and comparing its effect with IgG2a Mab also to FcR11 (2E1), MacIntyre et al (1988) demonstrated that tripartite binding by C1KM5 could induce calcium mobilization in human monocytes, but divalent binding by its F(ab') sites could not. A much less effective response was obtained by cross-linking the 2E1 $F(ab')_2$ fragments. O_2^- production could not be induced by C1KM5 unless the monocytes were primed by IFN-gamma, and not at all by cross-linked 2E1 fragments. Preliminary work with other types of cell (including B lymphocytes and PMN) suggested a different response to C1KM5, indicating that the epitope on monocytes may be cell-type specific or that the mechanism of activation is not precisely the same.

In some instances FcR binding triggers a cell-mediated cytotoxic event (Fanger et al 1989). FcR111 could not mediate this event on PMN, but could do so on NK cells, in which they are not PI-linked, indicating the cell-type specificity of the receptor subtype functions, as may be regulation of receptor expression; IFN-gamma induction of FcR1 on PMN can be inhibited by the synthetic

glucocorticoid dexamethasone (DEX) (Petroni et al 1988), but DEX enhances the effect of IFN-gamma on FcR1 expression in monocytes (Girard et al 1987). FcR in RA will be considered in a later section.

In this project use has been made of the HAGG system for stimulating FcR in both PMN and MNC without discriminating between the receptor sub-types, in the presence or absence of SH-blockade. Some preliminary work has been carried out also using purified IgG and the monoclonal antibodies 1V.3, 3G8 and also 32+22, an FcR1-specific Mab.

Table 2. Human Fc-gamma-R Sub-Types.

Receptor	Cell Type	IgG Specificity	Mabs	Approx. Mol Wt.
FcR1	Monocyte	$IgG_1=IgG_3>IgG_4$	32.2	70
	U937;HL60		FR51	
	Macrophage			
	NK cell			
	*PMN			
FcR11	Monocyte	$IgG_1=IgG_3>IgG_2$	IV.3	40
	PMN	and IgG_4	KuFc79	
	eosinophil		2E1	
	B cell		C1KM5	
	U937;HL60			
	K562;Daudi;			
	Raji			
FcR111	Macrophage	$IgG_1=IgG_3$	3G8	50-70
	PMN		B73.1	
	eosinophil		Leu11a,b,c.	
	NK cell			

Note: * = only PMN preincubated in IFN-gamma.

(Abbreviated from:- Anderson and Looney 1986; Unkless et al 1988; Fanger et al 1989)

D. REACTIVE OXYGEN.

Stimulation by the systems so far described, using specific ligands such as FMLP or HAGG, using non-surface-receptor ligands such as fluoride or PMA results in O_2^- production and thereby also to H_2O_2 from activation of NADPH oxidase.

i. NADPH Oxidase.

The NADPH oxidase complex is the source of O_2^- generated when phagocytes undergo the respiratory burst, and as such is a key element in the bactericidal response of these cells. It has an absolute requirement for extracellular oxygen, and in anaerobic conditions is completely inactive (Curnutte and Babior 1975). It is also dependent on a fully operational proton pump (Henderson et al (1988). Its function is to convert molecular oxygen into O_2^-, which it does at the expense of NADPH supplied by the hexose monophosphate shunt (HMPS).

HMPS

$$1\ G6P + 12\ NADP^+ \text{----------} > 6\ CO_2 + 12\ NADPH + 12\ H^+ + P_i$$

NADPH oxidase

$$12\ NADPH + 24\ O_2 \text{----------} > 12\ NADP^+ + 12\ H^+ + 24\ O_2^-$$

The CO_2 produced by the HMPS is hydrated to H_2CO_3 and this subsequently supplies the protons for secretion (Borregaard et al 1984).

Activation is thought to be of an electron transport chain, which includes a cytosolic subunit with an unidentified role, but which may be translocated to

the plasma membrane on activation; a non-covalently-bound FAD associated with a 45-48KD protein which may be a substrate for protein kinase C; and a cytochrome b. The evidence for these assumptions has been gathered largely from analysis of PMN, both whole and fractionated, from animals or from man. The membrane components suggested have midpoint potentials which make a logical 'chain' for electron transport;

$NADPH/NADP^+$ = -320mV

$FADH_2/FAD^+$ = -280Mv

cyt b/cyt b^+ = -245mV

O_2/O_2^- = -160mV

Investigations of O_2^- production necessarily combine both that of the stimulatory pathways to activation, and the enzyme complex itself. There seems to be no dispute that, whatever the activation mechanism, or the stimulus employed, the enzyme complex activated is the same one. In 1979, Tauber and Goetzl first isolated NADPH oxidase activity from human PMN fractions. They identified NADPH oxidase activity in both the plasma membrane and also in another particulate fraction. The work of Clarke et al (1987) found enzyme activity in the plasma membrane fraction, but three times as much in the fraction containing specific granule membranes. They also found that there was a soluble factor required, with magnesium, for activation of the membrane bound fractions, a factor which was noted by Tanaka et al (1988) either to be inactive or not present in the cytosol once activation had taken place. Curnutte et al (1987a and b) found a similar requirement for magnesium with the cytosolic cofactor, but their isolation procedure separated activity in two fractions of molecular weights 250KD and 50KD. The molecular weight of the cytosolic factor identified by Clarke et al was 10KD, which precluded its being protein

kinase C, because the smallest active fragment of PKC they found was 50KD. Activation occurs simultaneously with changes in protein phosphorylation (Andrews and Babior 1983; Gennaro 1985; Hayakawa et al 1986).

The most important discovery for the elucidation of this enzyme complex has been the finding that patients with chronic granulomatous disease are deficient in both O_2^- production and in some of the proteins suggested as elements in the oxidase complex. The disease has been genetically subdivided into groups, depending on the factor missing in the fractionation of PMN. The X-linked disease has been associated with normal levels of cytosolic factor, but no cytochrome b_{558}, as has a group of autosomal recessive-linked disease (Curnutte et al 1987). There is another autosomal recessive group, which does have the cytochrome but has been found to be deficient in the cytosolic factor (Curnutte et al 1988), and other subgroup deficiencies may come to light. Carriers of the disease demonstrate only partial efficiency of O_2^- production.

Activation of the oxidase by FMLP, PMA or by Con A was found to be coincident with phosphorylation of a 45KD membrane protein (Ohtsuka et al 1987), an event lacking in some autosomal recessive CGD patients (Segal et al 1983). Hayakawa et al (1986) identified phosphorylation of a 48KD protein associated with NADPH oxidase activation, which was not found to the same extent in CGD patients, and found labelling seen with PMA stimulation, of a cytosolic 48KD protein which was not found in the CGD patients. It seems that phosphorylation of a 45-48KD protein occurs in conjunction with NADPH oxidase activation, and is considered as a substrate for the PKC. However, when activation of this kinase is blocked by an isoquinoline derivative (Bass 1987), activation of NADPH oxidase still occurs in response to the receptor-mediated ligand FMLP, indicating either that another kinase activated by FMLP stimulation can phosphorylate the 45-48KD protein, or that oxidase activation is not dependent on phosphorylation alone. Diphenyliodonium (DPI) inhibits

PMA-stimulated O_2^- production. Inhibition of NADPH oxidase by I^{125}-labelled DPI was seen to inhibit NADPH reduction of FAD, and of cytochrome b_{245}, whilst labelling a 45KD protein (Cross and Jones 1986), suggesting that DPI inhibited the PKC phosphorylation site.

The location of the enzyme complex is dependent on the ability of preparative techniques to separate fractions, plasma membrane and specific granule membrane in particular, without inducing a fusion event or the activation of resting cells. (For example, Curnutte and workers (1987) point out membrane aggregation as a hazard of their preparative procedure). The location of subunits of the oxidase in resting cells is therefore not fully resolved. Amit et al (1988) found almost no cytochrome b_{559} in plasma membrane, and found activity not dependent on its presence at all. Borregaard et al (1983) located the cytochrome to the specific granule membrane and reported translocation of 75% of it to the plasma membrane on activation, and in 1984 reported that the flavoprotein was found not to be an intergral membrane protein in specific granule membranes, whereas the cytochrome b was. It is possible to inhibit degranulation and still invoke O_2^- production, with fluoride for instance (Curnutte et al 1979), so there may be no obligatory subunit residing solely in specific granule membranes.

Lipids form an essential part in the NADPH oxidase complex. Tauber and Goetzl (1979) used deoxycholate instead of phospholipids in their suspension buffer, finding no activity with the absence of both. Activation of NADPH oxidase in whole cells has demonstrated a role for arachidonic acid which has yet to be clarified. Whilst it activates NADPH oxidase in membrane preparations (Tanaka 1988; Bromberg and Pick 1983; Amit et al 1988; Clark et al 1987) it is toxic to whole cells if added exogenously (Cohen et al 1986). As a preferential substrate in PLA_2 cleavage of phosphatidylcholine, an event activated by opsonized zymosan-stimulated cells for instance (Maridonneau-Parini et al 1986), and as a product of diacylglycerol catabolism after PLC

activation (Ohta et al 1985), it is an important metabolite in two pathways known to be linked to NADPH oxidase activation. Saturated and monounsaturated fatty acids were found to activate the oxidase in membrane preparations without the nonspecific increase in oxygen consumption that arachidonic acid induced (Tanaka et al 1988). Removal of fatty acids by the addition of albumin inactivated the enzyme system. These results may suggest a role for lipids in regulating the juxtapositioning of the subunits of the enzyme complex. In membrane fractions the oxidase has also been found to be activated by negatively-charged detergents such as SDS (Cox et al 1987), a process dependent on an unidentified cofactor and inhibited by increasing concentrations of phospholipids (Aviram and Sharabani 1989). PLC and PKC were also activated in membrane preparations in the presence of a cytosolic cofactor by negatively-charged phospholipids, and preapplication of phosphoinositides prevented the stimulation of NADPH oxidase by SDS (Aviram and Sharabani 1989).

An effect of low doses of stimuli such as FMLP, PMA,(Bass 1987) LPS (Sasada 1983) PAF (Ingraham et al 1987) or TNF-alpha (Berkow et al 1987), is to enhance the O_2^- response induced by a second, different, stimulus, even when the first, as in the case of TNF-alpha or PAF, does not induce O_2^- itself at any dose. Bass and workers identified the PMA priming effect as the translocation of PKC to the membrane; the work of Sasada's group showed that the effect of PMA priming was to increase the rate of NADPH oxidase activity and to lower the binding constant for NADPH on subsequent stimulation by a different ligand. This suggests that there are regulatory steps at the oxidase end of O_2^- production as well as at earlier stages of signal transduction.

The role of the cytoskeleton in NADPH oxidase activation or regulation is unknown but the enzyme does co-sediment with the actin-rich cytoskeletal membrane vesicle fraction (Jesaitis et al 1988). As mentioned, the inclusion of

cytochalasin B before FMLP stimulation is common practice, and has an enhancing effect on O_2^- production (Simchowitz et al 1983; Lemeyer et al 1979; Dahlgren 1988). Whether this is solely due to an effect on FMLP-receptor regulation, or if it also includes regulation of the NADPH oxidase complex has not yet been determined.

Recently work has suggested that the elements for O_2^- production can be found and activated in certain populations of B lymphocytes and in some EBV-transformed B cell lines (Maly et al 1988;1989). The tiny amount of O_2^- elicited from normal B cells was in response to extremely large doses of stimuli, however it has to be admitted that the active enzyme system is present in these cells.

The consiquence of NADPH oxidase activation is the generation of reactive oxygen species to the outside of the cell and in the phagocytic vacuole.

ii. Free Radicals and Reactive Oxygen Species.

a. Production.

Oxygen-derived free radicals are normal metabolites in a number of biochemical pathways where they may form an unwelcome byproduct to be removed as soon as possible or they may be essential end products. Some are highly reactive and rapidly initiate peroxidation in proteins and lipids.

The formation of free radicals is described diagrammatically below

$O_2 + e^- \text{ ------} > O_2^-$ 1.

$2O_2^- + 2H^+ \text{ ------} > H_2O_2 + O_2$ 2.

$\xrightarrow{\text{Metal}^{n+}}$

$O_2^- + H_2O_2 \text{ ------} > O_2 + OH^- + OH\cdot$ 3.

$I^- + H_2O_2 \text{ ------} > HOI + HO^-$ 4.

$\xrightarrow{\text{MPO}}$

$H_2O_2 + Cl^- \text{ ------} > OCl^- + H_2O$ 5a.

$OCl^- + H_2O_2 \text{ ------} > H_2O + Cl^- + {}^1O_2$ 5b.

$OCl^- + RNH_2 \text{ ------} > RNHCl + HO^-$ 5c.

$OCl^- + RNHCl \text{ ------} > RNCl_2 + HO^-$ 5d.

(from Fantone and Ward 1985)

The formation of oxygen-derived free radicals and their metabolites begins with the one electron reduction of oxygen to form O_2^- (equation 1). In the presence of SOD this is dismutated to hydrogen peroxide (H_2O_2) and oxygen, which may also happen spontaneously, if quite slowly (equation 2). Catalase can convert H_2O_2 to water and oxygen. However, hydroxyl radicals (OH·) can be produced by the Haber-Weiss reaction; in the presence of transition metal ions called the Fenton reaction (equation 3). If H_2O_2 becomes available to myeloperoxidase from phagocyte granules, hypochlorite can be produced (equation 5a). A chain of reactions may follow (equation 5b-d) in which chloramines are produced - the chain length depending on what is available in the surrounding environment. Optimal pH for MPO hypochlorite-generating activity is acidic, but in alkaline conditions it catalyses the conversion of H_2O_2 to oxygen and water. Singlet oxygen, an intermediate intracellular product which is detectable in vitro as chemiluminescence, has an indeterminate role physiologically. The iron-iodide system for the formation of radicals (equation 4) may be of limited physiological relevance being dependent on iodide ions - which are not widely distributed.

In all aerobic cells radicals can be removed by catalysts such as superoxide dismutase (SOD), catalase and glutathione peroxidase. In purine breakdown, the conversion of xanthine to uric acid is catabolized by xanthine oxidase, with release of O_2^-, and this enzyme may be released by damaged cells following ischaemia. In electron transport chains, O_2^- may be released; in mitochondria they are, for instance, a byproduct in mitochondrial oxidative phosphorylation and these organelles contain SOD and catalase to render it harmless (Fantone and Ward 1985). Van Kuijk et al (1987) demonstrated a protective role for PLA_2 in the selective removal of peroxidized fatty acyl chains from phospholipids for reduction with glutathione and glutathione peroxidase. In phagocytic cell specific granule and plasma membranes O_2^- is the end product

of the short electron transport chain, NADPH oxidase, previously described, which is designed specifically for its formation as an important factor in destruction of phagocytosed particles (Curnutte and Babior 1975). O_2^- may be released as a direct initiator of chemical modification designed to damage or kill phagocytosed particles (Babior et al 1979), but much of it is also dismutated and released as H_2O_2.

Peroxide products differ depending on the stimulus; those released by FMLP and heat-aggregated IgG being about 90% and 30% catalase-inhibitable (i.e. H_2O_2) respectively (Maslen et al 1987). In MNC activated by heat-aggregated IgG it has been shown that the major lytic agent in destruction of unsensitized red blood cells is OCl^-, and that inhibition of O_2^- with SOD had much less effect than inhibition of H_2O_2 with catalase or OH· with mannitol (Geffner et al 1987). Both ascorbate and MPO provide examples of agents the control of whose activity may bring about dramatically different effects. When azurophilic degranulation occurs in conjunction with the production of reactive species in activated phagocytes, MPO is released which, with chloride ions, can form highly toxic OCl^-. Even in the presence of high (>0.2uM) concentrations of free iron, MPO can compete successfully and totally for H_2O_2, resulting in a complete absence of OH· formation (Winterbourn 1986), and MPO-derived oxidants can inactivate PMN enzymes such as lysozyme, beta-glucuronidase, collagenase and gelatinase, only elastase being resistant (Vissers and Winterbourn 1988). Winterbourn also mentioned that ascorbic acid can act as an electron donor in the Haber-Weiss reaction, substituting for O_2^- in the reduction of ferric to ferrous ions. This raises the point that a deficiency in the ascorbate transport mechanism would not only deplete the intracellular environment of an important free radical scavenger, but would also promote in the extracellular surroundings a plentiful supply for the Haber-Weiss reaction. It also demonstrates that MPO, which has been described as a system for producing

lethal bactericides (OCl^-;N-Cl) and as an enzyme activator, may also serve a protective role. Although OCl^- may be a most potent effector of free radical damage, MPO deficiency is not usually fatal, but a deficiency in the O_2^- - generating system, such as is found in chronic granulomatous disease, results in recurrent infections which are eventually insurmountable.

b. Functions.

As well as their destructive role, radicals may be necessary to activate certain enzymes when appropriate; cyclo-oxygenase has been shown to require peroxides for activation for instance (Hemler et al 1979), with peroxide levels governing the preferred final product (Warso and Lands 1983), and collagenase released from specific granules on PMN degranulation requires modification by OCL^- to activate it (Weiss et al 1985). Others, such as the plasma anti-elastase enzyme, are inactivated by free radicals (Test and Weiss 1986). So a certain level of peroxidation is necessary for some functions of cells and tissues.

c. Damage.

Normally, highly reactive radicals are well contained. For instance, when introduced in vitro to a monolayer of vascular endothelial cells which have been invaded by Candida hyphae, PMN can selectively destroy the hyphae from around and within the endothelial cells with no damage to the monolayer (Edwards et al 1987). But stimulated PMN can kill suspensions or monolayers of endothelial cells in vitro, by a H_2O_2-dependent mechanism (Weiss et al 1981), which is insensitive to SOD, and not blocked by MPO inhibition.

Damage by free radicals is deleterious to most of the normal functioning of cells and an investigation of the exact molecular nature of the damage caused to proteins has been undertaken by Davies and workers (1987a-f). They subjected a number of different isolated proteins, and bovine serum albumin in particular,

to different free radical-generating systems with and without selective scavengers, and followed this treatment with exposure to red blood cell extract. They concluded that exposure to free radicals led to the proteins being degraded at a faster rate, and by a different (non-ATP-requiring) system, compared to unexposed proteins. Most of the damage incurred by exposure to radicals could be prevented by mannitol (an OH· inhibitor) as opposed to SOD, and damage was greatest in the presence of OH· or OH· with O_2^-, rather than with O_2^- alone, and in the presence of oxygen rather than nitrogen. In examining what damage the pretreatment induced which increased the rate of proteolysis, an increase in hydrophobicity correlated well and this was induced in bovine serum albumin by formation of covalent bonds by exposure to OH· and by formation of non-covalent bonds by exposure to O_2^-. This could be prevented by urate, a physiological scavenger, as efficiently as by mannitol. As the exposure to free radicals was undertaken in the absence of lipids, the damage was not an indirect effect of lipid peroxidation followed by a chain reaction. In work on exposure of proteins to lipid peroxides, it was found also that the proteins decreased in solubility - demonstrating an increase in hydrophobicity (Roubal and Tappel 1966).

In vivo lipids form the essential hydrophobic barrier which retains the cells' controlled intracellular environment, whilst also providing a fluid environment in which enzymes, regulatory proteins and other lipids can move both laterally across the cell surface and rotationally, from the cytoplasmic to the extracellular side. This potential for interaction of membrane constituents is far greater than could be expected from a static membrane and may include complex interactions of control of transport and regulation mechanisms within the bilayer. The functional integrity of the membrane can be compromised by lipid peroxidation, which may result in cross-linking of phospholipid fatty acid side chains, in their fragmentation, or in alteration of fatty acid mobility by alteration of covalent

bonding. Lipid peroxides are not as reactive as free radicals, therefore they are longer lived, and, due to the inherent mobility of the membrane, literally in a position to have more widespread effects.

d. Iron.

In biological systems, iron is kept tightly bound both in transport by transferrin molecules and in storage bound to ferritin or in haemosiderin. However O_2^- has been found to utilize iron from physiological concentrations of ferritin in liver microsomes to form lipid peroxides (Koster and Slee 1986). Other workers have shown ascorbate to stabilize the ferritin shell, preventing accessibility of iron (Bridges and Hoffman 1986). Iron is also present in proteins such as the cytochromes and haemoglobin. Iron may be utilized for the Fenton reaction while still protein-bound. In a cell-free system McCord and Day (1987) found transferrin-chelated iron as efficient as EDTA-chelated iron in bringing about OH· production from a xanthine-xanthine oxidase system. Lactoferrin from specific granules could act as a source of iron (Ambruso and Johnstone 1981). Copper ions can also be utilized in the Fenton reaction and they, too, are found as an integral part of certain proteins, (e.g. caeruloplasmin). Caeruloplasmin is very labile in vitro (Winyard et al 1987) which may suggest that it could release its copper ions and promote the Fenton reaction in vivo. Its role is difficult to determine, but it is generally thought to have a ferroxidase role. One of the laboratory parameters for RA is a high serum copper level (Mussalo-Rauhamaa et al 1988). The availability of iron may be of importance in ischaemic damage in particular, in which an accumulation of damaged red blood cells may make localized iron levels high, and reperfusion restore oxygen levels to that required for PMN respiratory burst activity. In rat lung (Ward et al 1983), in which complement was activated by infusion of cobra venom, damage to the vascular endothelium was found in the presence of PMN. Chelating iron with lactoferrin (which is released from PMN specific granules) protected the lungs from

damage. Protection was almost total, regardless of the presence of iron, if the rats were first pretreated with an OH· scavenger, dimethylsulphoxide. The injury period was self-limiting and no damage was incurred if the rats were first made neutropaenic.

e. Protection.

In vivo, in an acute inflammatory response an invasion by phagocytes eventually retreats leaving no lasting damage in its wake which supports the theory that phagocyte reactive products are normally confined either to the phagocytic vacuole or to a contained microenvironment such as that created by the cell closely adhering to an extracellular substrate. It is likely that the modifications seen to occur in vitro when free radicals are investigated do occur naturally, but in a manner which can be controlled by scavenger enzymes and contained by repair mechanisms. The protection mechanisms described are dependent on one another to maintain health and a deficiency in any one area will affect the whole. SH groups form part of this system. There are intra- and extra-cellular mechanisms for limiting free radical damage. However glutathione peroxidase is the only extracellular peroxidase (Maddipati et al 1987). In an interesting study designed to examine the relative role of various antioxidants in human plasma, Wayner and co-workers (1987) found the following proportional contributions to the total antioxidant activity; urate 35-65%, plasma proteins 10-50%, ascorbate 0-24% and alpha-tocopherol 5-10%. However, the serum SH groups were preferentially lost (oxidized) first, even before urate. This implies a major role for these groups in preventing peroxidative damage and they are found to be present in lesser amounts in the plasma from patients with R.A. (Lorber et al 1964) who also have lowered levels of serum albumin - the major contributor of SH groups in plasma. O'Donnell-Torney and workers (1987) found that in human cell-line and normal human fibroblast cultures pyruvate scavenged H_2O_2 and that the cells secreted

pyruvate until the intra- and extra-cellular concentrations were the same, and were in the normal concentration range found in human plasma.

Ascorbate and alpha-tocopherol represent water soluble and lipid soluble protectants respectively. Ascorbate acts both intra- and extracellularly, whilst alpha-tocopherol resides in membranes. Ascorbate relies on an active transport mechanism to cross the plasma membrane, which has been found to be inhibited by activated complement in the fibroblast 3T6 cell-line (Padh and Aleo 1987). In disease states in which complement is activated, cells may be quite deficient in this important radical buffer whilst serum ascorbate levels are quite normal. As just mentioned, ascorbate has been thought to stabilize the ferritin shell, but it can also promote radical formation under certain conditions (Winterbourn 1986). The effects of ascorbate deficiency seen in scurvy may be of relevance in joint damage; ascorbate is essential for adequate hydroxylation of collagen by prolyl hydroxylase. In scurvy inadequately formed arrays of collagen fibres lead to fragile blood vessels and skin lesions (Stryer 1981). Deficient collagen fibre arrays are seen in rheumatoid articular cartilage. The physiological role of ascorbate may be sensitive to its immediate environment which would dictate whether or not its activity were to scavenge free radicals or promote their formation or to be available for keeping the iron in prolyl hydroxylase reduced. Also, oxidative damage to cartilage matrix leads to reduced levels of hydration which may make it less accessible to this water soluble protectant.

Alpha-tocopherol, or vitamin E, has a cyclic structure which acts as a free radical trap, breaking possible chains of radical formation in the vulnerable hydrophobic fatty acyl region of membranes. It is found to protect cells in vitro from chemicals which induce oxidation and/or lipid peroxidation, such as that caused by the anticancer agent adriamycin (Pascoe et al 1987a; 1987b). This effect was mediated by maintaining cellular glutathione, which in turn

maintained intracellular SH levels. The extracellular antioxidant role of alpha-tocopherol was the least contributary in plasma of those assayed and it seemed likely that the other extracellular protectants probably maintained alpha-tocopherol in its reducing state, for its crucial role in maintaining membrane integrity (Wayner et al 1987).

Lipid peroxidation may not only initially alter the fluidity of the membrane and its hydrophobicity and alter the availability of substrates for such enzymes as phospholipases and the mobility of substrate, regulatory and other enzymatic proteins, but also may cause a chain of peroxidation from lipids to proteins. Peroxidative damage of membrane proteins may result in loss of structure and function and in their rapid recycling, and may occur independently of lipid peroxidation, SH groups being particularly vulnerable.

Although damage by reactive oxygen metabolites may not be causative of rheumatic disease, they certainly contribute to the damage seen in the vasculature and joints in particular, in rheumatoid arthritis.

E. RHEUMATOID ARTHRITIS.

In rheumatoid arthritis the balance between the inflammatory response and the immune response which is found in normal tissues is defective. The primary target of this disease is diarthrodial joints. The alterations found locally in acute inflammation arise in the articular joints, persist as the condition becomes chronic, and changes occur both inside and outside the local area as the disease progresses.

i.The synovial Joint.

Diagram C illustrates the normal joint structure.

Changes seen in The Joint in Rheumatoid Arthritis:

A. Bone - Eroded at the cartilage-pannus interface.

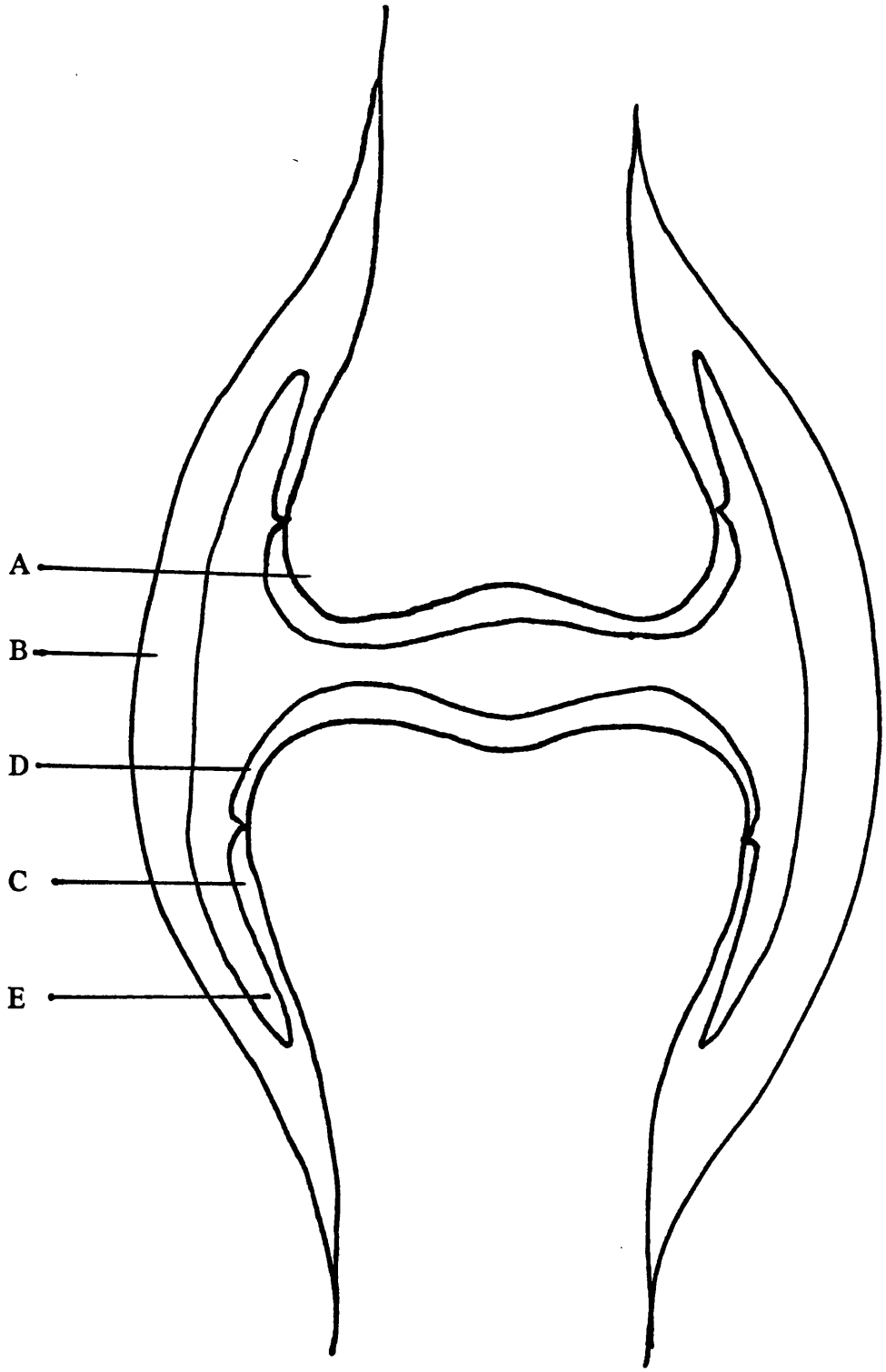
B. Capsule - Ruptured.

C. Synovium - Hypertrophic synovial and fibroblast cell layers with increased vascularity of oedematous tissuewith immune cellular infiltrate; forming invasive pannus encroaching on bone and cartilage.

D. Cartilage - Thinned and eroded.

E. Synovial fluid - Volume increased, viscosity and complement components reduced and immune cellularinfiltrate present.

Diagram C. The Rheumatoid Joint.



Normal synovium is nourished by the vasculature. The thin layer of synovial cells, or intima, is composed of A and B cells, which may be the same cell type at different stages of maturation. These cells supply the proteoglycans, core proteins and hyaluronic acid which give the fluid its characteristic viscocity, and also provide nutrients for the chondrocytes in the avascular cartilage, and remove debris. They rest on a lower, or subintimal, layer of less vascular connective tissue. The synovium ends where it meets the cartilage covering the opposing bone ends of the joint. This cartilage is aneuronal as well as avascular and is composed of type II collagen which surrounds the chondrocytes which synthesize it. The collagen is arranged parallel to the surface in the superficial layer, being less well organised further down, near the site of synthesis. It retains well-hydrated proteoglycans which give it its cushioning properties (Dieppe at al 1985).

The features of the rheumatoid synovial pannus in some respects may resemble lymphoid tissue, as the invading T lymphocytes and plasma cells can aggregate to form lymphoid follicles which secrete antibodies, namely rheumatoid factor. This forms a major part of the evidence for immune system involvement in the persistence of symptoms which typify RA (Dieppe et al 1985).

Activation of complement in synovial fluid by rheumatoid factor aggregates or immune complexes releases leukoattractants so a persistence of infiltrating PMN may be involved in alteration of and damage to joint structure and to the local vasculature, and eventually may lead to more widespread effects.

Inter-cell regulation can be found with HAGG-stimulated PMN and MNC (Appelboom et al 1988); low concentrations of HAGG induced a factor from MNC which inhibited adherence of PMN which were also directly inhibited by

high concentrations of HAGG. This factor was found in RA tissues and fluids. Blackburn et al (1987) also found a low molecular weight factor (10KD) in RA synovial fluid which enhanced immune complex-mediated degranulation and phagocytosis in PMN, also found in the supernatant from cultured monocytes. Soluble or fixed immune complexes induce TNF-alpha secretion by monocytes which is a powerful priming agent for PMN (Debets et al 1988). Regulation of FcR function can be seen to be complex and to have the potential for involvement in immune dysfunction in disease states such as RA. The attachment (PI-linked or transmembrane protein) may be important in chronic inflammatory conditions because elastase, which is released from activated PMN, cleaves PI-linked receptors (Tosi and Berger 1988). Both enzyme release and O_2^- production in PMN were markedly inhibited by hydrocortisone treatment (Roos et al 1976). This may occur with steroid therapy in RA. This disease is a condition in which the immune system is persistently activated, and down regulation of FcR function maybe attributed to either premature release of FcR111, or internalization of FcR11.

Infiltration of cells may be controlled by a number of interacting factors, amongst which are the products of the cylo-oxygenase pathway and lipoxygenase pathway of arachidonic acid metabolism (Zeitlin 1981). Prostaglandin E_2 (PGE_2) is primarily a powerful vasodilator, but it also potentiates the activity of other factors such as bradykinin and histamine (Keele and Neil 1978). Indirectly it affects the chemotactic activity of leukotriene B_4 (LTB_4), which is ineffective if cells are unable to exit from the vasculature. These agents may also modulate the role of other regulators of the immune response which are active in inflammation, such as TNF-alpha (Renze et al 1988) and IL-2 (Flescher et al 1989) by PGE_2, and be regulated by others in turn, such as IL-1 (Gilman et al 1988). The interactions of cytokines which may

be involved in the immunomodulation of inflammation are reviewed by Balkwill and Burke (1989).

ii. Mediation of Damage.

As has been described in the previous section, damage to biological tissues can and does occur in the presence of free radicals; their action with respect to joint damage is under investigation, and so is their possible role in promoting the chronic state. When induced by a soluble stimulus the respiratory burst of neutrophils is not accompanied by the release of azurophilic granules. An important consequence of this could be that there a lack of competition from MPO for hydrogen peroxide, which can thus be freely available for the Haber-Weiss reaction with subsequent OH· formation. Also, it has been suggested (Thomas et al 1983) that some of the chloroamine products of MPO activity protect PMN from cytolysis and render them inactive, thereby controlling their contribution to inflammation and protecting the environment from the uncontrolled release of their contents.

In an inflammatory response in which infiltration of IgG occurs into the synovial fluid or its production occurs in the synovium, the invading phagocyte population is not activated by monomeric IgG. The presence of antigens causes small soluble complexes to form. These can activate PMN and be disposed of by phagocytosis. But there are at least two mechanisms for perpetuating the normally antigenically-induced response of PMN without the intrusion of a foreign antigen; free radicals alter the antigenic properties of IgG molecules which may cause them to fix complement without the presence of foreign antigen (Hewitt et al 1987) and the antibody-complement complex can activate the PMN respiratory burst. Also RF secreted by the rheumatoid pannus into the synovial fluid can bind circulating immunoglobulins which form part of the vascular infiltrate, to form complement-activating immune complexes, which

not only activate PMN but also present to the phagocyte as an insoluble stimulus.

Anaemia in the R.A. patient may exacerbate the inflammatory condition if MPO forms an important part of free radical regulation and thereby inflammatory control. A feature of anaemia in experimental animals is a reduction in MPO, which is a iron-containing enzyme, with no parallel drop in NADPH oxidase activity (Murakawa et al 1986). Work by Blake's group on the possible role of iron in synovitis and joint damage, in rats with adjuvant induced arthritis, using desferrioxamine (DFX) to remove iron (Andrews et al 1987a), and using diet-induced mild iron-deficiency (Andrews et al 1987b) established that it was indeed iron removal by DFX, at a concentration which did not affect haemoglobin levels, which prevented the joint symptoms found in the controls (swelling, bone erosion), and in both papers showed that the immunological features, such as the acute local inflammatory response, raised serum levels of acute phase reactants and granulomas in peripheral lymph nodes, were still present. This suggests that the joint as such may be an iron-sensitive environment.

Blake et al (1989) also investigated the human knee joint. In the inflamed joint, there was found to be no means of compensating for the increase in pressure within the resting joint, and work done brought about a further increase in pressure with resulting exclusion of capillary flow and hypoxia. Hypoxic damage and that due to pressure itself was exacerbated by reperfusion when the joint was rested and pressure was relieved. A review by Levick (1990) discusses this work and supporting evidence of hypoxia and acidosis from others. Membrane proteins are damaged and red blood cells become leaky under hypoxic conditions (Deuticke et al 1987), and iron released from damaged synoviocytes and capillary endothelial cells with oxyen supplied by the resumption of blood flow could combine to promote the formation of free

radicals either in the presence of PMN or MNC, or utilizing released enzymes such as xanthine oxidase from capillary endothelia (Allen et al 1987). Blake and workers found evidence of lipid peroxidation and a change in IgG consistent with free radical activity within the reperfused inflamed joint. Work with cat intestine has increased the evidence for reperfusion injury, implicating free radical formation, iron and PMN, particularly in the primary step of microvascular permeability (Granger 1988).

The rheumatoid joint is difficult to investigate for lack of an ethical control. Also, as the rheumatoid condition is immunosuppressive, there is an enhanced likelihood of infection when synovial fluid is withdrawn. The rat air pouch model provides a region of tissue which develops structural and histochemical similarities to the synovial cell layer. Morris et al (1987) found that antigenic challenge with bovine serum albumin induced an increase in the production of the iron binding protein apoferritin in parallel with the inflammatory response, in A cells ('macrophage-like' synovial cells) in the pouch lining. In a second challenge with blood further apoferritin production took place, with deposition of iron in secondary lysosomes in conjunction with an increase in structural rearrangement of cells to form a deep layer of lining cells, an organized connective tissue substratum and vascular proliferation. Blake et al (1984) noted that iron deposition correlated with persistent disease. These findings suggest that the inflammatory response and the response to iron load induce apoferritin synthesis, but that the iron may also induce features of the rheumatoid pannus. Other elements of the red cell cytoplasm were not excluded, and so although the evidence points to iron as the active agent in this pannus-like formation it is not conclusive.

Blake's work in the human knee supports the finding of Hewitt et al (1987) in the rat air pouch model that altered IgG is to be found in inflammation. This finding in itself may show that such products, whilst indicative of damage by

radicals, may not be sufficient alone to induce chronicity, and can normally be removed by phagocytes from the area. However, their significance in the presence of rheumatoid factor has already been described. Other factors may combine to induce tissue injury and a chronic disease condition, the presence of which may be promoted by free radicals. Fragmentation of the macromolecular complexes in cartilage by H_2O_2 has been found in vitro, using neonatal human articular cartilage (Roberts et al 1989). This may reduce hydration and reveal new antigenic sites and sites which attract phagocytic cells intent on removal of damaged tissue. Roberts et al noted that the effects found with human tissue were not seen in rat tissue. Ugai et al (1983) investigated the activity of PMN in the presence of rheumatoid articular cartilage in vitro, compared with both normal cartilage and that removed from joints of patients with other articular disease, such as osteoarthritis. Although they found that large numbers of PMN attached to immune complexes embedded in the articular surface of the rheumatoid samples compared to only a few attached on the other samples, in the presence of autologous synovial fluid (SF), either fresh or heat-inactivated, this attachment was not seen. The conclusion to be drawn from this anomalous result is not really clear. It suggests that SF contains a heat-stable protectant, and that PMN in vivo therefore do not indulge in the activity seen in its absence in vitro, even though attractive sites are present. Collagenase activity was localised in excised rheumatoid tissue to the pannus cartilage interface (Woolley et al 1977). Mohr et al 1981) found on electron microscopic analysis that PMN were present at the pannus cartilage interface in excised tissue from rheumatoid joints, and that they were deeply embedded in the cartilage matrix. At such a site they might be remote from a synovial fluid inhibitory factor. The rat pouch model suggests that the PMN production of reactive oxidants promotes the production of the pannus. Damage to the cartilage may be by cells in the pannus including synoviocytes, monocytes and macrophages. The localization of active collagenase suggests that an interaction with a factor (activator) in synovial fluid

may be required by these cell types, and it is at this pannus cartilage interface that damage to cartilage and to bone is seen to occur.

Free radicals may promote damage; they may also recruit more cells to the inflammatory site, either directly or by promoting the induction of chemotactic or other agents which contribute to damage or to inflammation. Cells found in acute inflammatory synovial fluid are initially PMN. These are later (about six hours later) replaced by mononuclear cells, both monocytes, and lymphocytes. In chronic states, PMN persist, and both MNC and PMN are seen to invade the intimal and subintimal layers of the synovium. The process of inflammation includes not only attracting these cells to the synovium, but also diverse effects on other cell types such as vascular endothelia, which will alter the permeability of the vasculature to allow exit of blood, and will alter the adhesive interactions between blood cells, platelets and endothelial cells. These interactions in turn promote synthesis of inflammatory mediators such as vasoactive amines and prostaglandins, and cytokines such as the interleukins and interferons.

iii. Aetiology and Pathogenesis of RA.

The products of the major histcompatability locus (HLA antigens) are expressed as antigenic determinants on all cells and identify them as constituents of the same organism. Normally, the class II, or Ia, antigens are expressed exclusively on cells of the immune system, and are required in particular for antigen presentation to T lymphocytes in the course of mounting a humoral immune response. HLA typing has led to the conclusion that certain tissue-types may predispose that element of the population to a certain disease, when they are exposed to the right "trigger", which may include environmental factors. For instance Bantu people living in rural areas have a very low incidence of RA, whereas those who live in urban areas have the same, higher, prevalence as their white contemporaries (Dieppe et al 1985).

In the caucasian population the incidence of HLADR4 is twice as high in patients with RA than in the normal population (Lanchbury et al 1988). Population studies have ascertained that environmental factors probably determine whether or not the antigenically identified susceptible group will in fact develop disease in the case of RA, and a number of studies suggest primary triggers. The most overt symptom of RA which must define it as an autoimmune disease is the production of antibodies to the Fc portion of the patient's own immunoglobulin G. However, patients who have no such antibodies (i.e. rheumatoid factor, RF, negative) often have no other symptom which distinguishes their disease from those who are RF positive, although their prognosis may be better. Antibodies to phagocytes have been identified and associated with damage to the vasculature (Specks et al 1988; Jennette et al 1988).

Sometimes a bacterial infection precedes the onset of RA, and one theory suggests that there is cross-reactivity of bacterial antigen and a component of the infected body. For instance monoclonal antibodies to Yersinia pseudotuberculosis cross-react with cells expressing HLA-B27 - a phenotype which occurs in a significant proportion of patients with ankylosing spondylitis. Another theory suggests that an infecting mycoplasma may adsorb antibody to itself and alter it in some way, inducing antibodies to it, that is, induction of rheumatoid factor. The influence of viral DNA expression in lymphocytes or expression of viral antigen on cell surfaces is also under investigation (Mills 1989). These pathogenic triggers may lie dormant for years, and in the susceptible individual require a further environmental activator for the disease to manifest itself.

FcRs are important in immune complex clearance, and currently under investigation is any link between FcR activity and HLA haplotype (Salmon et al

1986a;1986b; Lawley et al 1981). Most clearance of immune complexes takes place in the spleen and liver where there are macrophages bearing large amounts of FcR111. In a patient with a disease involving excessive clearance of IgG-associated platelets, treatment with Mab 3G8 (anti-FcR111) was temporarily extremely effective (Clarkson et al 1986). There is considerable reproducable inter-person variation in the normal expression of FcR11 on platelets, which affects their function in vitro. This could be genetic and could have important implications in disease states (Rosenfeld et al 1987). It has been found that FcR can be expressed on endothelial cells in vitro, after infection with Herpes simplex virus (Cines et al 1982). If this is also the case in vivo it might be of relevance in diseases where immune deposits are thought to be involved in mediating vascular damage, and introduce a new theory about the consiquence of infection in immune regulation, other than cross-reactive or genetically-linked autoimmunity.

It is likely that there is more than one phenotype which predisposes an individual to develop what is classified by the single diagnosis RA, under particular circumstances. The variations in symptoms, clinical outcome and response to treatment may reflect this but may depend on any number of other, unidentified, genetic factors.

iv. Non-Steroidal Anti-Inflammatory Drugs.(NSAIDs)

This broad category of drugs is used in RA as a first line in control of the symptoms attributable to inflammation but not erosive disease, such as pain and swelling and as such they improve motility and a feeling of well-being in the patient. They are quick-acting and their effects of short duration, only lasting while the drug is at an optimum concentration in the circulation. Patients taking NSAIDs provide the control patients for investigating second line therapy, as the disease process is not thought to be affected by these drugs and there are no

drug-free RA patients. NSAIDs include several types of acid, which may contribute to their differing effects in vitro.

NSAIDs are lipophilic acids, which readily insert into the plasma membrane, especially under acidic conditions (Lombardino et al 1975). Most NSAIDs in plasma are protein-bound and when binding sites on albumin are saturated they may bind to other proteins, with unknown effects (Schlegel S.I. 1987). Dosage varies both with the particular drug and with the patient, an empyrically-determined dose being a balance between effect and side effects. NSAIDs are readily absorbed through the gut where gastrointestinal problems (bleeding, ulceration) are a particular problem. The range of dose is from the tens of milligrammes to several grammes a day. The drug level and availability in plasma may differ. Thus high concentrations used in work in vitro, may not reflect that available physiologically.

An effect of NSAIDs is the inhibition of cyclo-oxygenase and thereby the production of stable prostaglandins (PG) (Vane 1971). Aspirin irreversibly acetylates PG synthetase, thus routing arachidonic acid metabolism through the lipoxygenase pathway. Other NSAIDs inhibit PG synthesis but not by acetylation, and the effects in vitro may be reversed by washing (Moskowitz 1986). NSAIDs directly inhibit vasodilation due to PG. PG also has influences such as suppressing the production of TNF-alpha by human MNC (Spengler et al 1989), and IL-2 by mouse spleen cells as seen with indomethacin treatment (Wolf et al 1985). The repression by NSAIDs of these actions of PG and the preferential channelling of arachidonic acid to produce lipoxygenase products may be equally as important for NSAID action as the inhibition of the direct effects of PG inhibition. It may even be considered that the important effects of NSAIDs are those brought about in cell membranes by drug which has not been bound by serum proteins or utilized for prostaglandin modulation.

NSAIDs have effects other than PG inhibition, and their differing effects depending on the stimuli used for the cellular response under examination may suggest selective effects on various activation pathways. In vitro Bell et al (1986) found piroxicam, inhibited opsonized zymosan-stimulated O_2^- from MNC in a dose dependent manner, whereas four other NSAIDs tested had no effect below 10^{-4}M. Inhibition of FMLP binding and O_2^- generation in MNC by piroxicam, was not seen in activation by other stimuli such as opsonized zymosan and PMA (French et al 1987). These seemingly different results for the response to opsonized zymosan may be a dose effect; the first work using 25ug/ml opsonized zymosan and the second using 2mg/ml. Using PMN taken from RA patients (from peripheral blood and from synovial fluid) and from controls after piroxicam administration Biemond and workers (1986) found O_2^- production depressed after either opsonized zymosan (concentration not specified) or PMA stimulation, and in PMN membrane preparations after pretreatment of whole cells piroxicam was effective at inhibiting the NADPH oxidase activation step. Paradoxically piroxicam inhibition of FMLP-induced O_2^- production from PMN has been found to be greater in the presence of PG than without it (Abramson and Weissman 1989).

Sodium salicylate may protect partially against the inhibitory effect of IAP on the PMN O_2^- and aggregatory response to FMLP (Abramson and Weissman 1989), but in common with indomethacin it has been found in vitro to have no effect on proteoglycan synthesis, which can be derepressed by piroxicam interacting with an inhibitory factor in RA synovial tissue (Herman et al 1989). Thus the effects of NSAIDs would appear to be more diverse than simply inhibiting PG sythesis and their other effects may be dependent on the particular drug and dose and on the pathway being modulated. In some instances differing findings between drug effects may be irrelevant to their antiinflammatory role or may indicate that there are different routes which bring about the same end.

may indicate that there are different routes which bring about the same end. They may also reflect only an in vitro situation not found in vivo depending on the dose of drug available at the site of action, namely the joint, as well as that available to peripheral blood leukocytes. So far only inhibition of PG has been found to correlate with potency (Flower et al 1972). Diverse modes of action may be clinically useful in that patients who receive no relief from one drug, or who incur unpleasant side effects, may benefit from a different NSAID. It is significant that despite these diverse effects on cell function NSAIDs have no clear effect on the progression of rheumatic disease.

v. Gold Compounds.

In this country gold is usually administered as the injectable gold "salt" sodium aurothiomalate (GST), or is given orally as the lipid-soluble auranofin (AUR). In either case the effect of the drug is long-lasting and takes a correspondingly long time to take effect (4-5 weeks or longer). The difference in solubility may allow different sites of activity. Mechanisms of action may also be different; patients with adverse reactions to intramuscular gold being able to benefit from AUR (Manthorpe et al 1988). Intramuscular injections of sodium aurothiomalate are usually given weekly in increasing amounts until a total of 1g has been given, after which the injections are given at wider intervals, decided empyrically. A second course of gold is not usually effective and so avoidance of a flare is important. AUR is given once or twice daily in oral doses of less than 10mg (British National Formulary 1988). The serum concentrations reflect the lipid solubility of AUR, Herrlinger et al (1982) finding 600ug/l AUR and 800ug/l GST in patients on these respective therapies for at least 3 months, with all the GST in the plasma, and about 40% of the AUR associated intracellularly. These workers found AUR five times more readily taken up than GST by leukocytes in vitro when incubated in physiological concentrations. Gottleib (1986) found weak binding of AUR SH groups, and Crook and Snyder (1986)

suggested that the distribution of AUR could be explained by sequential sulphydryl exchanges. Preferential binding to thiophosphines and thioesters may radically affect cell function, and includes interaction with DNA. These workers found that all doses tested increased the activity of phospholipase C in vitro, suggesting also that the co-administration of NSAIDs may counteract the increase in intramembrane enzymic activity.

In rat adjuvant arthritis injected gold was not found to have any effect on the arthritis nor did it suppress non-specific inflammation (Jessop and Currey 1968) indicating caution for extrapolation of work from animals. In vitro, gold can affect IL-1 production in various cell types, having a biphasic, dose-dependent effect on human mononuclear cell cultures (Epstein et al 1989), differentially affecting proliferation depending on the stimulus, IL-1 production and IL-2 receptor expression (Blitstein-Willinger 1987). It has been suggested to have a selective effect on collagenase activation (Martel-Pelletier et al 1989), activating that from chondrocytes and not that from monocytes, the suggested benefit of this being a reduction in the active life of the enzyme. However, AUR was not seen to activate collagenase. In mouse calvaria (Klaushofer et al 1989) 1uM AUR demonstrated PGE_2-mediated induction of bone resorption, and at higher doses (3uM) inhibiting that induced by PGE_2 and other agents, and having selective cytotoxic effects on bone cells. The inhibitory effect of AUR in vitro on PMN chemotaxis and LTB_4 synthesis has been found to be dose-dependent (Elmgreen et al 1989), and similar dose dependence has been found with its effect on PMA-stimulated PMN O_2^- production and the levels of phosphorylation induced (Hurst et al 1989).

Both gold and Dpen lower RF levels in vivo (Jaffe 1976). Gold (sodium au-glucose) interacted in vitro with selenium on glutathione peroxidase (Dillard and Tappel 1986), an effect seen with Dpen also, which suggests that both the gold

and the SH moieties of metabolised GST and AUR may be involved in the effects seen in vivo and in vitro.

AUR decreased chemotaxis in normal PMN and those from RA patients (Movat 1976). Dpen had no effect. The relevance of investigating a parameter of cell function in establishing the mode of action of a drug may be gauged in the light of comparison with different drugs. The adverse effects of gold therapy are closely mirrored by those of Dpen (Day and Paulus 1987). It has been suggested (Epstein 1989) after thorough scrutiny of the literature and the statistical data in particular, that the clinical evidence does not reveal a net beneficial outcome of gold therapy and that its use may be outdated. However on an individual basis significant benefits may be seen in particular patients for many years (Davies J. personal communication).

vi. D-penicillamine.

Although Dpen is readily absorbed through the fasting gut, like the gold compounds it may take several weeks to show any beneficial effect in RA and a correspondingly long time after cessation of drug therapy for side effects to disappear. As with the gold compounds, Dpen is introduced over several months in increasing doses until benefit is noted. It is given orally, a typical maintainance dose being 500mg daily.

DPen can reduce oxidized SH and so may inactivate or activate receptors and enzymes, depending on the particular role of accessible SH groups. This lack of specificity could account for some of its side effects as well as its beneficial properties. Its beneficial actions in RA have not been precisely determined but thought to be relevant are formation of a) disulphide links b) thiazolidine rings and c) metal complexes and chelates (Howard-Lock 1986).

thought to bring about clinical improvement in scleroderma by the thiazolidine ring formation with collagen preventing or dissociating cross-links (Steen et al 1982). This could also be the case with collagen cross-linked after damage by hydroxyl radicals. Metal chelates or complexes formed with Dpen may contribute to the reduction seen in serum copper levels, an effect seen in common with gold. However, a dietary supplement of copper in RA had no effect on the effects of Dpen (Multicentre Trial Group 1973). Complexed to copper Dpen has SOD activity (Youns et al 1977), and as a weak iron chelator it may be protective in the rheumatoid joint. Dpen is ineffective in ankylosing spondylitis, in which copper levels are also raised, which suggests that any beneficial effect of copper depletion or sequestration by Dpen may be on the inflammatory aspects of the disease which are not significant in AS, rather than immunomodulatory ones. It has also been found to prevent continued periarticular bone erosion, but the mechanism for this important finding remains undefined (Gibson et al 1976).

In vitro, Dpen selectively affected survival of monocytes over lymphocytes in mixed cell cultures (De Vries et al 1982), and inhibited lymphocyte proliferation; an effect much more rapid in the presence of copper sulphate (Lipsky and Zeis 1980). Subsequently Dpen with either caeruloplasmin or copper ions inhibited T-cell proliferation and helper function, effects inhibitable by MPO or catalase (Lipsky 1984). Cuperus et al (1987) suggested that Dpen reacts with MPO in the presence of HOCl to form a stable inactive form of the enzyme. Monocyte accessory cell function has been found to be normalised in vitro in RA patients on Dpen compared with the deficient response of patients on NSAID alone. The NSAID response was significantly improved by the addition of SH (2-mercaptoethanol) whereas it had no effect on the normal or Dpen cells, and PHMPSA incubation (SH blockade) of normal cells was inhibitory (McKeown et al 1984). Defective suppressor cell function of IgM

improved by the addition of SH (2-mercaptoethanol) whereas it had no effect on the normal or Dpen cells, and PHMPSA incubation (SH blockade) of normal cells was inhibitory (McKeown et al 1984). Defective suppressor cell function of IgM synthesis was noted in patients on NSAIDs, partially corrected by the addition of 2-ME, whereas the response of Dpen was already normal, and not affected by 2-ME (Brown-Galatola 1989).

v. SH-Containing Drugs.

When treatment of RA by non-steroidal anti-inflammatory drugs fails to prevent inflammation, or significant progressive erosion requires other treatment, the second line therapy includes gold-containing drugs and D-penicillamine (Dpen), antimalarials. All the effective gold-containing compounds contain SH, as does Dpen. None of these drugs is targetted to a specific tissue which may contribute to their toxic side effects. Why these should prevail in some patients and not in others is not predictable. Similarly tolerance varies from drug to drug with any one patient, which may suggest that despite their having some frequent side effects in common, such as skin rashes, mouth ulcers or temporary proteinurea, different drugs may bring them about by different mechanisms.

vi. Other Anti-rheumatic Drugs.

Further therapy may include the use of antimalarial drugs such as chloroquine and hydroxychloroquine, and treatment of more resistant disease by steroids or immunosuppressants. These will not be discussed here.

With all antirheumatic drugs the effects are a balance between beneficial effects and unwanted side effects which seem to emanate from the lack of specific targetting, a situation which cannot be resolved until the targets have been identified.

F. SULPHYDRYL GROUPS.

i. The Role of SH Groups.

Modification of cell functions and molecular structure by free radicals and their derivatives has been described already in this introduction and modulation of these effects by sulphydryl groups has been indicated. SH can act as a buffer against oxidant activity and maintainance of the reduced state may be necessary for normal functioning of membrane proteins. Albumin acounts for 86% of SH in serum (Thomas and Evans 1975), and in RA serum albumin levels are decreased and globulins raised, with lowered SH on both, as is found in other disease states such as cancer and bronchitis. Serum thiols on albumin can be oxidized by activated PMN (Hall et al 1984). Any deficiency in the level of albumin or the reduced state of these SH may leave other SH, such as those exposed on cell surfaces, vulnerable to oxidation. Such plasma proteins as activated complement depend on free SH for normal function (Lambris 1988). C3b normally undergoes covalent binding at numerous sites through a cysteine residue and defective C3b activity may modulate immune function (Ochs et al 1986). Also PMN adhesion may be dependent on the surrounding SH buffering system, through the leukocyte antigen CD18 molecules which have disulphide-rich regions (Schwartz and Harlan 1989). The intracellular milieu is not a closed system, and events outside the cell will be reflected within it. To maintain SH in their reduced or oxidized state for normal functioning the main intracellular SH buffer is glutathione, where it is also important for reducing non-SH species such as organic peroxides (Stryer 1981). It is maintained in its reduced form by glutathione peroxidase, which requires NADPH supplied by the hexose monophosphate shunt. Glutathione peroxidase can be found in cells and in plasma as two distinct enzymes (Takahashi et al 1987). In normal tissues

the level of GSH peroxidase is high enough to inhibit peroxide activation of cyclooxygenase (Hemler et al 1979). Mammalian cells also contain thioredoxin and its reductase to act as a further buffering system (Holmgren 1985). The section devoted to damage by free radicals and other oxidants has indicated the importance of serum SH in buffering against oxidative damage (Wayner et al 1987) and the vulnerability of SH on proteins (Davies et al 1987a-f). Levels of the peroxidases are low in RA; selenium supplementation can increase the levels except in PMN (Tarp U. 1986). The significance of the low serum levels of reduced sulphydryls found in RA (Lorber et al 1964) cannot be ascertained without an understanding of what the role(s) of SH may be both extra- and intracellularly in maintaining healthy cell function. An indication of the involvement of immune regulation in RA is the finding that serum SH levels correlate with in vitro synthesis of IgM and IgG (Brown-Galatola 1989), and that cells from patients on SH-containing drugs seem to function in vitro in a manner paralleled by treatment of cells from RA patients not on these drugs with SH-containing reagents such as 2-ME (McKeown et al 1984).

ii Effects of SH Modulation In Vitro.

Work has centred on altering SH groups and monitoring the effects, particularly because the most useful second line antirheumatic drugs, such as D-penicillamine and gold compounds contain SH groups. For investigating altered availability of SH, impermeant SH blockers have been used such as parachloromercuribenzene sulphonate (PCMBS) to demonstrate a lack of effect on PMN phagocytosis (Tsan et al 1976a), and adhesiveness (Giordano et al 1973), the cell permeant agent N-ethylmaleimide (NEM) inhibiting both of these events and totally abolishing O_2^- activity by all stimuli tested (Akard et al 1988). Parahydroxymercuriphenylsulphonic acid (PHMPSA) has been used to show lack of inhibition of FMLP binding (Hurst et al 1987). Cell permeant 2-

mercaptoethanol (2-ME) has been used (McKeown et al 1984), as also has dithiothreitol (DTT) to reduce SH (Adunyah and Dean 1986).

In cytosolic preparations, peroxidation of glucocorticoid receptors inhibited their normal activity, a situation reversed by DTT (Tienrungroj et al 1987). Discussing the role of cysteine residues, Malbon et al (1987) noted that proteins from G protein-mediated receptors in particular, changed their electrophoretic mobility on SDS PAGE after reduction, suggesting altered conformation. Similarly, Dpen treatment has been found to alter the position of alpha-1 antitrypsin on isoelectric focusing by irreversible modification of a free cysteine residue (Whitehouse et al 1989). Pedersen and Ross (1985) found in phopholipid vesicles containing beta-adrenergic receptors and G_s proteins, that DTT activated the receptor and that this indirectly potentiated GTP binding to G_s. This suggests that reduction of disulphide bonds could form part of the receptor ligand interaction causing a G-activating conformational change. NEM blocked GTP binding and autokinase activity in p21 ras, an oncogene product with extensive sequence homology with G proteins in the GTP-binding region (Hattori et al 1986). In human fibroblast cultures insulin activation of the hexose monophosphate shunt was found to be mimicked by serum in conditions of low extracellular glucose. This was sensitive to H_2O_2, an effect which, in turn, could be reversed by DTT (Germinario and Vlachopoulou 1987). Blocking external SH groups had no effect on transport nor did it alter the effect of H_2O_2. These results suggest that hexose transport activation involves oxidation of intracellular SH groups. However, D'Amore and Lo (1986a and b) found that glucose transport activation in undifferentiated L6 rat myoblasts was dependent on extracellular SH groups at a site distal to the binding and transport site itself. These differing results may be a reflection of the different cell-types used. Both, nonetheless, demonstrate essential roles for SH groups in hexose transport.

In PMN, the activity of the HMP and O_2^- production could be impaired simultaneously by external SH blockade with PCMBS; however phagosytosis still occurred, unless the blockade was of intracellular SH using NEM (Tsan et al 1976a; 1976b). The PCMBS-inhibited glucose metabolism was preventable by pretreatment of cells with a high concentration of cysteine, which suggests that the inhibition seen was an indirect one perhaps affecting the GSH buffering system. Giordano et al (1973) found that PCMBS did not affect adherence or motility, whereas NEM did in a cysteine-reversible manner; the presence of serum required a tenfold increase in dose for the same effect as in phosphate buffered saline. SH-mediated effects have been found also in monocyte accessory cell function (McKeown et al 1984; Fidelus et al 1987); in platelet release of calcium (Adunyah and Dean 1986), and aggregation (Sugatani et al 1987) and on the biregulation of platelet alpha-adrenergic receptors by PGE_1 and adrenaline, in which only inhibition by adrenaline was prevented by NEM (Jakobs et al 1982); in intracellular calcium mobilization by $[GSH]_i$ in rat hepatocytes (Thor et al 1985); in T suppressor cell activity and <u>in vitro</u> Ig synthesis (Brown-Galatola 1989); in PMN adhesion (Schwartz and Harlan 1989) and respiratory burst (Rajkovic and Williams 1984); in hexose metabolism (Germinario and Vlachpoloulou 1987); and in lymphocyte activation and proliferation (Fidelus et al 1987). These diverse examples of SH involvement in cell function, and the benefits seen clinically in treatment of RA with SH-containing drugs make further investigation necessary to establish the relevant effects for disease modification to allow better targetting and a reduction in side effects.

iii. SH in this project.

This project has used blockade of PMN and MNC with the impermeant SH-blocker PHMPSA to determine whether or not SH groups are involved in the external events of signal transduction.

PMN have been studied for changes in internal calcium flux, and in azurophilic granule release. Assays for the production of O_2^- and H_2O_2 have been used to monitor these phagocyte-specific functions, allowing the use of mixed mononuclear cells for technical simplicity to study monocytes, and allowing a comparison of PMN and monocyte activation. Cells from RA patients on NSAIDs or on SH-containing drugs have been used as in vivo models of a disease control group and SH-treated disease groups respectively and those from healthy volunteers used both as normal controls and for investigation in their own right. It was hoped that as well as demonstrating any SH involvement in the activation pathways investigated that the experiments as a whole might add usefully to knowledge of the pathways under scrutiny.

Note:- Membrane Fluidity.

Membrane fluidity has been measured to investigate cell membranes in health and disease. Fluidity of the plasma membrane is a function of its constituents, their relationship to one another, their structure and their activity. Alterations in plasma membranes have been found in disease states such as RA (Beccerica et al 1988), muscular dystrophy (Wilkerson et al 1978) and in leukaemia (Inbar et al 1973);at different stages of cell maturation (Roozemond and Urli 1979) and on ligand-binding (Heron et al 1980). Experimental probes have been used which penetrate the membrane to varying degrees and which undergo rotational and translational motion within the confines of the membrane as dictated by the

fluidity of the membrane region into which they have been incorporated. Incorporation is assumed to be in the "spaces" between the lipid moieties of phospholipids. This can be detected by an alteration in emission intensity of vertically and horizontally polarised fluorescence from which the microviscosity of the membrane can be calculated. From the calculation a change in microviscosity may be detected under given circumstances.

A major restriction on the usefulness of this technique is that the complex plasma membrane is made up of multitudinous proteins, glycoproteins, plycolipids and phospholipids in microdomains. Probes such as diphenyl hexatriene (DPH) can give an overall picture of change throughout the membrane, small regional changes not being identifiable. However, other probes may be more specific and may measure fluidity at different depths in the membrane.

In this project it was not known to what PHMPSA would bind not what the effects(s) of this would be. DPH was used to determine if any general effect could be detected, by measuring fluidity in cells with and without preincubation in PHMPSA. The method followed was that used by Beccerica et al (1988) for their investigation of fluidity in lymphocytes. Unfortunately the work was discontinued after a short time as the polarising lenses required alteration and it was not possible to regulate temperature as precisely as thought necessary. Reference to the results has been made in the discussion.

CHAPTER TWO

MATERIALS AND METHODS

1. MATERIALS.

Equipment Suppliers.

Spectrophotometer:	LKB, Uppsala, Sweden.
Automated Micro ELISA Reader:	Dynatech Instruments, California. U.S.A.
Microscope:	E Leitz (Instruments) Ltd., Bedfordshire. England.
Water Bath:	Grant (Instruments) Ltd., Cambridge. England.
Centrifuge:	MSE Scientific Instruments, Sussex. England.
Vortex Mixer:	Gallenkamp, Leicestershire. England.
Concentrator:	Amicon Corporation, Massachusetts. U.S.A.
Spectrofluorometer:	Shimadzu Corporation, Kyoto. Japan.

Computer:	Amstrad plc.,
	Essex. England.
Glassware:	Richardsons of Leicester,
	Leicestershire. England.
Plastics:	Elkay Ltd.,
	Hampshire. England.
	and
	Luckham Ltd.,
	West Sussex. England.

All reagents and buffers supplied by Sigma Chemical Co Ltd., Poole. Dorset. except those listed below:

Hypaque 90	Winthrop-Breon Laboratories, New York. U.S.A.
Phosphate buffered saline tablets:	Oxoid Ltd., Hampshire. England.
Potassium and Sodium Salt	BDH Ltd., Bristol. England.
Hanks buffer:	Gibco Europe Ltd., Paisley. Scotland.
Ficoll 400	Pharmacia, Milton Keynes. England.

Cell Separation Reagents

1. Ficoll-metrizoate gradient; 50ml Hypaque 90

+ 2.95ml dist H_2O

+ 238.275ml 9% Ficoll 400

Filter sterilized and stored at room temperature in the dark.

2. Phosphate Buffered Saline; 1 PBS tablet (Oxoid)

with Glucose (PBSG) + 0.16g Glucose

to 100ml with dist H_2O

Made fresh as required and not stored.

3. PBSG with Calcium (PBSG*) as above +0.3mM Calcium chloride

IgG Purification Buffers

1. 10/150 Buffer; 0.1M Na_2HPO4 (A)

(Stored at 4°c) 0.1MKH_2PO4 (B)

Added B to A with mixing until

pH 7.4 - diluted resulting

solution x10 in dist H_2O and

added 8.77g/l NaCl

2. 1M Acetic Acid; 6ml Glacial Acetic Acid

(Stored at 4°c) + 94ml dist H_2O

Hydrogen Peroxide Assay Reagent

0.02g Phenol Red +0.0056g (200 U/mg) Horseradish peroxidase to 100ml with PBSG.

Made up and used immediately as required.

(Desiccated horseradish peroxidase stored at -20^{0}c.)

Superoxide Assay Reagents

0.197g Cytochrome C to 100ml with PBSG. [150μM]

Made up and used immediately as required.

(Desiccated cytochrome C (type 3 from horse heart) stored at -20^{0}c.)

Superoxide dismutase was dissolved in dist H_2O and stored at 4^{0}c

Myeloperoxidase Assay Reagents

1.Citrate Phosphate Buffer; (A) 2.101g citric acid

(CPO) to 100ml with dist H_2O

(B) 3.58g $Na_2HPO_4 12H_2O$

to 100ml with dist H_2O

Mixed A and B approximately 50/50

and titrated to pH 5.5

Added 50ul Triton X100 /100ml

2.MPO Reaction Buffer; 100ul 1M HCL

+ 900ul 90% ethanol

+ 0.0244g O-Dianisidine

+ 10ul H_2O_2

+ 31.25ml CPO

Lactate Dehydrogenase Assay Reagents

1.Tris/HCL; 110ml 0.05M HCL

+ 200ml 0.05M tris pH to7.5

2.Pyruvate Buffer; 1.2ml 0.63mM Pyruvate

in 0.05M tris/HCL

3. Substrate; 5mg NADH in 640ul Tris/HCL

Calcium Flux Assay Reagents

1. Total release reagent; 3mM Diethylenetriaminepentacetic

acid + 0.05% Triton X100

2. Chelator; 1.14g EGTA + 1.454g Tris

to 10ml with dist H_2O

3. Hank/hepes buffer; 1litre Hanks buffer (Gibco - phenol - and sodium carbonate-free)

+ 2.383g hepes. pH to 7.4 Stored sterile at 4^0c.

2. METHODS.

A. Preparation of Stimuli

i. Heat Aggregated IgG

Plasma from a single donor with an IgG-secreting myeloma was used throughout this project, from which was isolated IgG for use, after aggregation, as a stimulus of cell activation.

Lipid was removed from a thawed aliquot of myeloma plasma by centrifugation and 2ml volumes of the resulting plasma were diluted x2 with 10/150 buffer (see materials section) and loaded onto a 5x1cm Protein A affinity column. Unbound material was washed through with the same buffer and the bound IgG subsequently eluted with 1M acetic acid. After thorough washing of the column with 10/150 buffer until neutral pH of the eluent was restored, the process was repeated with further aliquots of plasma until approximately 10ml of the original plasma had been used. The aliquots of IgG in acetic acid were kept at 4^0c until after the final collection and then were dialysed against PBS at 4^0c, with two changes of buffer in the first hour and further dialysis overnight against a large volume of buffer. When neutral pH was restored, the concentration of the protein was established spectrophotometrically. After concentrating the preparation to 15mg/ml, 500ul aliquots were frozen and stored at -20^0c until required.

For each experiment a fresh aliquot of IgG was thawed and heated at 65^0c for 30 minutes to induce aggregation, after which it was diluted with PBSG to the required concentration.

The components of this preparation were already established (Maslen 1985), and in this work inter-batch variation was not seen; this was established by

overlapping use (not shown) of each batch and noting its effect on stimulating the same preparation of cells.

The unaggregated IgG was also used, from an aliquot stored at $4^{o}c$.

ii. Phorbol Myristate Acetate (PMA):

PMA was diluted to 1mg/ml in dimethylsulphoxide (DMSO) and stored at room temperature. Working dilutions were made in PBSG.

iii. N-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP):

FMLP was dissolved to 0.1M in DMSO and stored in small aliquots at $-20^{0}c$. Aliquots were thawed and diluted in PBSG immediately prior to use.

iv. Fluoride:

Sodium fluoride was dissolved in distilled water containing sodium chloride and was stored at room temperature, in the dark, in a polypropylene container. A concentration of more than 250mM did not stay in solution and so a working solution of 200mM fluoride was stored and used directly.

vii. Other Reagents:

i) 30mM calcium chloride in distilled water was kept at $4^{0}c$ and added to working buffers where indicated.

ii) 10mM EGTA in distilled water was kept at $4^{0}c$, and was added to washing buffer (PBSG) to prevent aggregation of cells prepared for all assays except hydrogen peroxide and calcium flux. It was also present in preincubation mixtures (see later), but was always omitted from the final assay procedures.

iii) 50mM Parahydroxymercuriphenylsulphonic acid (PHMPSA) in distilled water was stored in the dark at 4^0c and diluted in the cell preincubation mixture.

iv) The Islet Activating fraction of Bordetella pertussis toxin (IAP) was stored, 5ug lyophilized powder /ml H_2O, at 4^0c and was added directly to preincubation mixtures to the required concentration.

B. Collection of Blood.

Venous blood was collected from consenting patients who attended the hospital out-patients clinic with an appropriate medical profile as deduced from the notes and as identified by the clinician on the day, and from normal healthy volunteers among the hospital and laboratory staff. The blood was used as soon as it had been collected. All collection, preparative and test procedures were carried out in polypropylene containers to minimize inappropriate activation of cells by either plastic or by glass, and to minimize cell loss due to adherence.

C. Cell Separation.

A one-step ficoll-metrizoate density centrifugation technique (Ferrante and Thong 1978) was used for separating neutrophils (PMN) and a mixed mononuclear cell fraction (MNC).

Whole, heparinized blood (125U heparin/10ml blood) was layered onto a ficoll-metrizoate cushion in a polypropylene centrifuge tube at room temperature and left for 10 minutes, then spun at 500g for 30 minutes. The resulting layers were separated and the PMN and MNC bands were washed once in PBSG with EGTA (see materials section). The PMN pellet was resuspended for 10 seconds in ice cold distilled water, to lyse contaminating red blood cells, and an equal volume of double strength PBS was added to restore tonicity. Both PMN and MNC pellets were washed a further two times in PBSG, resuspended in a 1ml volume and counted. This method of isolation resulted in a fraction which was >98% pure PMN by morphology and a similarly purified mixed mononuclear cell fraction of which it was assumed approximatly 20% were monocytes. Trypan blue exclusion established viability, as >95%. Preincubation Procedures: These were identical for PMN and MNC.

D. Sulphydryl Blockade with PHMPSA:

Cells were resuspended in PBSG with 10μM EGTA at a concentration of $5x10^6$cells/ml and PHMPSA was added to give a final concentration of 50uM. Control cells were preincubated without PHMPSA. Incubation was carried out in a 37^0c water bath for 30 minutes, following which the cells were washed once and resuspended in PBSG without EGTA to the test concentration.

E. IAP Blockade of G-Proteins:

Cells were resuspended to $5x10^6$cells/ml in PBSG with EGTA and IAP was added to give a final concentration of 500ng/ml. Incubation at 37^0c in a water bath for 60 minutes was followed by one wash and resuspension in PBSG for assay. Control cells were preincubated without IAP.

F. Indo-1 AM uptake for Calcium Assay:

PMN were preincubated at $17.5x10^6$cells/ml in PBSG without EGTA and with 15uM Indo-1 AM, incubated at 37^0c for 30 minutes, followed by washing three times in PBSG and resuspension in 1 ml PBSG.

Monocytes were preincubated at $20x10^6$cells/ml in Hanks/hepes buffer and with 10uM Indo-1 AM, incubated at 37^oc for 30 minutes, followed by three washes and resuspension in 1ml Hanks/hepes buffer.

Assays

1. Hydrogen Peroxide:

(The assay was based on that of Pick and Keisari (1980)).

Duplicate sets of 1.5ml polypropylene tubes were indelibly labelled.

To one set was added 10ul 3M Sodium hydroxide.

To the second set was added the reaction mixture as follows;

800ul Phenol red with horseradish peroxidase (or without enzyme for the control)

100ul stimulant or PBSG as control

100ul cell suspension

Where the test contained calcium, this was added to the phenol red assay reagent with the appropriate volume of PBSG omitted.

The reaction mixture was thoroughly vortex-mixed both before and after the addition of cells. The tubes were incubated in a 37^{o}c water bath for 30 minutes, vortex-mixed again, put on ice for 5 minutes, and centrifuged at 500g at 4^{0}c for 5 minutes. The supernatants were tipped into the appropriate duplicate tubes containing sodium hydroxide to raise the pH of the reaction mixture to 12. At this pH the colour change seen is proportional to the amount of hydrogen peroxide produced, as seen by performing the assay with hydrogen peroxide in the place of cells (Figure 1). This effect was seen to be linear to 30uM.

After another vortex-mix, 200ul aliquots of supernatant were read on an automated micro-ELISA reader at 610nm and the results obtained from the

standard curve, Figure 1 ; the O.D. reading being the change in colour seen after deduction of the enzyme-free "blank".

All results given in the figures and tables have had the quantity of spontaneously-produced hydrogen peroxide deducted.

Cell-free experiments established no interaction of reagents except for fluoride which was therefore not used in this assay.

In earlier work with this assay (Pick and Keisari 1980) use was made of catalase to abolish oxidation of phenol red in controls. These workers could not totally abolish activity. Later work on the nature of the oxidizing species (Maslen 1985) established that other peroxides were produced in a stimulus-dependent fashion. In this project catalase was not been used and the products assayed were probably a mixture of peroxides.

2. Superoxide:

(Babior et al 1973)

Duplicate sets of 1.5ml polypropylene tubes were prepared. One set was put to one side. To the second set was added the following:

700ul Assay reagent

10ul Superoxide dismutase (50 units) or 10ul PBSG

90ul PBSG

100ul cell suspension

100ul stimulus

When calcium was included it formed part of the assay reagent diluent, and where more than one stimulus was added this replaced part of the volume of PBSG.

The tubes of reaction mixture were thoroughly vortex-mixed before and after addition of cells and incubated at 37°c for 30 minutes in a water bath. After this the tubes were mixed again, placed on ice for 5 minutes, mixed and centrifuged at 500g for 5 minutes. The supernatants were tipped off into the clean set of duplicate tubes and mixed. 200ul aliquots were read on an automated micro-ELISA reader at 550nm and the results were expressed as nMol cytochrome C reduced by $1x10^6$cells as found using the following formula:

Absorbance at 550nm = 29.5*x concentration x 5.1**

Where * = extinction coefficient of reduced cytochrome C (Sigma)

and ** = the length of the lightpath through the assay mixture in the ELISA plate (millimetres).

No interference of the assay reagents was found, using a xanthine/xanthine oxidase system to generate superoxide.

3. Myeloperoxidase:

(Segal et al 1980)

This reaction measured the reduction of o-dianisidine in the presence of H_2O_2 by MPO, as seen by a change in absorbance at 560nm.

PMN ($4x10^6$cells/ml) were stimulated variously in 1ml PBSG for 30 minutes in polypropylene tubes in a 37°c water bath. The tubes were centrifuged at 500g for 5 minutes at 4°c and the supernatants removed. Aliquots of the supernatants were used as follows:

50ul Supernatant was added to 3.2ml MPO reaction buffer and incubated, after mixing, for 5minutes at 37°c. 1ml perchloric acid was added to stop the reaction, and after further mixing the test was read at 560nm. against a cell free blank.

The results were expressed as a percentage of total MPO released from cells lysed with PBSG + 0.2% Triton X100 at the beginning of the stimulation period.

4. Lactate Dehydrogenase:

(Wrobeloewzki and LaDue 1955)

This assay measured the release of the cytosolic enzyme by the rate of reduction of NADH in the presence of pyruvate as seen by the loss of absorbance at 340nm, the rate being proportional to the amount of enzyme in the reaction where the amount of substrate is not rate-limiting.

Cells were preincubated in either PBSG, IAP or PHMPSA for 1 hour and the supernatant removed for assay.

100ul of supernatant was added to 1ml (2.5mg/ml) sodium pyruvate and 20ul (2.5mg/ml) NADH, mixed and the loss of absorbance read every 15 seconds for 5 minutes.

The results have been expressed as a percentage of the rate of enzyme activity from cells lysed with 0.15% triton X100.

Allowance was also made for nonspecific loss of absorbance by performing the assay without adding NADH.

5. Release of Internal Calcium:

(Bijsterbosch et al 1986)

In its acetomethoxy- form Indo-1 AM is taken up by the cell and metabolized whereupon it binds avidly to free calcium, in combination with which when excited at 355nm it emits fluorescence at 400 nm in direct proportion to the amount of calcium to which it has bound.

For these experiments, no EGTA was added to the washing buffer.

75ul PMN ($2.6x10^6$) were incubated with 4ul (15uM) acetomethoxy ester of Indo-1 for 30 minutes at 37°c, with or without PHMPSA (50uM) and 110ul PBSG followed by three washes in PBSG and resuspension in 1ml PBSG.

200ul monocytes ($3x10^6$) were incubated with 10uM Indo-1 in Hanks/hepes buffer instead of PBSG, followed by preincubation, washing and resuspension in Hanks/hepes buffer.

The 1ml cell suspension was added to 1.9ml PBSG (PMN) or 1ml Hanks/hepes (monocytes), brought to 37°c in the spectrofluorimeter, and stimulated for a given amount of time. The total amount of cell calcium was established by addition of 0.05% Triton X100 in the presence of 10uM diethyleneaminepentacetic acid, and minimal calcium reading was obtained by addition of 100ul of 10mM EGTA/40mM TRIS solution.

6. Statistics.

Statistical significance was found using the Mann-Whitney U test for unpaired data or the Wilcoxon test for paired data. Where n= <5 results were expressed as the mean +/- one standard deviation.

Chapter Three

RESULTS

SECTION 1.

i. AGE

The ages of the controls were compared with those of the RA NSAID group and the NSAID group ages with the patients on second line therapy.

Group.	Mean	Median
Normal	37	36
NSAID	53	50
Second line	60	63

Unpaired analysis of the data showed that the difference between the normal and NSAID group was significant ($P<0.001$) but that between the NSAID group and those on second line therapy was not. Therefore any differences is responses seen between the normal and NSAID group might be a function of the aging process, but the differences between the NSAID group and those on second line therapy can reasonably be attributed to the therapeutic effect of the drugs.

Selection of Patients.

Patients were attending Dr Joan Davies' Out-patient's Clinic at the Royal National Hospital for Rheumatic Diseases. They were selected on the basis of their clinical notes and drug therapies:-

i.e. NSAID= No steroids for at least 6 months; no Dpen or other second line therapy.

Dpen= on Dpenicillamine for at least 6 months ; no previous second line therapy for 12 months, or steroids for at least 6 months.

Aur= on auranofin for at least 6 months; no previous second line therapy for 12 months, or steroids for at least 6 months.

Patients were all sero +ve for rheumatoid factor at the last visit and showed signs of active disease when attending the Clinic at the time of blood collection.

ii. Lactate Dehydrogenase Assay.

The work of Wroblewski and LaDue (1955) on myocardial infarction, demonstrated that the cytosolic enzyme lactate dehydrogenase (LDH) was released from damaged cells. This has led to the enzyme being used as a measure of the permeability of plasma membranes.

To ascertain the degree of permeability induced in the cells by the preparative procedures, and also to see if PHMPSA or IAP affected permeability, release of LDH was measured in three types of experiment:

In the first PMN and MNC were isolated and then measured immediately for LDH release.

In the second cells were isolated and then given an hour's preincubation in PBSG, PHMPSA or IAP, followed by washing and assay for LDH.

In the third cells were isolated and then incubated in a 37°c water bath for 30 minutes with either PBSG, fluoride, FMLP or PMA, before being assayed.

The results in Table 3 show that after the initial isolation procedure PMN released negligible amounts of LDH (3%); MNC released slightly more (13%). The hour-long preincubation did not raise the amount of LDH released, but the cells preincubated in PHMPSA showed a slight toxic effect, release from both cell types being almost doubled. Usually, for other assays (such as those for superoxide or calcium flux), cells were preincubated in PHMPSA for 30 minutes only, and the incubation time used here was only normal for IAP and its controls.

The third type of experiment, in which various stimulatory ligands were tested showed that these ligands induced no noticeable rise above that induced by the extra incubation time i.e. the PBSG control level of release. The results are the mean of three experiments done in duplicate, and brackets denote standard deviation.

Table3. : Release of Lactate Dehydrogenase from Normal PMN.

Treatment % LDH Released

(where release in the presence of Triton x100 = 100%)

	PMN	MNC
a. No treatment;	3 (2)	13 (7)
b. 1 Hour Preincubation;		
PBSG	6 (2)	10 (4)
PHMPSA(50uM)	11 (5)	19 (10)
IAP(500ng/ml)	7 (4)	10 (6)
c. 30min Stimulation;		
PBSG	12 (6)	16 (3)
Fluoride(20mM)	11 (1)	19 (3)
FMLP(5uM)	8 (2)	18 (3)
PMA(2ng/ml)	10 (1)	19 (6)

SECTION 2.

HYDROGEN PEROXIDE ASSAY.

This colorimetric assay for cell activity provided a vehicle for measuring SH-blockade with PHMPSA in both MNC and PMN using various stimuli. It was also used to investigate the effects of exogenous calcium ($calcium_e$) on stimulation, both with and without PHMPSA, and for comparison of the effects on cells obtained from patients and from normal healthy volunteers.

This assay also measures other, unspecified peroxides, for which a catalase control can be included. However it was decided to omit these controls for two reasons: Firstly because cell-free experiments with catalase and H_2O_2 (not shown) determined that catalase did not inhibit H_2O_2 totally, and secondly because their omission made more cells available for extra experiments in each assay; this was particularly important for obtaining sufficient MNC results. The results have therefore been reported as peroxide, rather than hydrogen peroxide.

In the peroxide assays:-

1. Concentrations of cells were $2x10^6$per ml unless otherwise stated.
2. The stimulated quantities of H_2O_2 displayed in the figures have already had the spontaneous levels subtracted.
3. + calcium = 1.5mM calcium chloride in PBSG used to make up the horseradish peroxidase-phenol red reagent.
4. Unpaired analysis of the data, given on the figures, was obtained using the Mann Whitney U test for non-parametric data. Paired analysis was undertaken using the Wilcoxon test.
5. Bars = One Standard deviation.

A standard curve was produced for this assay, using known concentrations of hydrogen peroxide (Figure 1), from which all the results were read.

As with all results, the curve for Figure 1 was derived from four separate experiments, each done in duplicate.

Hydrogen Peroxide Standard Curve

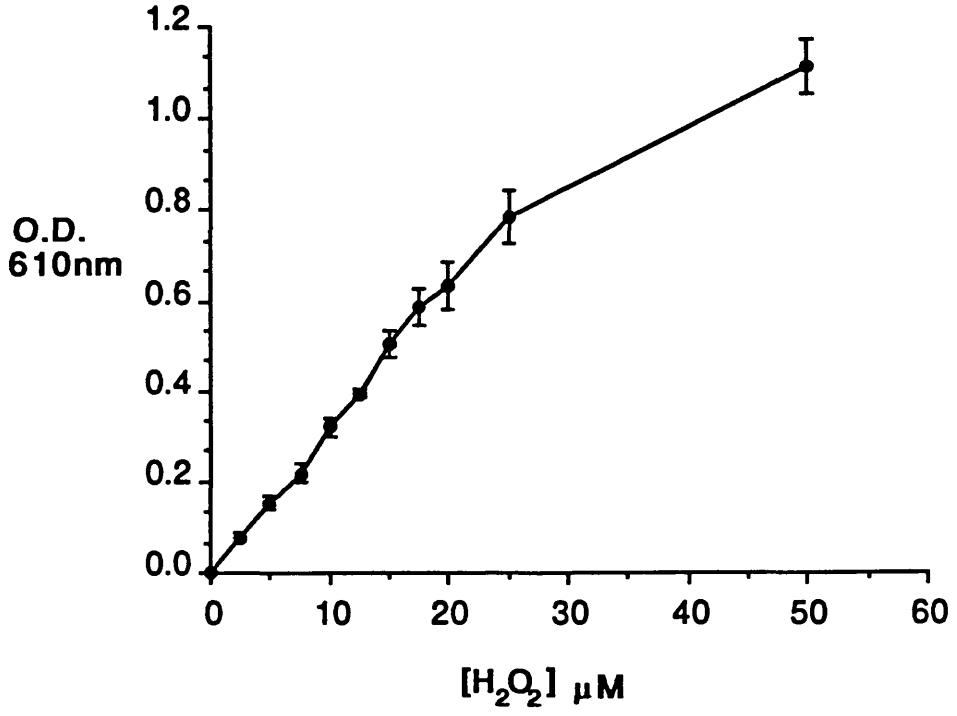
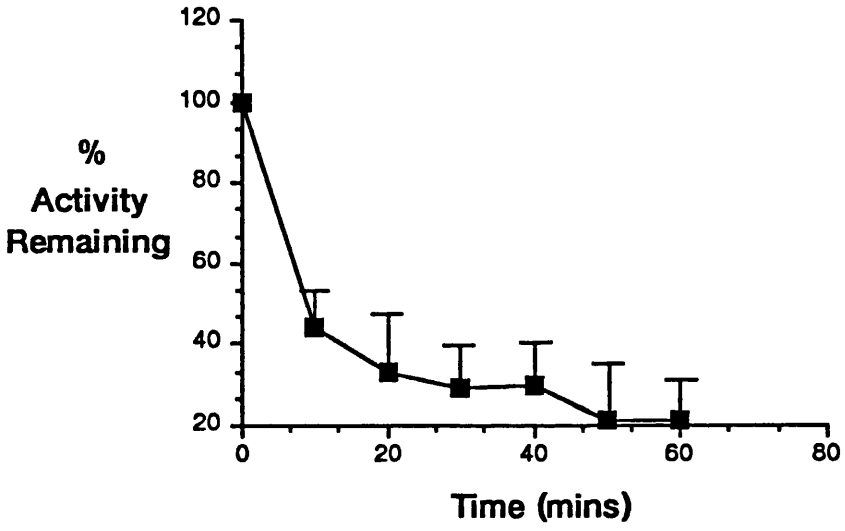


Figure 1

i. PHMPSA Inhibition.

A timecourse experiment was carried out to establish an incubation time for the blockade-effect of PHMPSA by using HAGG-stimulated production of peroxide by PMN (Figure 2). By 30 minutes the PHMPSA had achieved greater than 90% inhibition, and so this was chosen as the standard preincubation time for these experiments.

Timecourse to Show Effect of PHMPSA on HAGG-Stimulated PMN.



Note: HAGG = 500 µg/ml

Figure 2

ii. Dose responses.

Dose response curves for each stimulus were produced for both cell types as follows:

FMLP :	PMN	Fig. 3	MNC	Fig. 6
PMA :		Fig. 4		Fig. 7
HAGG :		Fig. 5		Fig. 8

From the standard deviation bars it can be seen that the deviation from the mean was sometimes quite large. This reflected the wide normal range of response.

From these curves the standard concentrations chosen were:

PMA 5ng/ml

HAGG 500ug/ml

FMLP 5uM

Peroxide Response of Normal PMN to FMLP Stimulation.

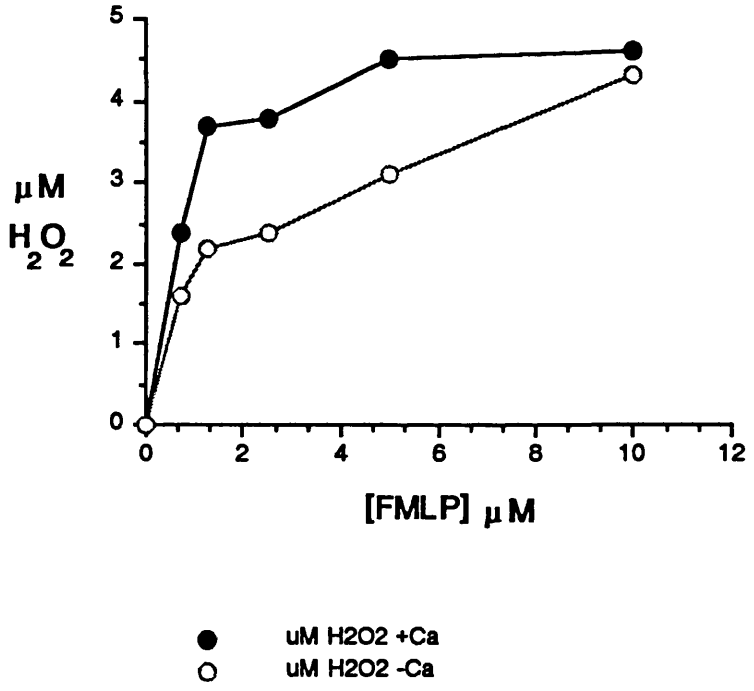
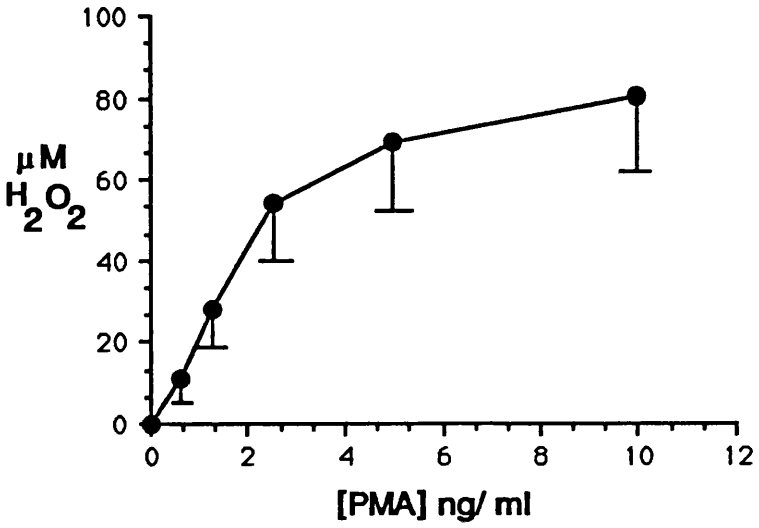


Figure 3

Peroxide Response of Normal PMN to PMA Stimulation.



Note: cells at $2.5x10^5$/ml

Figure 4

Peroxide Response of Normal PMN to HAGG Stimulation.

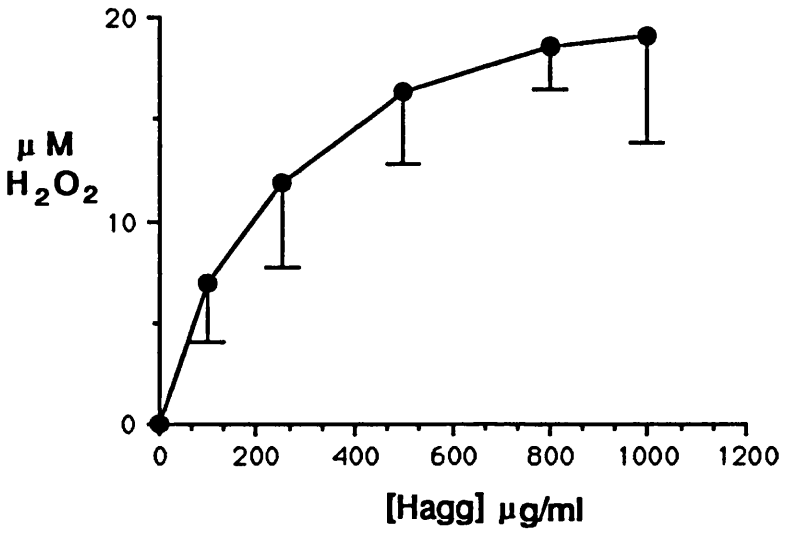


Figure 5

Peroxide Response of Normal MNC to FMLP Stimulation.

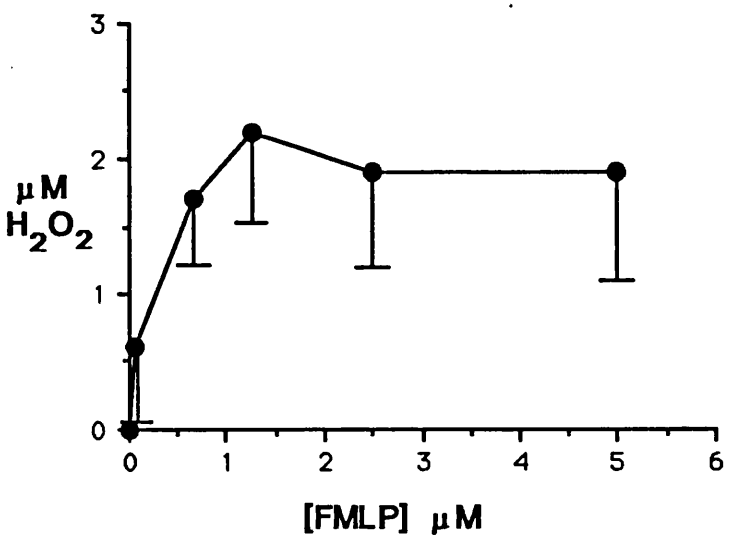


Figure 6

Peroxide Response of Normal MNC to PMA Stimulation.

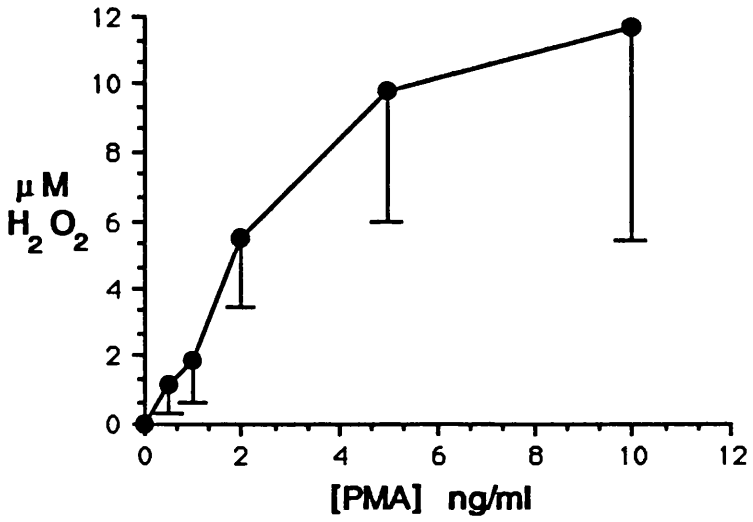


Figure 7

Peroxide Response of Normal MNC to HAGG Stimulation.

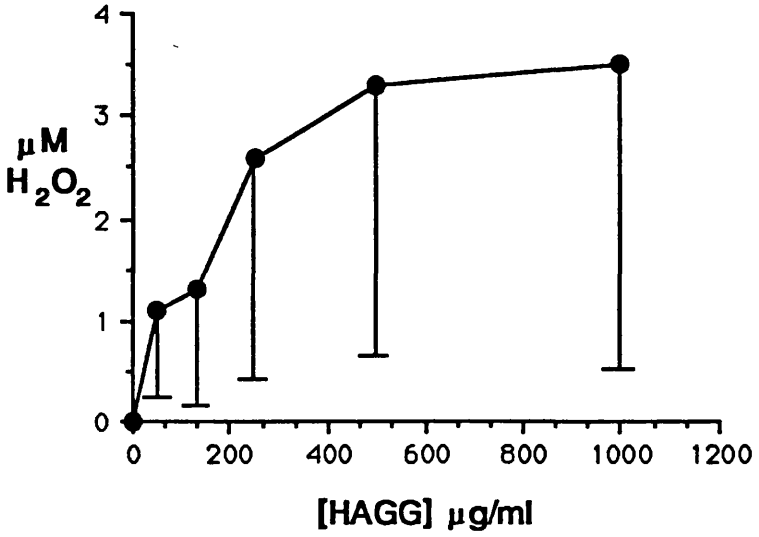
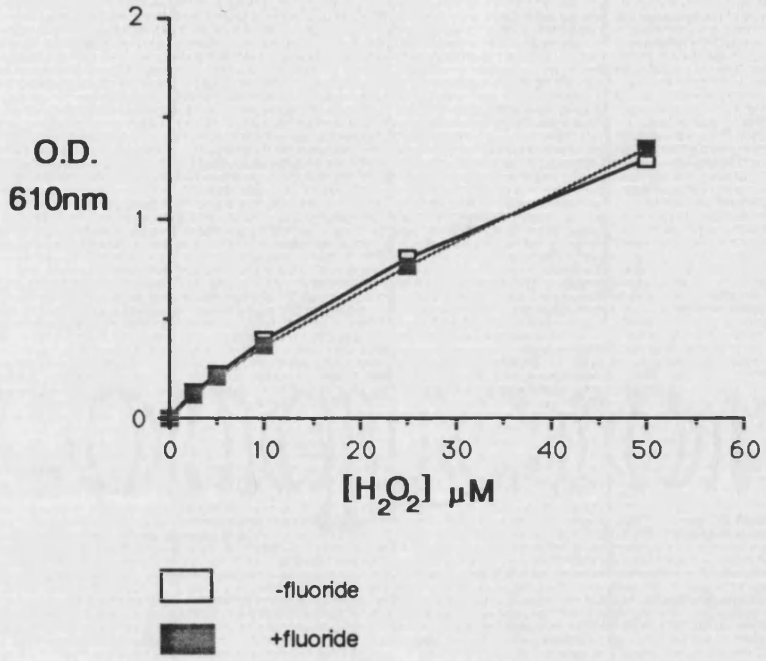


Figure 8

There was an effect of fluoride on the H_2O_2 assay which precluded obtaining any consistent stimulation. The cell-free experiment (using dilutions of hydrogen peroxide) did not demonstrate any observable interaction (Figure 9), and the reason for this problem was not found. However, the need to use this ligand caused later work to be undertaken using the cytochrome C assay for O_2^- production.

Effect of Fluoride on Hydrogen Peroxide Assay.



note: [fluoride] 20mM

Figure 9

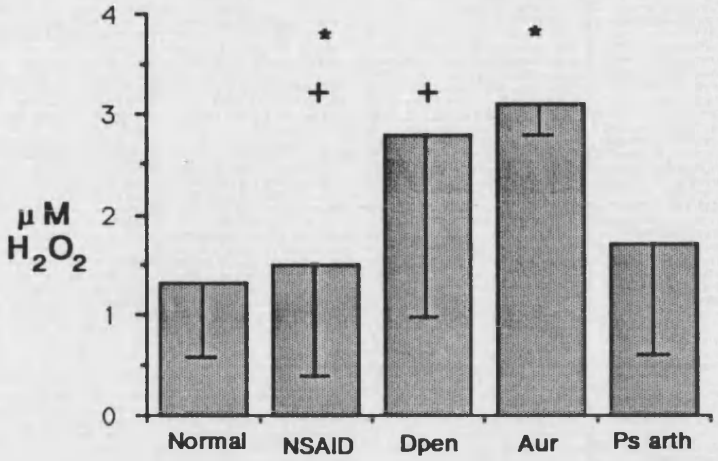
iii. Spontaneous Peroxide Production and the Effect of Exogenous Calcium.

Cells isolated from patients with sero +ve rheumatoid arthritis (RA) on various specified drug regimens - non-steroidal anti-inflamatory drugs, (NSAID); D-penicillamine, (Dpen); or Auranofin, (Aur) - were compared with each other and with those from normal volunteers. Comparison was made between the spontaneous production of peroxide by PMN and MNC (Figures 10 and 11 respectively) in the presence and absence of $calcium_e$. Patients with psoriatic arthritis were also used as another disease group to compare with rheumatoid patients on the same range of NSAID.

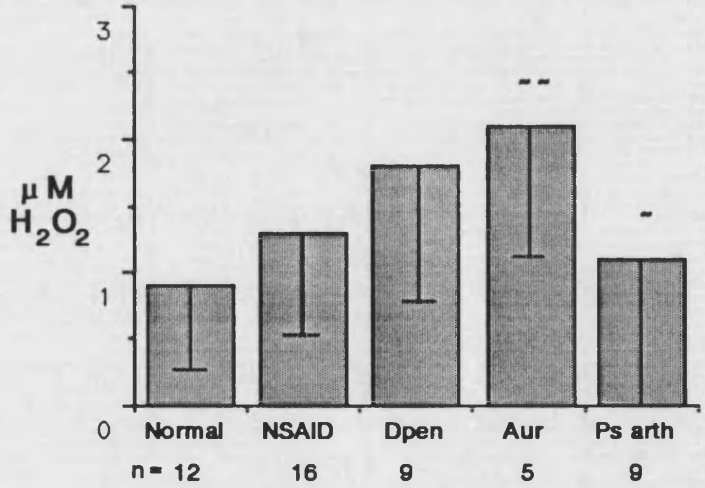
The paired data for spontaneous peroxide production in the presence and absence of $calcium_e$ were analysed, as were the values obtained from the different patient groups, compared to normals and to one another, in both PMN and MNC. Note: Where numbers were very low no statistics of significance have been calculated. Otherwise, where statistical significance has not been indicated on the figures there was none.

Spontaneous Peroxide Release by PMN.

A.In the presence of Calcium.



B. In the absence of Calcium.



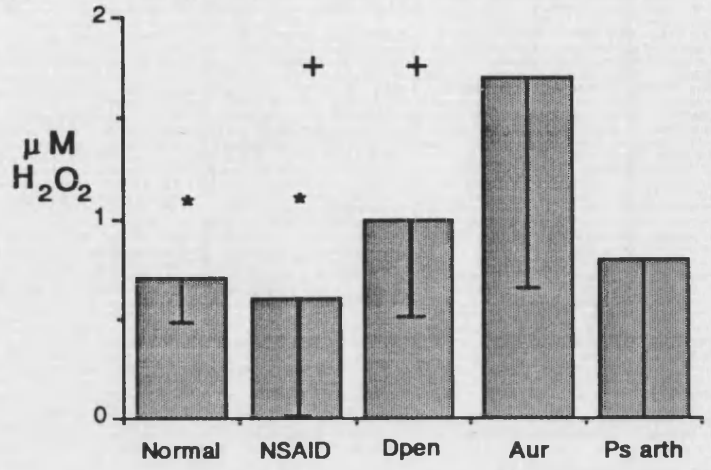
Paired analysis - and -- = effect of calcium P<0.001

Unpaired analysis + = P<0.05 * = P<0.01

Figure 10

Spontaneous Peroxide Release by MNC .

A. In the presence of Calcium.



B. In the absence of Calcium.

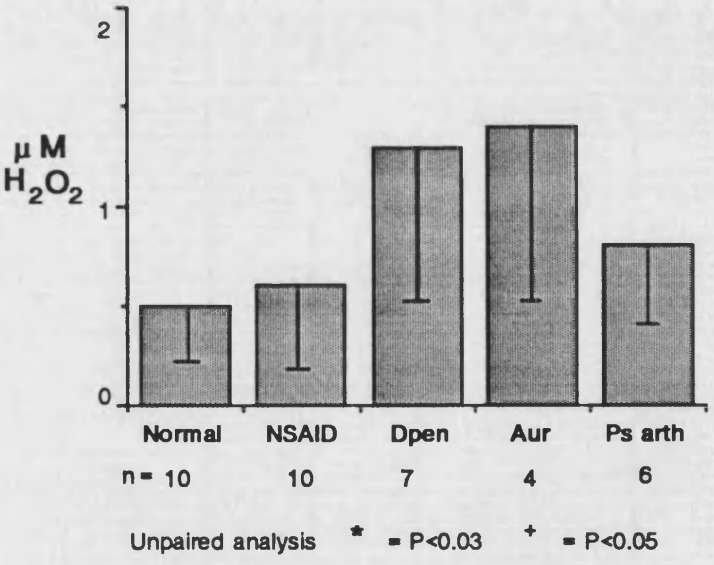


Figure 11

It can be seen from figure 10 that PMN from patients on second line drug therapies, Dpen and Aur, showed by unpaired analysis a significantly higher level of spontaneous peroxide generation than the NSAID group which did not differ from the normal group. In the presence of $calcium_e$ only, the Dpen and Aur groups released significantly more peroxide than the NSAID group ($P<0.01$).

Using paired analysis of PMN no additional effect in the normal, NSAID or Dpen group was seen when $calcium_e$ was present, but there was a significant enhancement of peroxide production by the psoriatic ($P<0.001$) and Aur ($P<0.001$) groups.

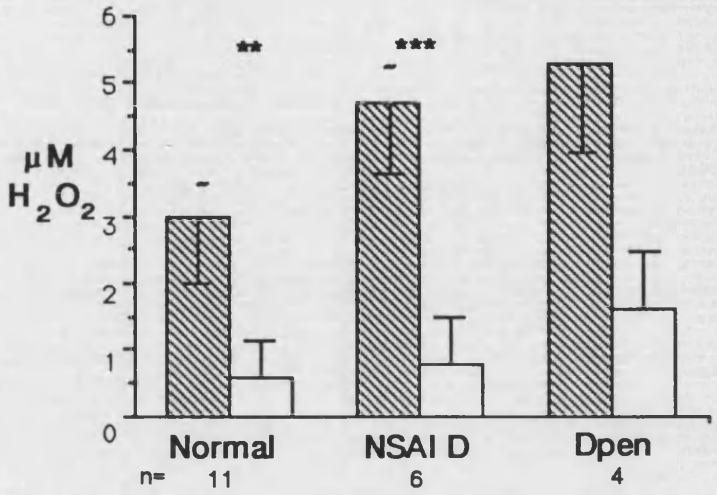
From the MNC data in figure 11, it can be seen that differences in spontaneous release were not significant in the absence of $calcium_e$, but that in the presence of $calcium_e$ the release by MNC from patients on Dpen was significantly higher than from the NSAID group ($P<0.05$), which was significantly lower than from the normal group ($P<0.03$).

iv. Stimulation of MNC and PMN:

Cells were preincubated in PHMPSA or PBSG (see Materials and Methods) and then production of peroxide measured in response to FMLP, PMA or HAGG, and in some cases the assay included stimuli with and without $calcium_e$. These results are given with significant statistical data in figures 12 to 15.

Effect of PHMPSA Preincubation on FMLP-Stimulated PMN.

A. In the presence of Calcium.



B. In the absence of Calcium.

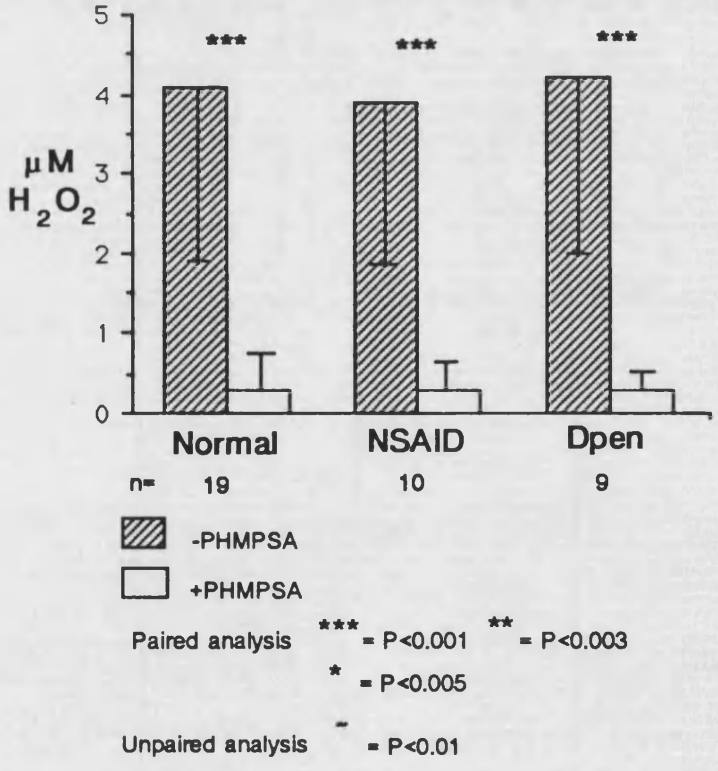
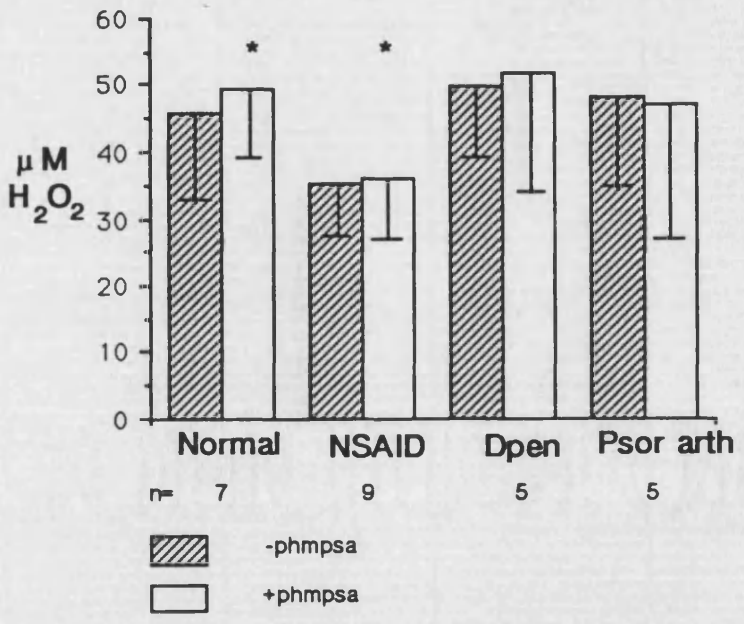


Figure 12

Effect of PHMPSA Preincubation on PMA-Stimulated PMN.

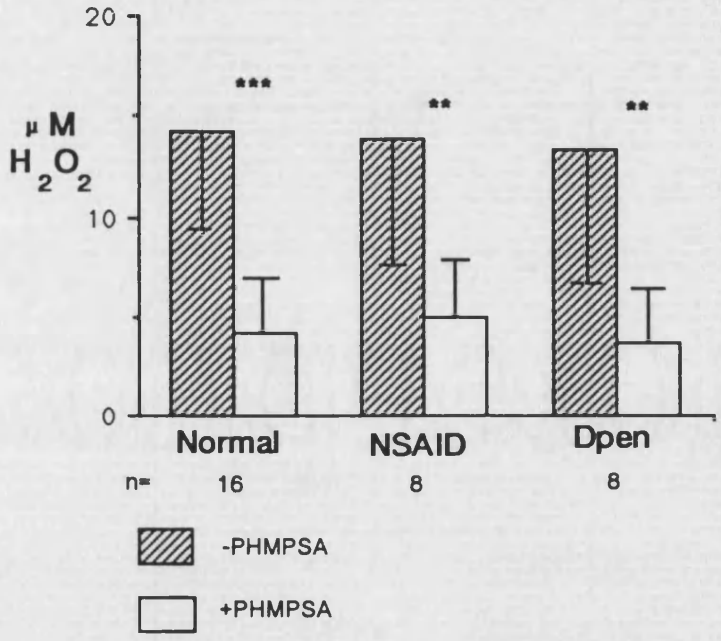


Unpaired analysis * = P<0.05

(In the absence of calcium)

Figure 13

Effect of PHMPSA on The Peroxide Response of HAGG-Stimulated PMN.



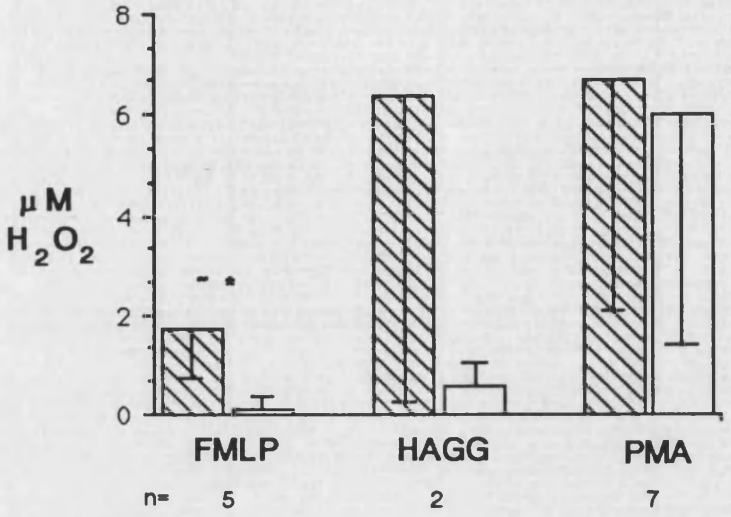
Paired analysis *** = P<0.001 ** = P<0.005

(In the absence of calcium)

Figure 14

Effect of PHMPSA on Normal MNC.

A. In the Presence of Calcium.



B. In the Absence of Calcium.

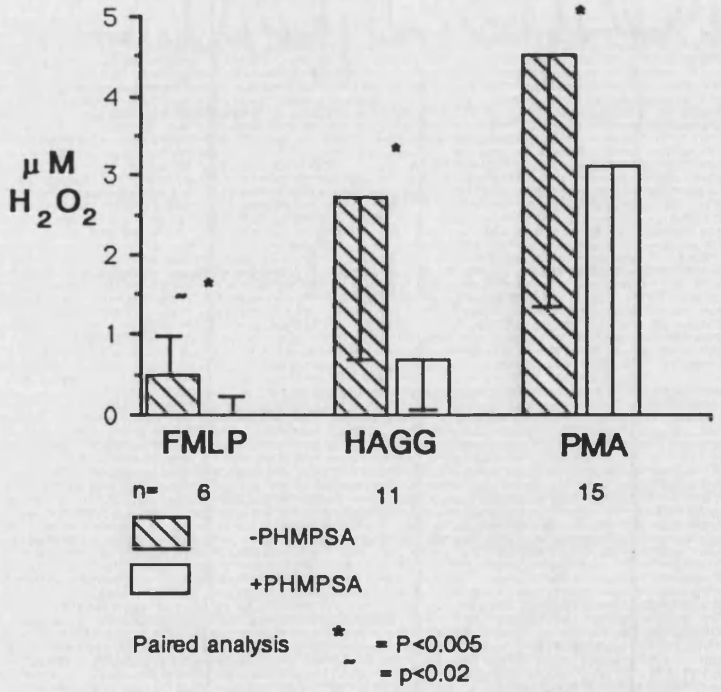


Figure 15

From the histograms it can be seen that the FMLP response in PMN from all sources and in MNC was greatly inhibited by PHMPSA preincubation (Figures 12 and 15). The HAGG response was also drastically reduced by PHMPSA (Figures 14 and 15). An interesting finding was that the PMA response, which remained unaffected by PHMPSA in PMN, was significantly lower in MNC preincubated in PHMPSA in the absence of $calcium_e$, an effect which was overcome by $calcium_e$ (Figure 15).

A comparison of the effect of $calcium_e$ on FMLP-stimulated normal PMN and MNC (Figures 12 and 15), showed that it had no effect on the PMN response whilst causing a significant elevation in response ($P<0.02$) from MNC.

SECTION 3.

SUPEROXIDE ASSAY.

The lack of a monitorable response of cells to the fluoride ion in the hydrogen peroxide assay, and the rather low sensitivity of the assay, caused work to continue using the superoxide dismutase-inhibitable one electron reduction of cytochrome C to measure O_2^-, a direct product of activation of the respiratory burst enzyme.

i. Dose Responses.

Dose response curves were produced for each stimulus (Figures 16 to 21), for which cells were at a concentration of $2x10^6$/ml except for the PMA-stimulated PMN which concentrated to $0.25x10^6$/ml. Cell concentrations remained the same throughout the O_2^- experiments. Results are the means of at least 3 separate experiments done in duplicate and the bars throughout the experiments represent one standard deviation. Calcium chloride ([300uM]) was added in all experiments.

The concentration of FMLP used for this assay (5uM) was extrapolated from that used for the hydrogen peroxide assay. However it has since been found to be supramaximal, especially for MNC, where in the range 10uM to 10nM the response remained at a plateau (not shown).

In a dose response curve with and without 5ug/ml cyt B (fig 17) it can be seen that the responses diverge at the higher range of doses such as have been used throughout most of this work. In 4 out of 8 experiments with PMN, cyt B elicited a O_2^- response itself. Where tested (n=3) this was partly inhibited (23%) by PHMPSA. In all 5 experiments with MNC cyt B invoked a O_2^- response, which again was inhibited by PHMPSA (n=3), by >40%. Cyt B was omitted from the majority of experiments using FMLP stimulation for simplicity of interpretation.

Superoxide Response of Normal PMN to FMLP Stimulation.

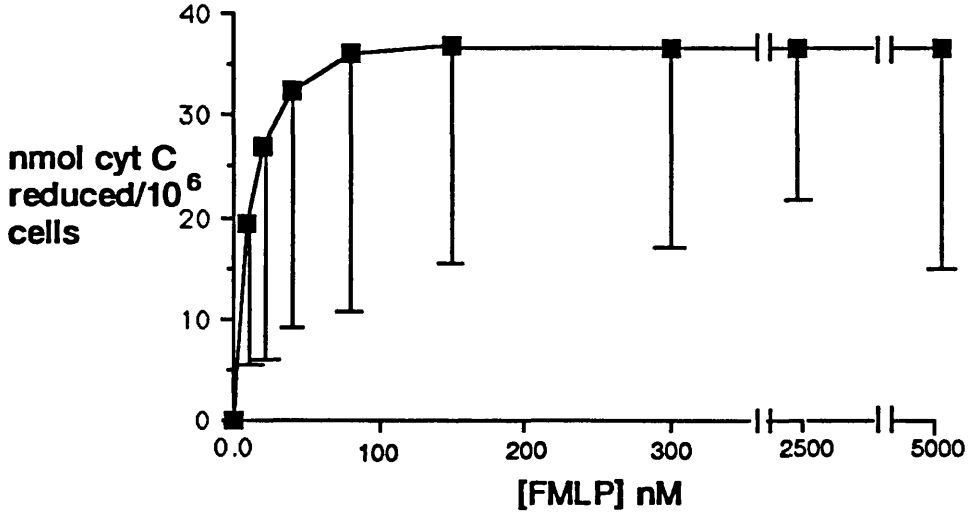


Figure 16

Effect of Cytochalasin B on the Superoxide Response of FMLP-Stimulated Normal PMN.

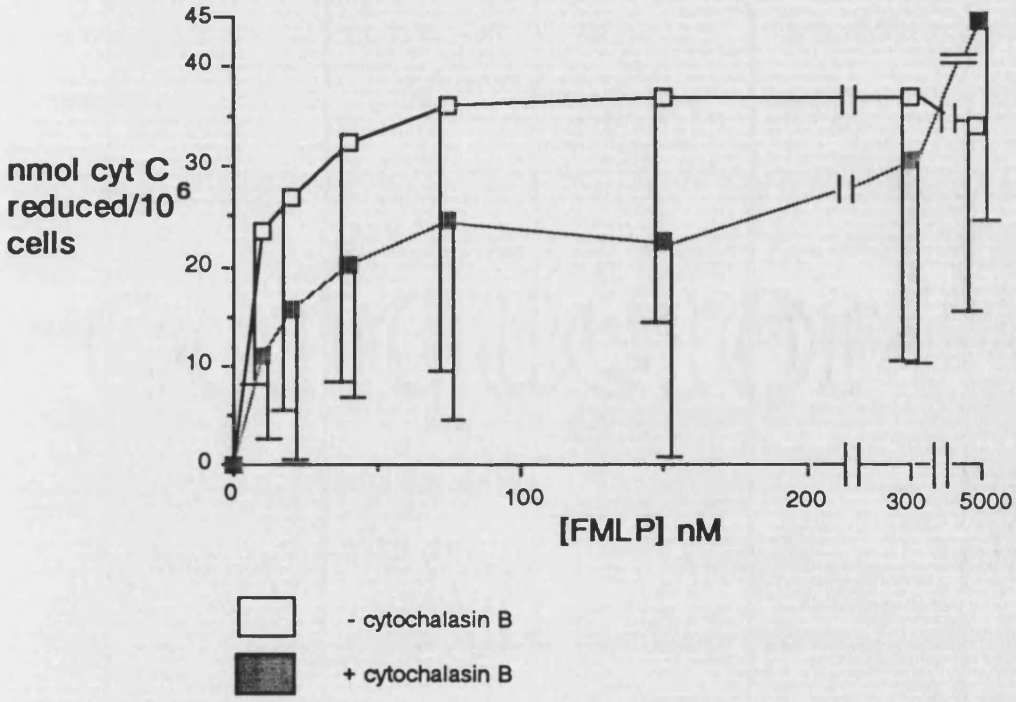


Figure 17

Superoxide Response of Normal PMN to PMA Stimulation.

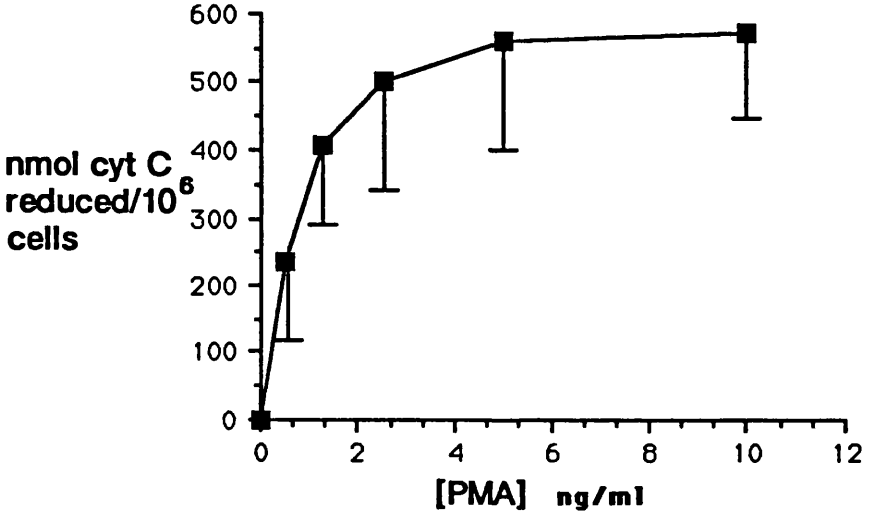


Figure 18

Superoxide Response of Normal PMN to Fluoride Stimulation.

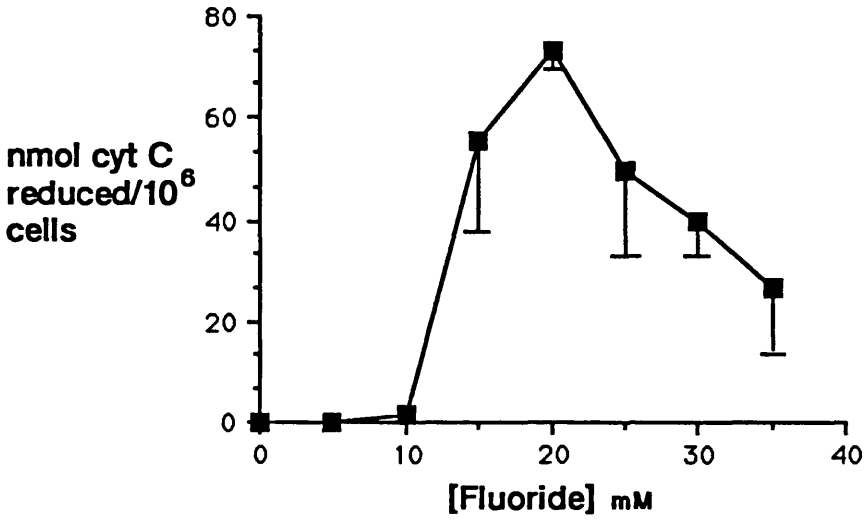


Figure 19

Superoxide Response of Normal MNC to Fluoride Stimulation.

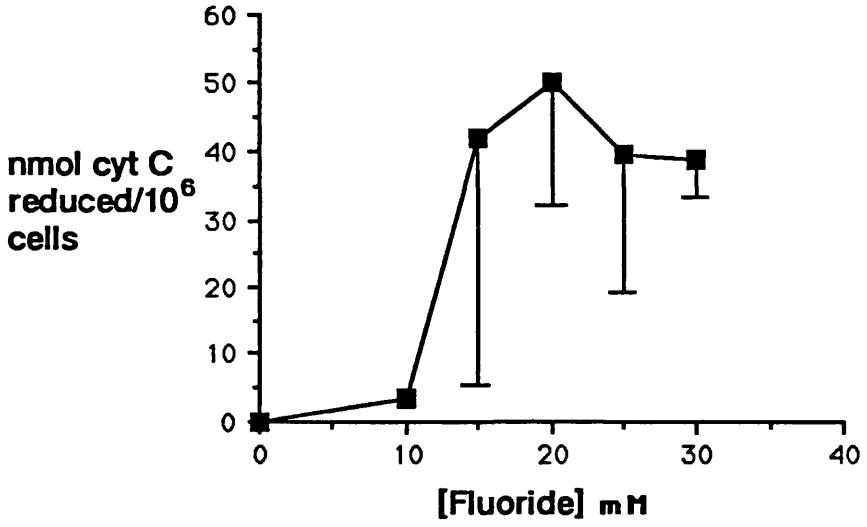


Figure 20

Superoxide Response of Normal MNC to PMA Stimulation.

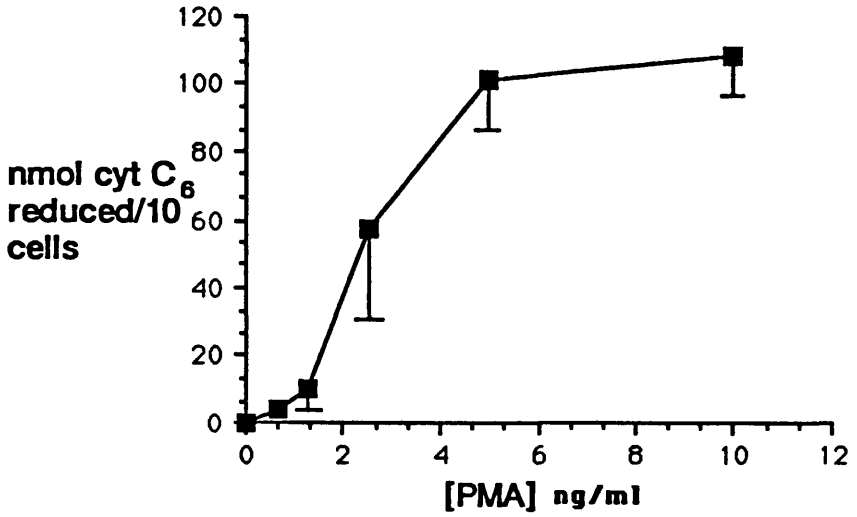


Figure 21

ii. Timecourses.

Timecourses for O_2^- production were measured, for PMN and MNC stimulated by PMA, by FMLP or by fluoride, or by PMA and fluoride together. One representative curve for each stimulus is given in Figures 22 to 25.

iii. Lag times:

The results demonstrate that both PMN and MNC had very rapid response to FMLP which was complete by one minute (Figures 22B and 24B). Both cell types exhibited the same lag of 5 minutes before the onset of the fluoride response (Figures 22A and 24A). The 2-3 minute lag time for the PMA responses was similar for both cell types (Figures 23A and 25A).

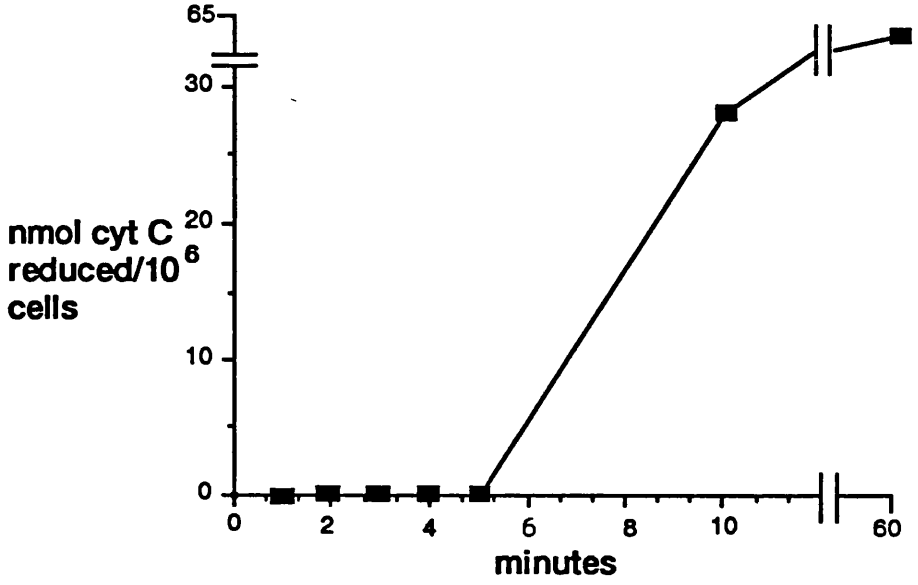
iv. Response curves.

The reaction of both cell types to FMLP was complete by the time 1 minute had elapsed. The large response of PMN to PMA reached a plateau after 40 minutes which may have been because of exhaustion of substrate, but as this was seen also in the MNC response after 30 minutes, it may be that the activity caused a negative feedback of toxic metabolites, including peroxides, which limited further activity. The fluoride response was complete in both cell types by 20 minutes, having an initial lag time of 5 minutes.

The combined effect of PMA and fluoride was examined. In PMN the lag time was less than that of fluoride alone, the final response being greater than for fluoride alone, but less than for PMA (Figure 23). MNC similarly demonstrated a reduction in lag time compared with fluoride alone (Figure 25). The co-stimulation was not timed beyond 10 minutes in these cells.

Timecourse for Superoxide Responses of Normal PMN to Fluoride or FMLP Stimulation.

A. Fluoride.



B. FMLP.

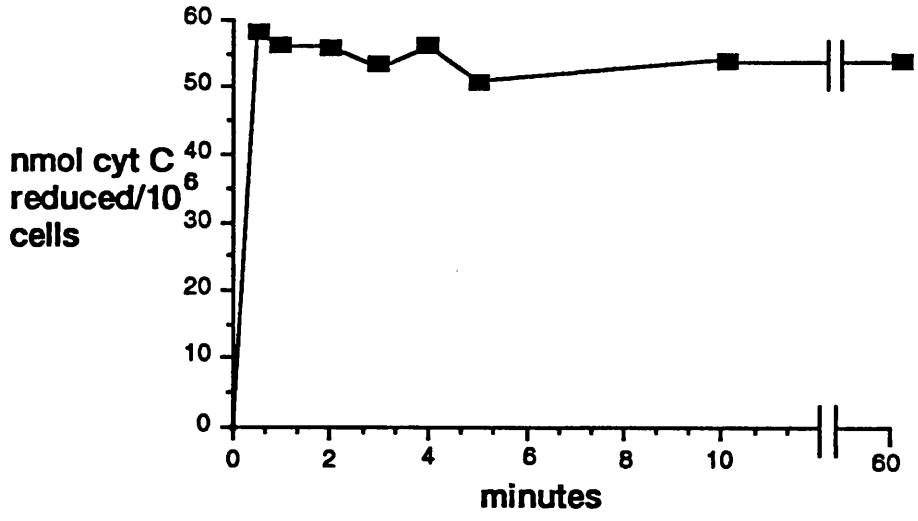
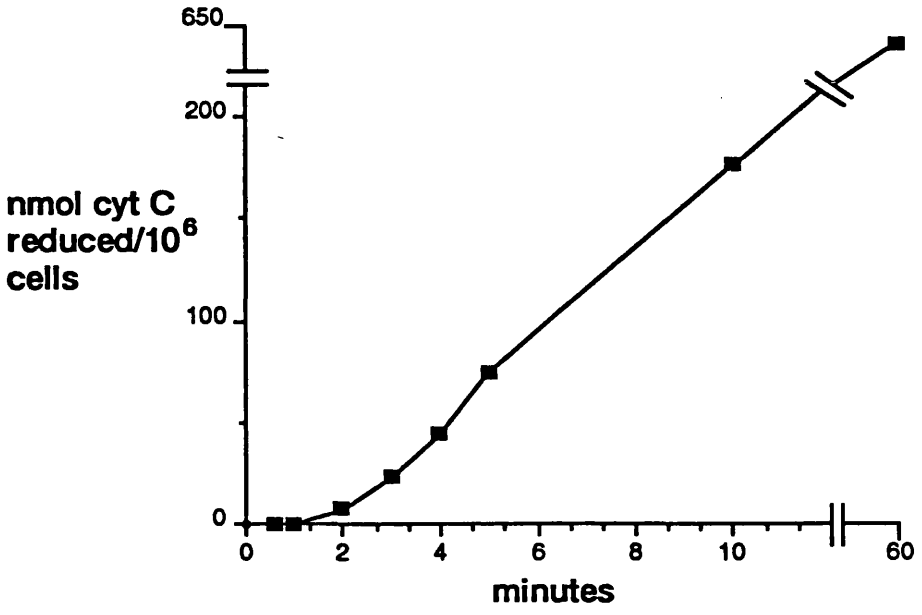


Figure 22

Timecourses for Superoxide Responses of Normal PMN to PMA or to PMA+Fluoride Stimulation.

A. PMA.



B. PMA+Fluoride.

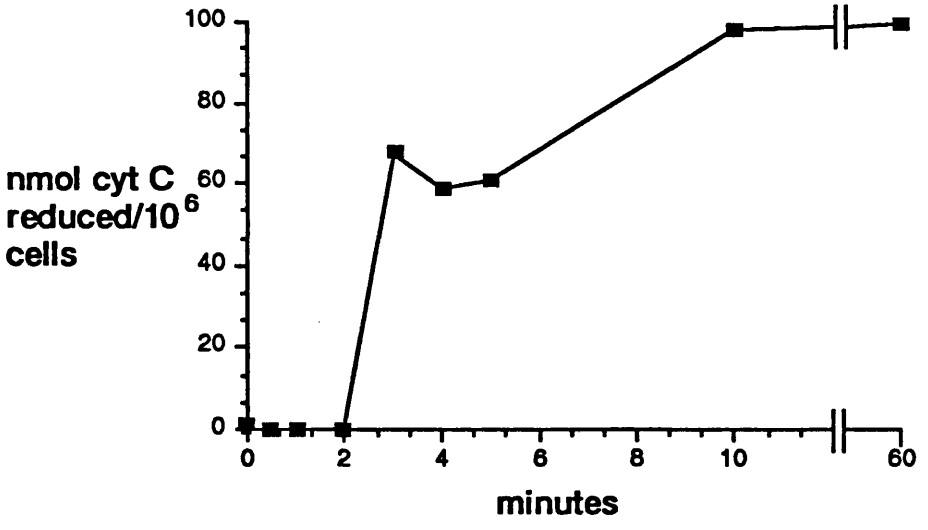
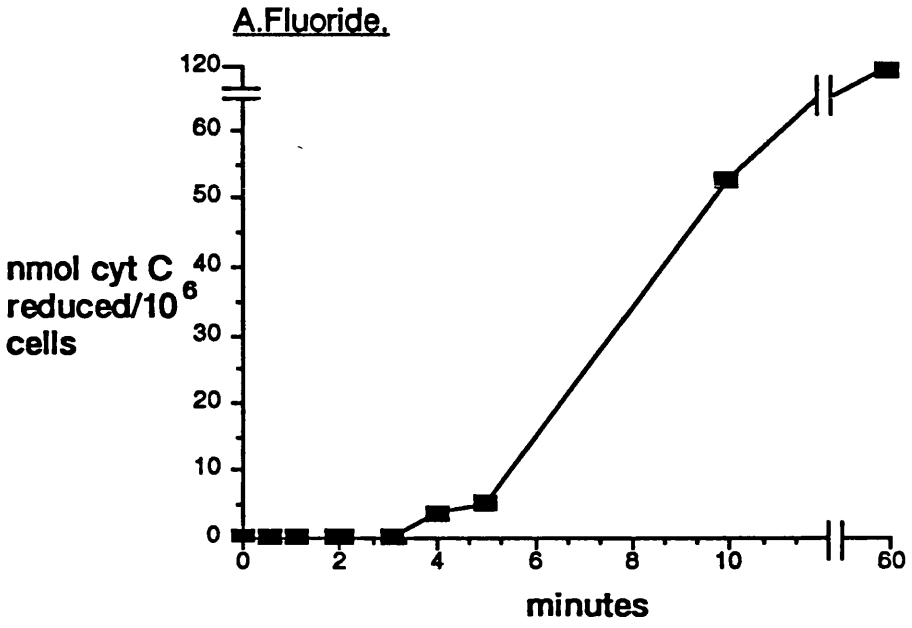


Figure 23

Timecourses for Superoxide Responses of Normal MNC to Fluoride or FMLP Stimulation.



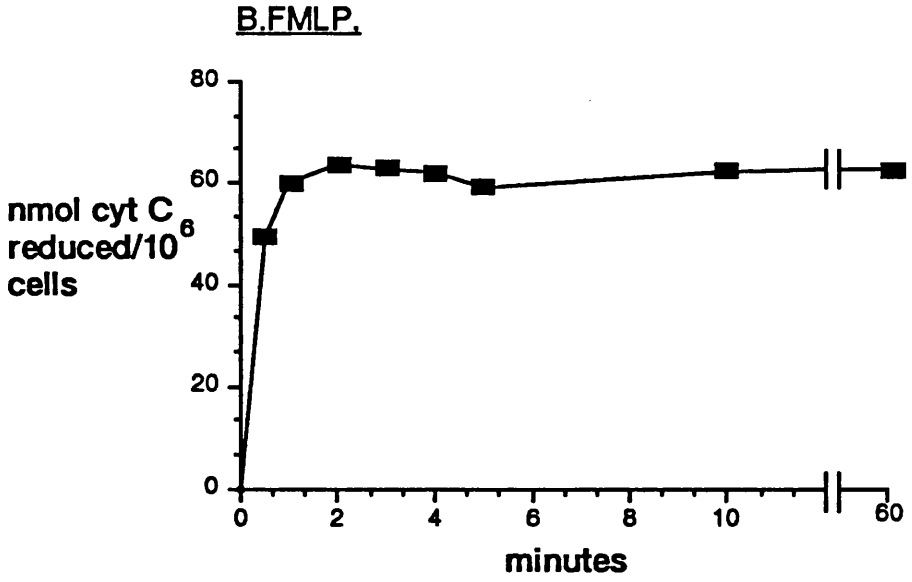
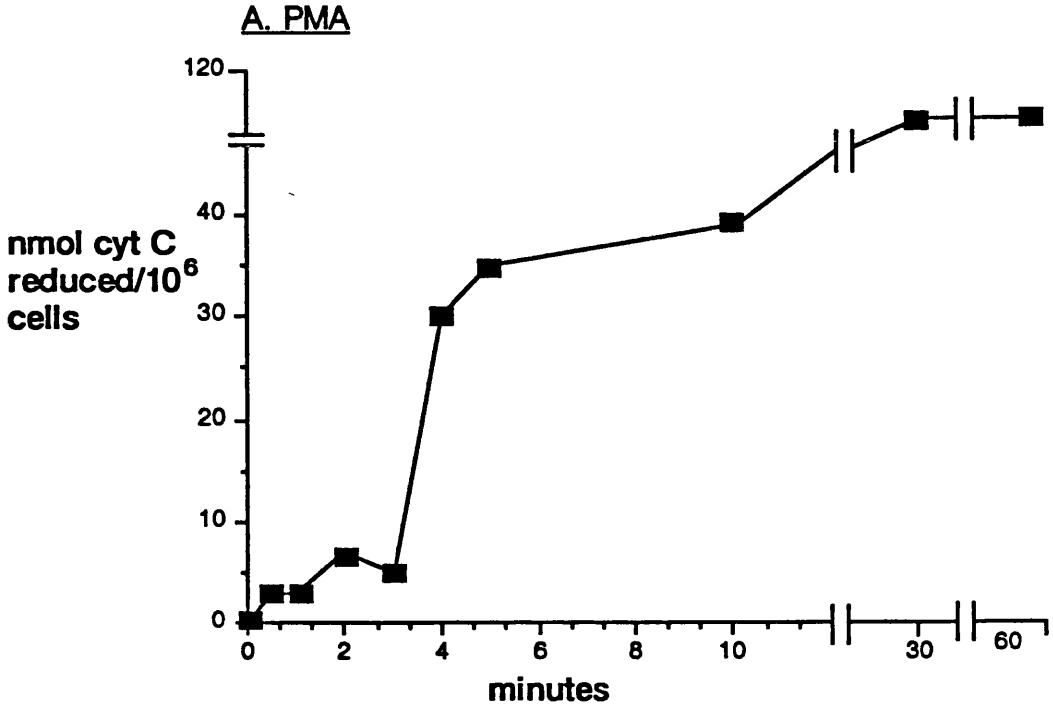


Figure 24

Timecourse for Superoxide Responses of Normal MNC.



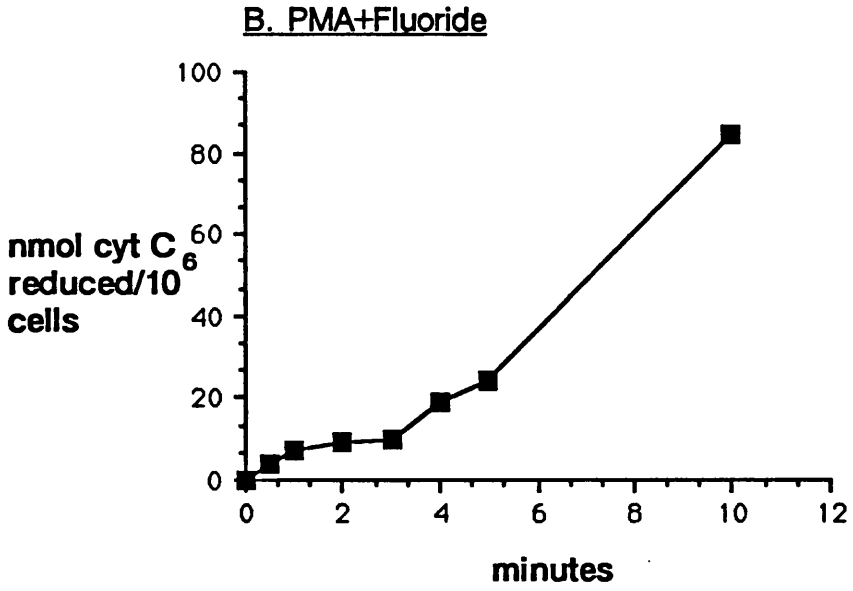


Figure 25

A. Preincubation with PHMPSA.

i. Spontaneous O_2^-.

From the histograms of the spontaneous release of O_2^- it can be seen that PHMPSA markedly inhibited this from cells of all groups except from Dpen PMN and from NSAID MNC (Figures 26 and 27). Mann-Whitney U analysis revealed no significant difference in the level of O_2^- released by the different groups with or without PHMPSA, nor in the effect of PHMPSA inhibition (Figure 28).

Effect of PHMPSA on Spontaneous Superoxide Release by PMN.

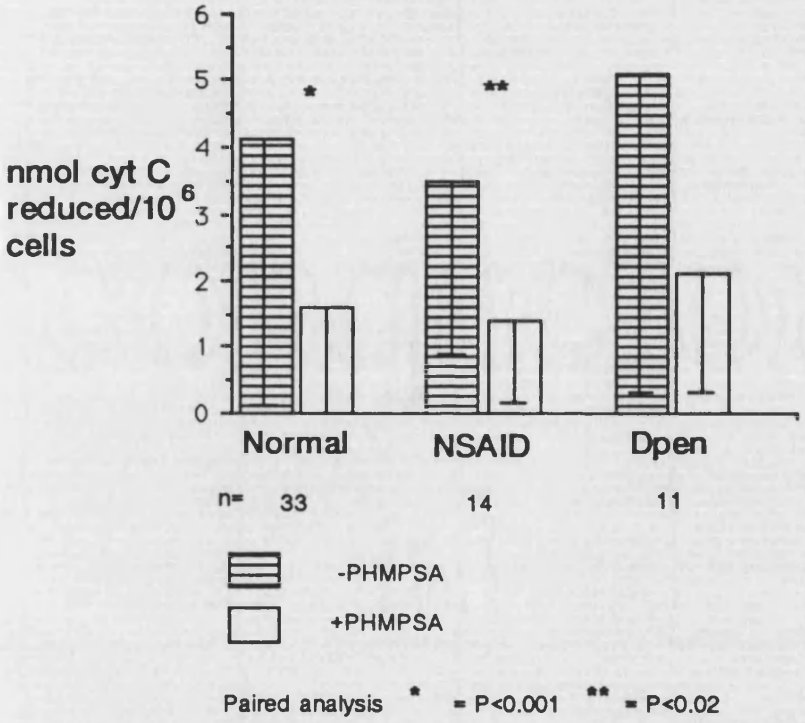


Figure 26

Effect of PHMPSA on Spontaneous Superoxide Release by MNC

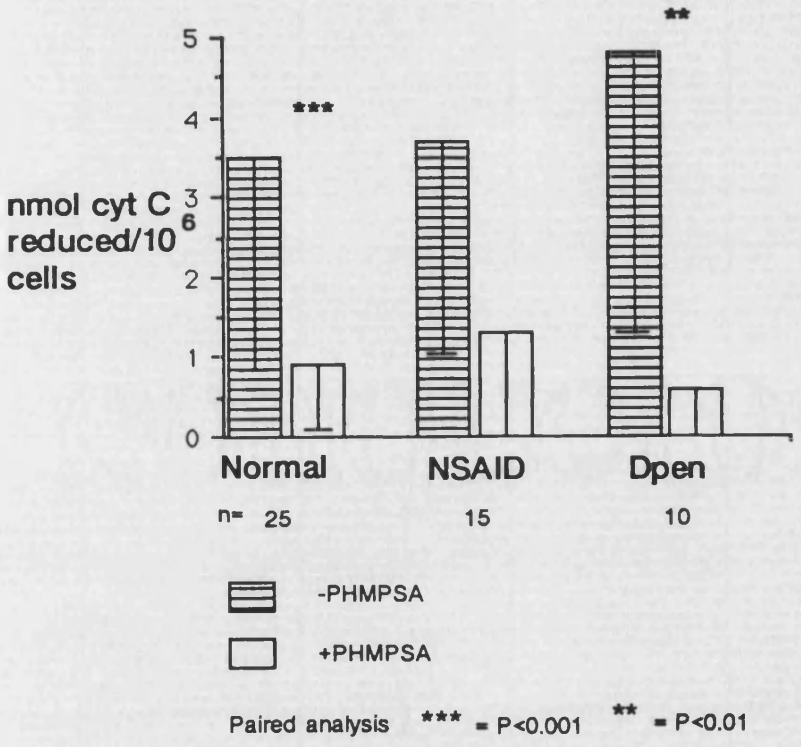


Figure 27

Percent Spontaneous Superoxide Production Remaining After PHMPSA Preincubation.

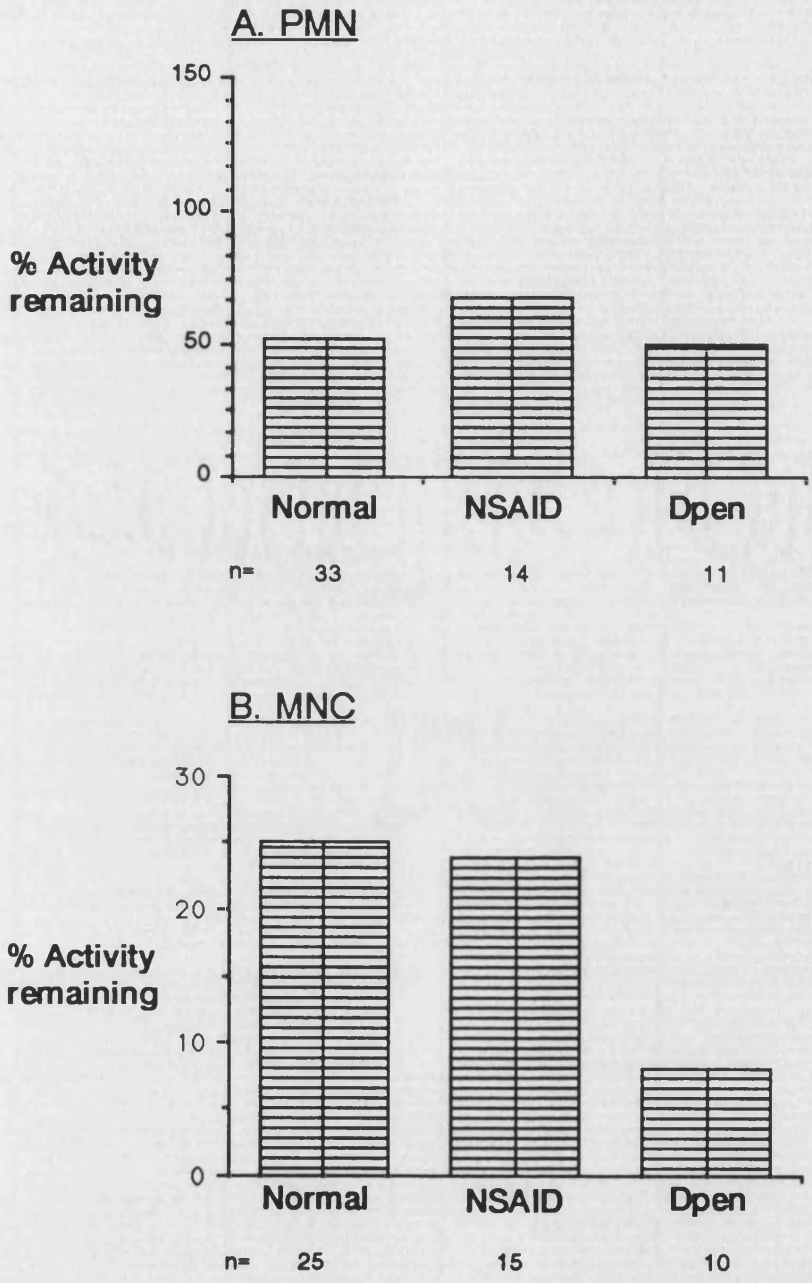


Figure 28

ii. Stimulated O_2^--Responses.

Comparison was made of O_2^- production in response to PMA and to FMLP by PMN and MNC from:

a) normal volunteers b) sero positive patients with rheumatiod arthritis on NSAIDs c) sero positive patients with rheumatiod arthritis on D-Penicillamine, each with and without prior preincubation with PHMPSA.

There was a significant inhibition of the FMLP response by PMN and MNC from all groups (Figures 29 and 31).

The PMA response of MNC was not affected by PHMPSA (Figure 32 note; no Dpen group), but the response of PMN was significantly enhanced in all three groups (Figure 30).

Mann-Whitney U analysis of the unpaired data, showed the NSAID group MNC response to PMA to be higher than the normal group both without ($P<0.03$) and with ($P<0.05$) PHMPSA preincubation. No other significant differences were found.

Effect of PHMPSA on the Superoxide Response of FMLP-Stimulated PMN.

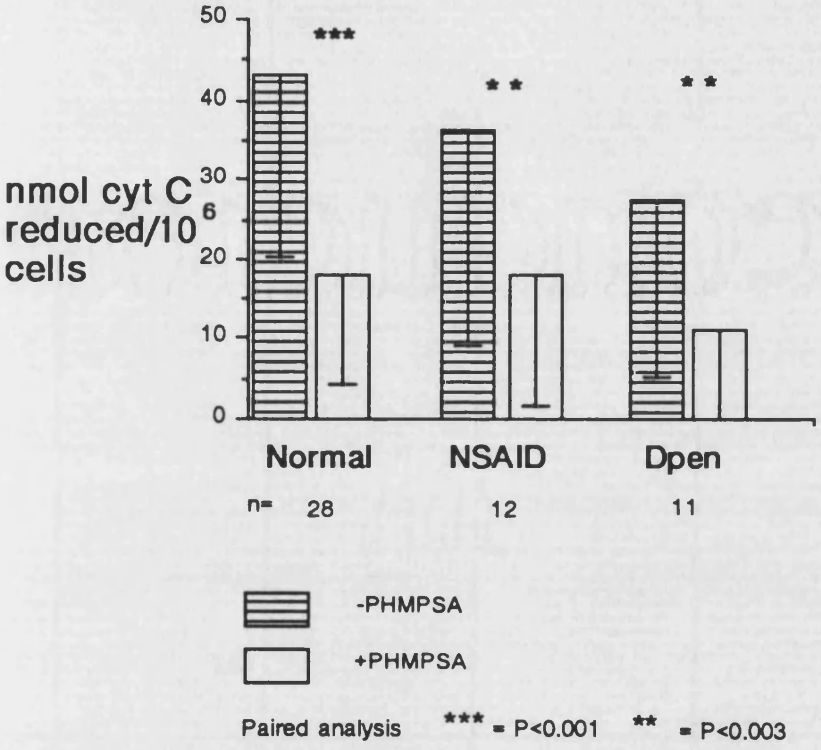


Figure 29

Effect of PHMPSA on the Superoxide Response of PMA-Stimulated PMN.

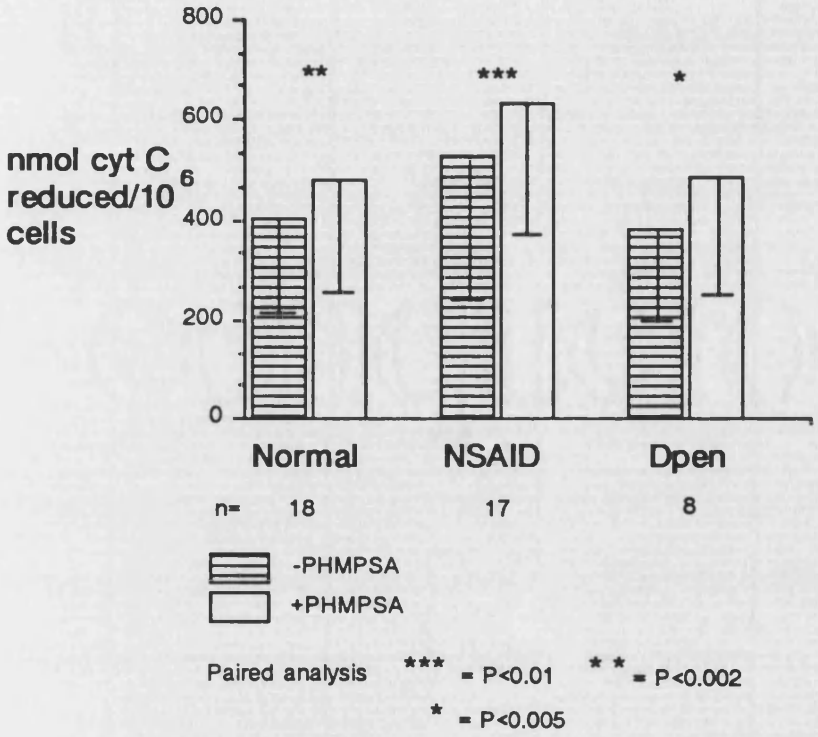


Figure 30

Effect of PHMPSA on the Superoxide Response of FMLP-Stimulated MNC.

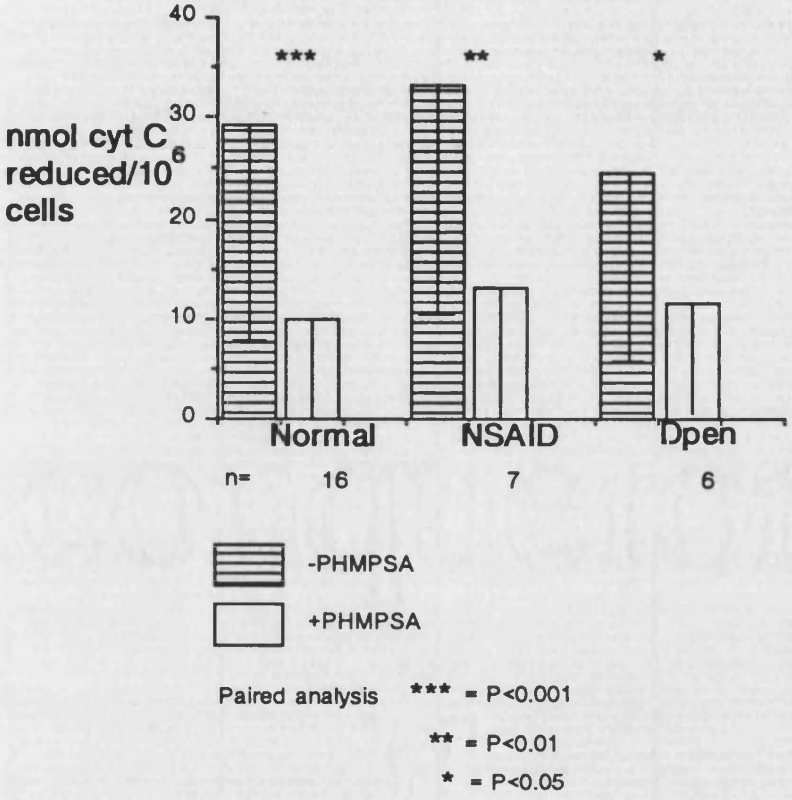


Figure 31

Effect of PHMPSA on the Superoxide Response of PMA-Stimulated MNC.

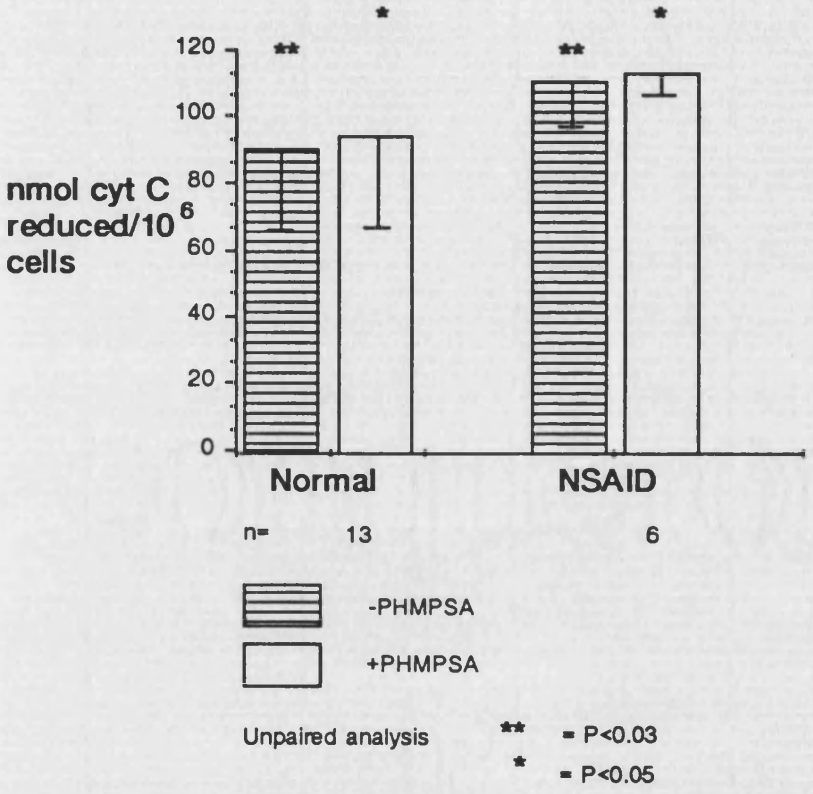


Figure 32

The response of PMN and MNC to fluoride stimulation was examined, with and without PHMPSA preincubation, in the three groups as for FMLP and PMA but the PMN data were also analysed for the possible effects of the sex of the donor.

In MNC from all groups PHMPSA significantly reduced the response (Figure 35), more in normals ($P<0.001$) than in the NSAID group ($P<0.002$) and least in the Dpen group ($P<0.04$).

In PMN there appeared to be no significant effect overall of PHMPSA on the fluoride response. However on dividing the results according to the sex of the donor of the cells, some interesting data emerged. The normal data were divided into males, premenopausal and postmenopausal women (Figure 33). The cells from male donors were significantly enhanced by PHMPSA ($P<0.05$) whereas those from either group of women were not affected. The patient groups were also divided (Figure 34). The results showed a significant enhancement of response after PHMPSA preincubation from NSAID and Dpen groups regardless of the sex of the donor.

Thus these data on the effects of sulphydryl blockade of the fluoride response demonstrate;

a) a difference due to the sex of the donor of normal PMN; b) a difference due to cell-type in all groups; and c) a difference in healthy PMN compared with those from rheumatoid patients.

Effect of PHMPSA on the Superoxide Response of Fluoride-Stimulated Normal PMN.

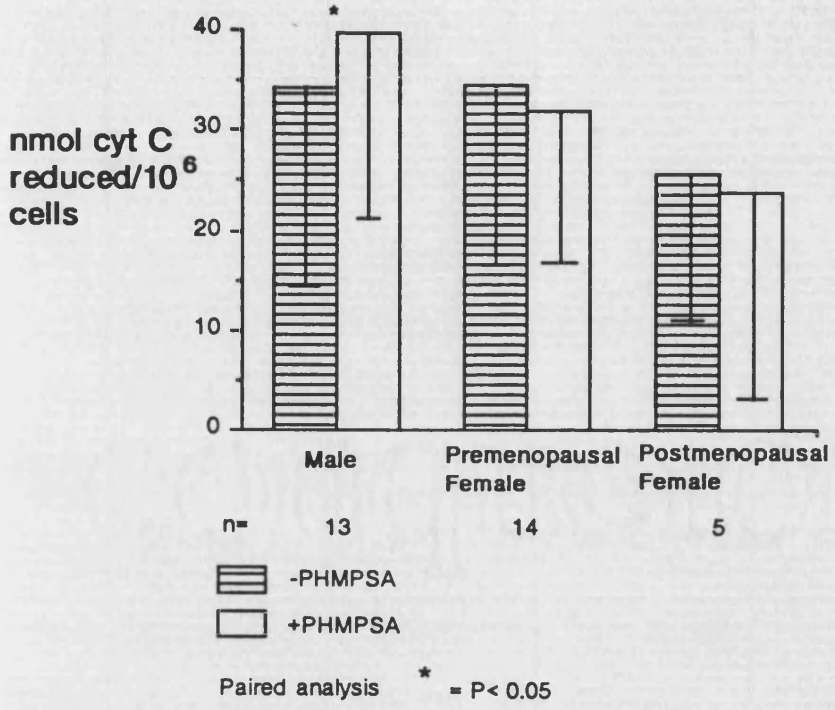


Figure 33

Effect ofPHMPSA on the Superoxide Response of Fluoride-Stimulated PMN from R.A. Patients.

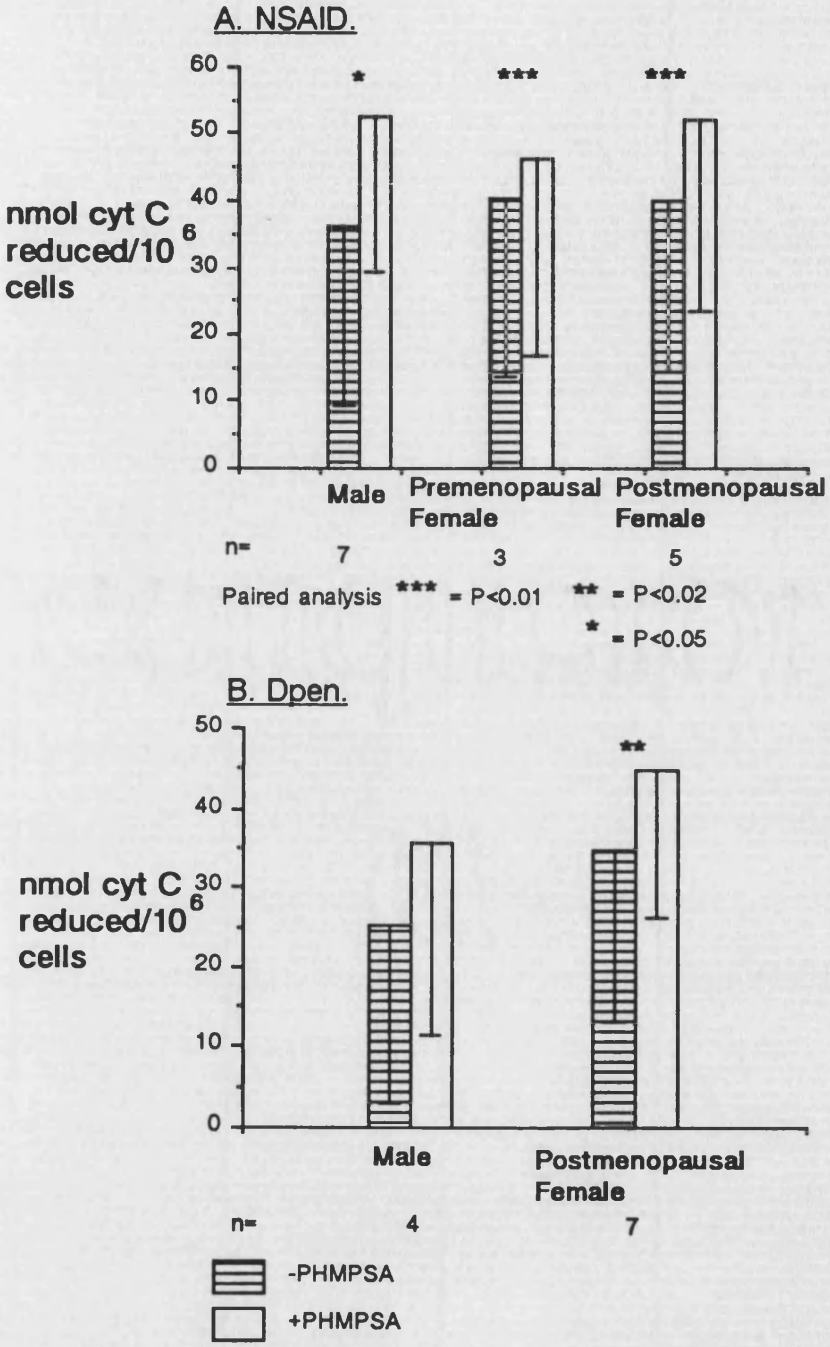


Figure 34

Effect of PHMPSA on the Superoxide Response of Fluoride-Stimulated MNC.

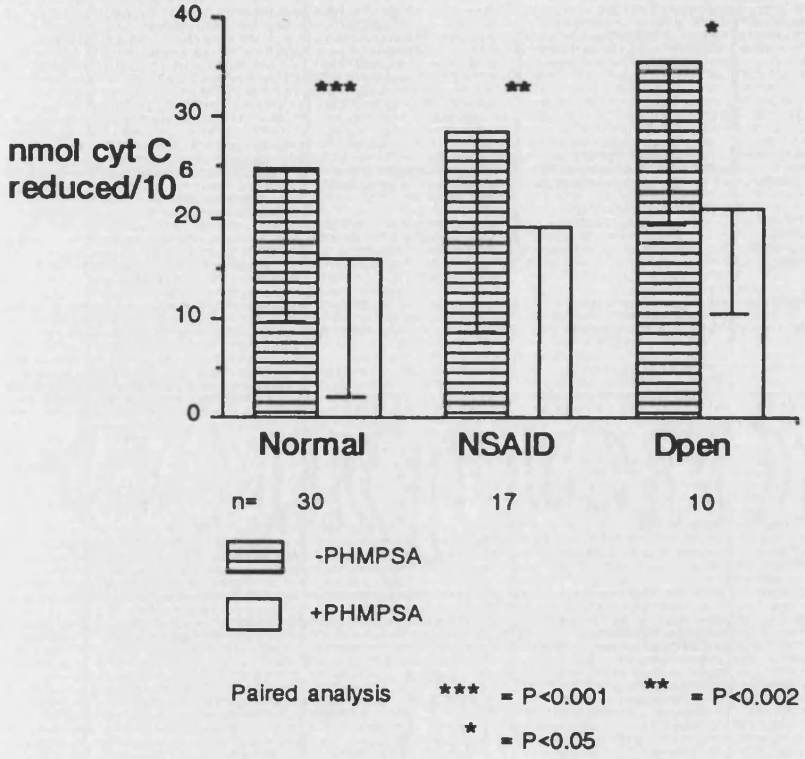


Figure 35

iii. Costimulation.

It was decided to investigate possible interactions of the different stimuli. Experiments were carried out in which cells were stimulated simultaneously with more than one reagent.

From Figures 36 and 37 it can be seen that the response with both PMA and fluoride together is considerably less than that found with PMA alone regardless of the presence or absence of PHMPSA, in both PMN and MNC, from either normal cells or those from the NSAID group. The PMN data given for the fluoride response were obtained from $2x10^6$cells/ml. The data for PMA and for PMA+fluoride were from $0.25x10^6$cells/ml. Fluoride affected PMN at a cell concentration at which no O_2^- response to fluoride alone was detectable.

Using FMLP and fluoride stimulation together (figure 38) the effect was seen to be cumulative.

Effect of Costimulation of PMN Superoxide Production with PMA and Fluoride.

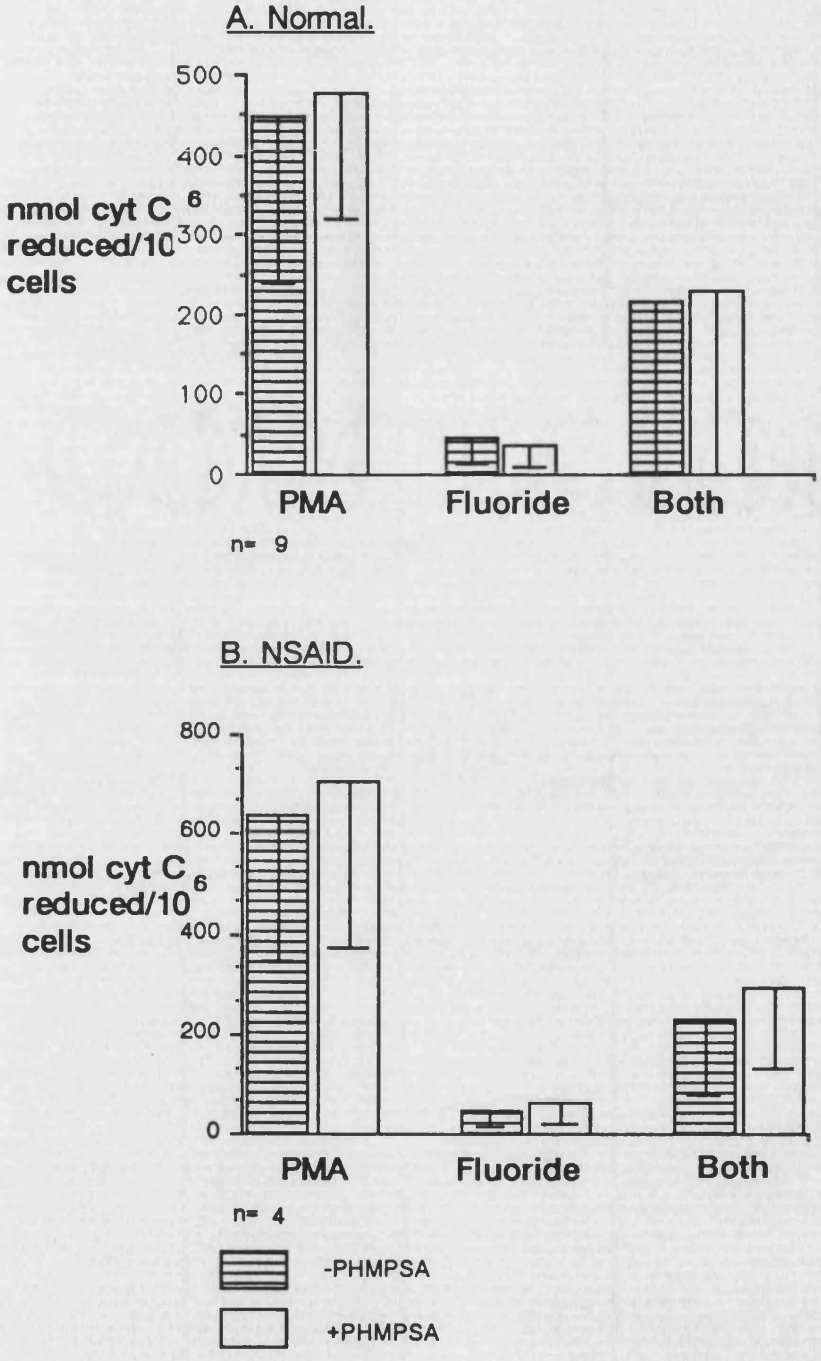


Figure 36

Effect of Costimulation of MNC Superoxide Production with PMA and Fluoride.

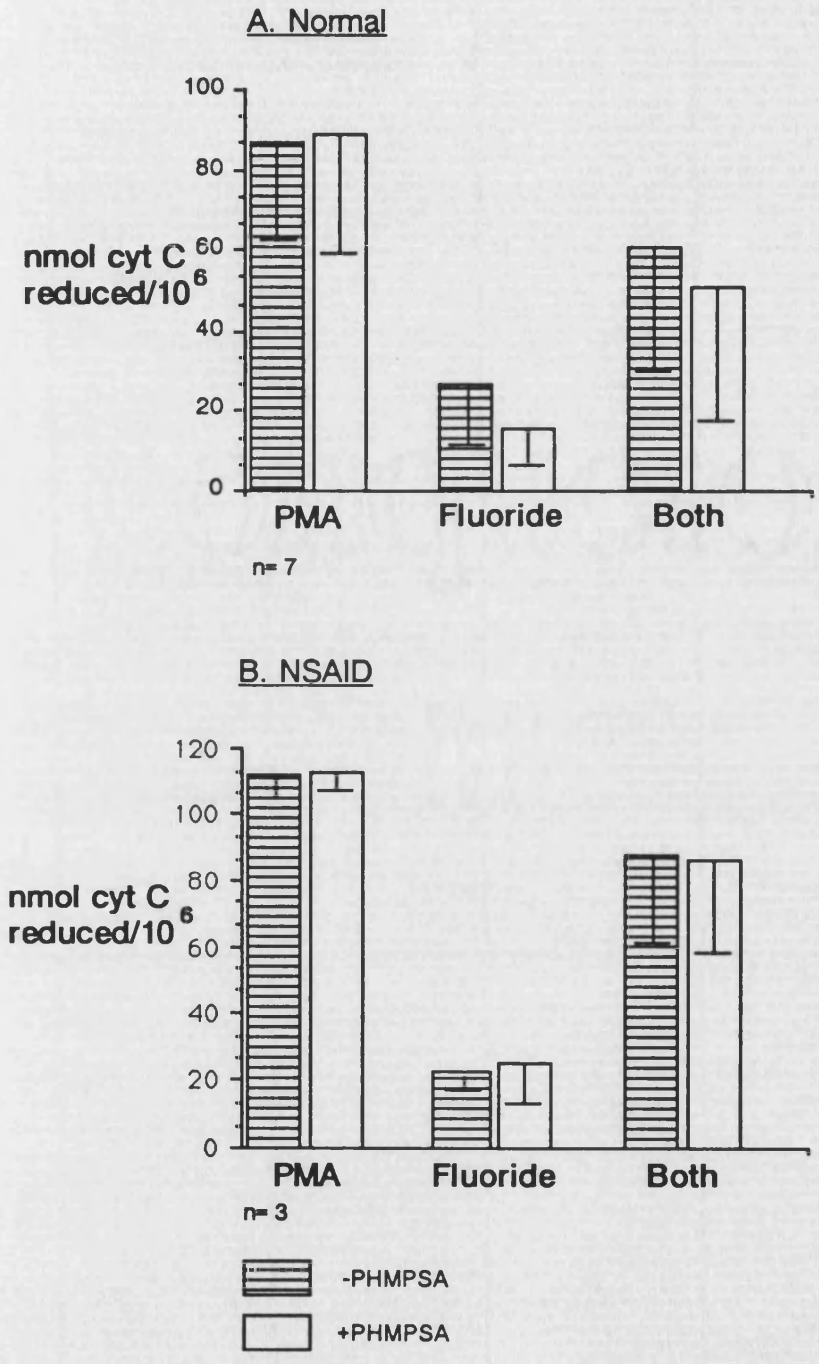
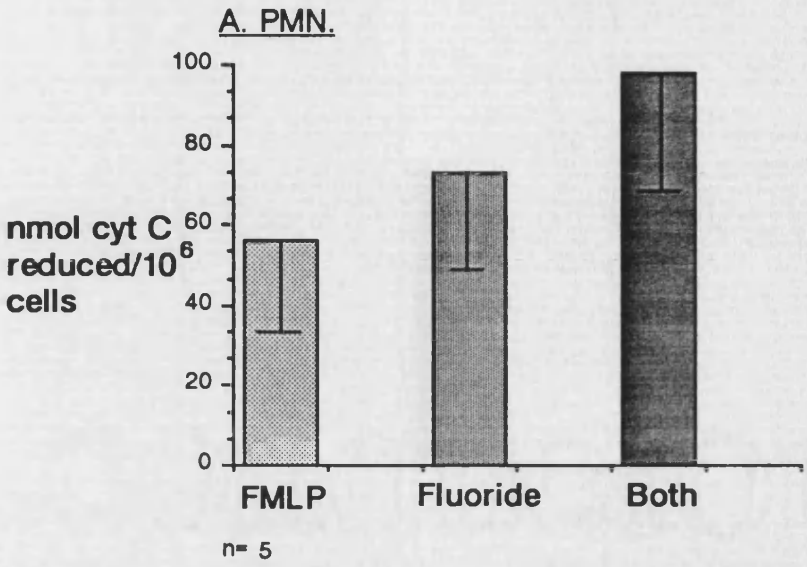


Figure 37

Effect of Costimulation of PMN and MNC Superoxide Production with FMLP and Fluoride.



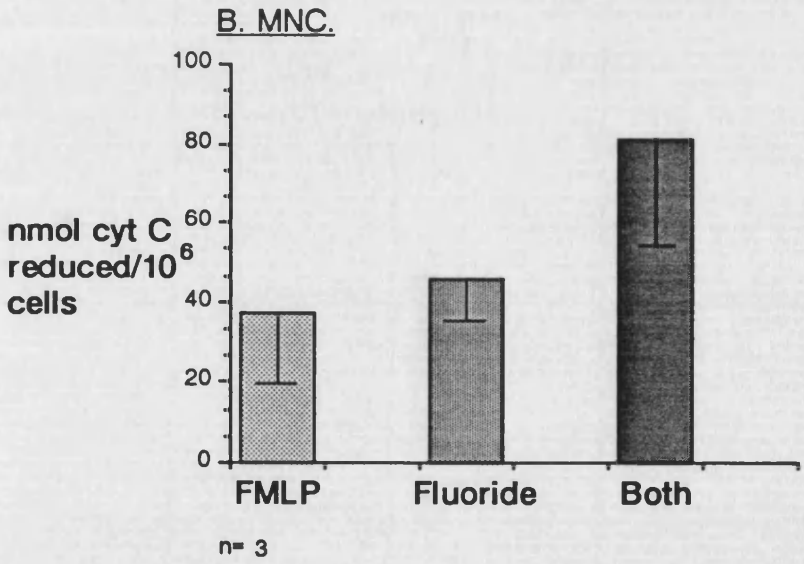


Figure 38

B. Preincubation with IAP.

Blockade with the Islet Activating Protein of Bordetella pertussis toxin was used in a preincubation step before stimulation of cells in the O_2^- assay to investigate the involvement of G Proteins in the activation pathways.

i. IAP and Spontaneous O_2^--Release.

The effect of IAP on spontaneous O_2^- release was analysed. From Figures 39 and 40 it can be seen that IAP reduced spontaneous O_2^- release significantly in all groups of PMN and MNC except the Dpen PMN. Analysis of the unpaired data did not show any significant difference between the levels of spontaneous release from cells of different groups, with or without IAP, nor did it show any difference between the effect of IAP on the PMN from any of the groups. It did however reveal a significant difference in the percentage of activity remaining after IAP preincubation of NSAID MNC compared with normal MNC ($P<0.05$) and no difference between the NSAID group and the Dpen group (Figure 41).

Effect of IAP on Spontaneous Superoxide Release by PMN.

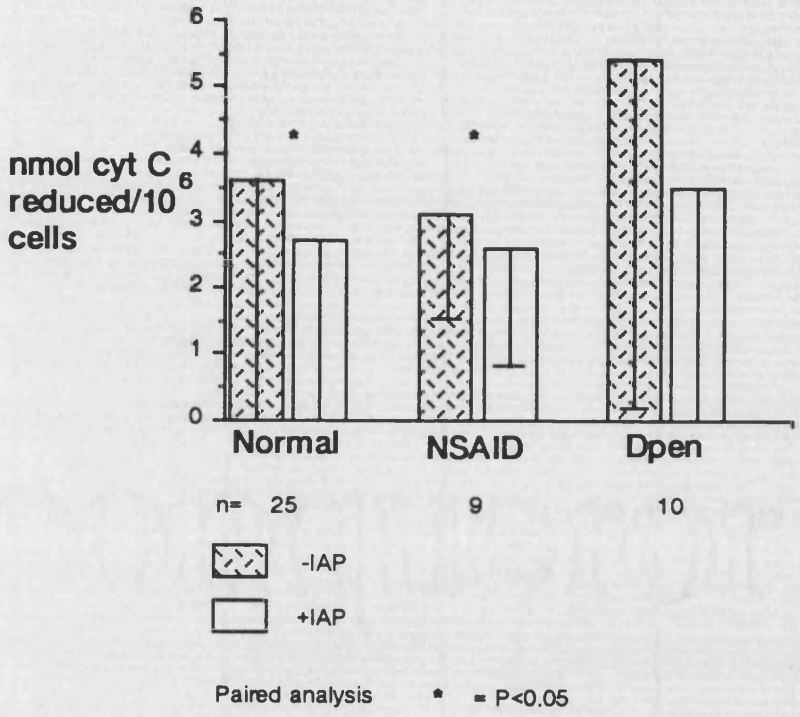


Figure 39

Effect of IAP on Spontaneous Superoxide Release by MNC.

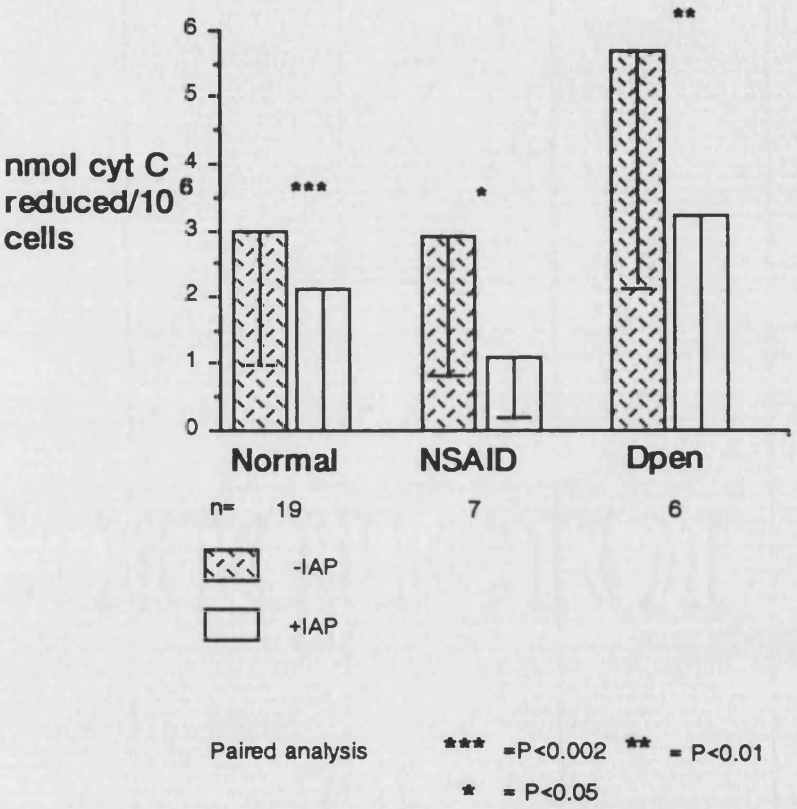


Figure 40

Percent Spontaneous Superoxide Production Remaining After IAP Preincubation.

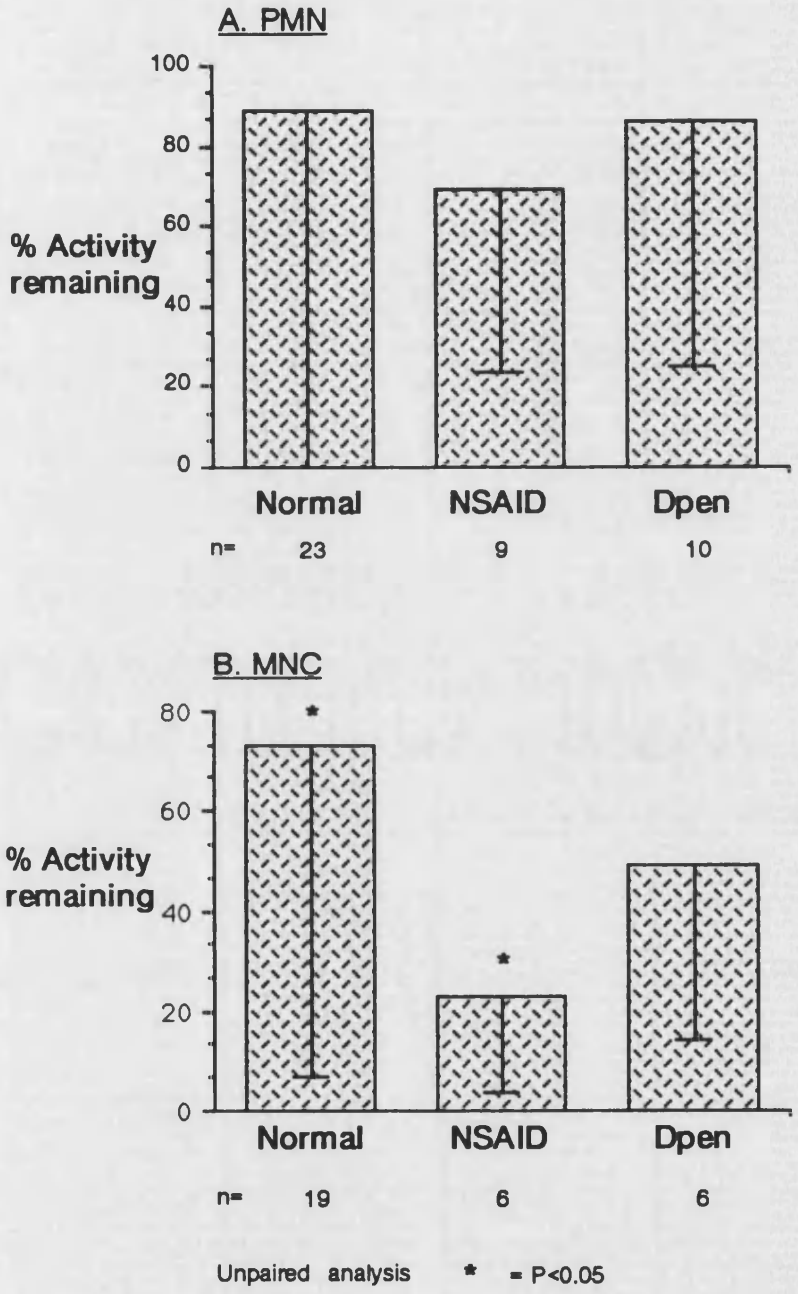


Figure 41

ii. IAP and Stimulated O_2^--Release.

The effect of IAP on stimulated levels of O_2^- from PMN and MNC and the statistical analysis of these data is shown in Figures 42 to 47.

Wilcoxon analysis of the paired data showed that IAP significantly reduced the amount of O_2^- released from PMN by FMLP stimulation in all three groups (Figure 42), the normal group most of all ($P<0.001$) the Dpen group slightly less so ($P,0.005$) and the NSAID group less still ($P<0.01$). It had no effect on PMA stimulation (Figure 43). The fluoride response of normal PMN was significantly inhibited ($P<0.001$), but neither that of the NSAID group nor the Dpen group was significantly affected (Figure 44).

MNC data showed a significant repression of the FMLP response from the normal group ($P<0.002$), numbers from the NSAID and Dpen groups being too small to analyse (Figure 45). No effect was seen on the PMA response (Figure 46). The fluoride response (Figure 47) was significantly reduced in the normal ($P<0.05$) and Dpen groups ($P<0.05$) but not in the NSAID group.

IAP also inhibited cyt B stimulation of MNC (n=3) by 38%, and PMN (n=3) by 36% (not shown).

Mann-Whitney U analysis of the unpaired data was carried out to examine the effect of IAP.

PMN: No significant difference was found between the effect on FMLP stimulation in comparison of any of the groups.

MNC: The inhibitory effect of IAP on the fluoride response was significantly greater in the Dpen group compared with the NSAID group ($P<0.05$).

The fluoride data was divided up into the sexes for analysis (not shown), but no difference was seen with IAP.

Effect of IAP Preincubation on Superoxide Production by FMLP-Stimulated PMN.

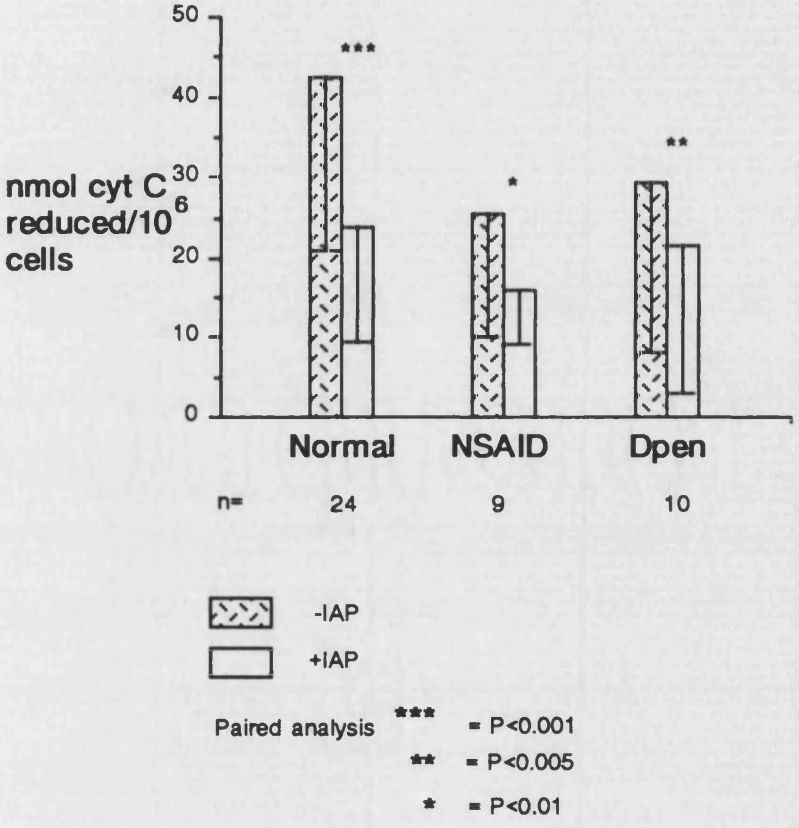


Figure 42

Effect of IAP Preincubation on Superoxide Production by PMA-Stimulated PMN.

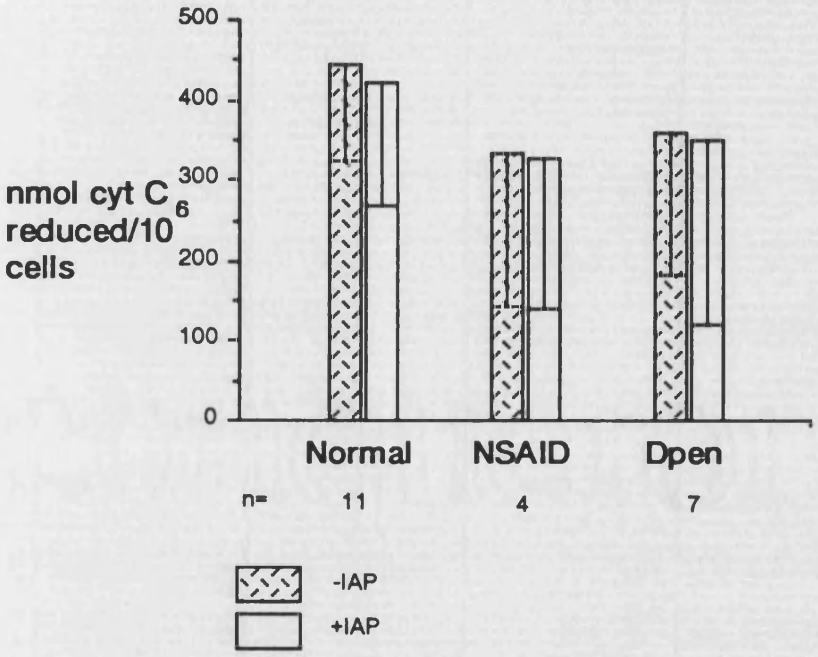


Figure 43

Effect of IAP Preincubation on Superoxide Production by Fluoride-Stimulated PMN.

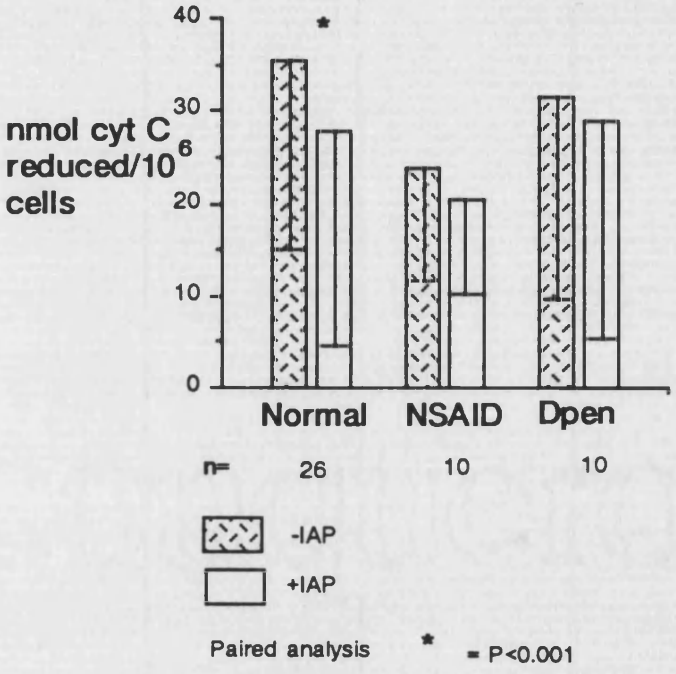


Figure 44

Effect of IAP Preincubation on Superoxide Production by FMLP-Stimulated MNC.

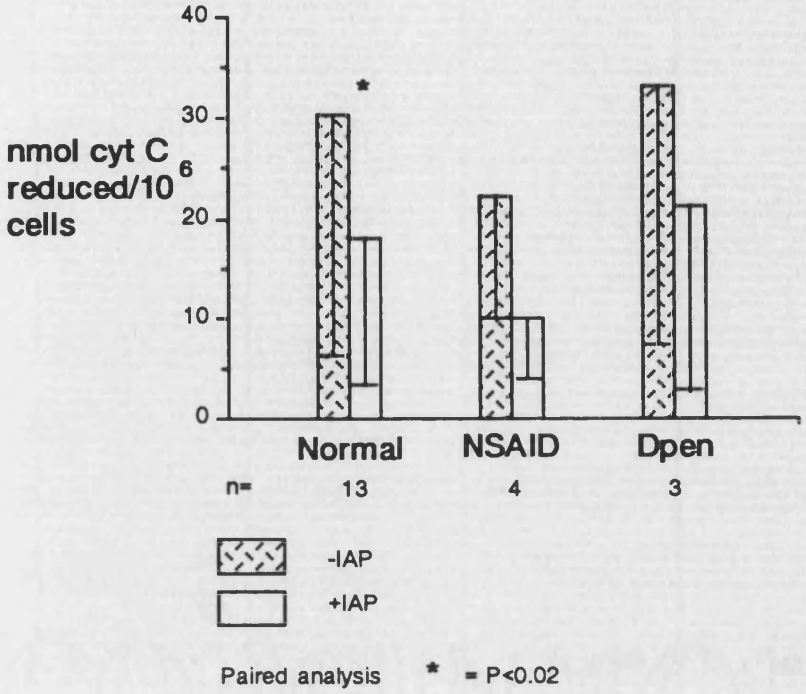


Figure 45

Effect of IAP Preincubation on Superoxide Production by PMA-Stimulated MNC.

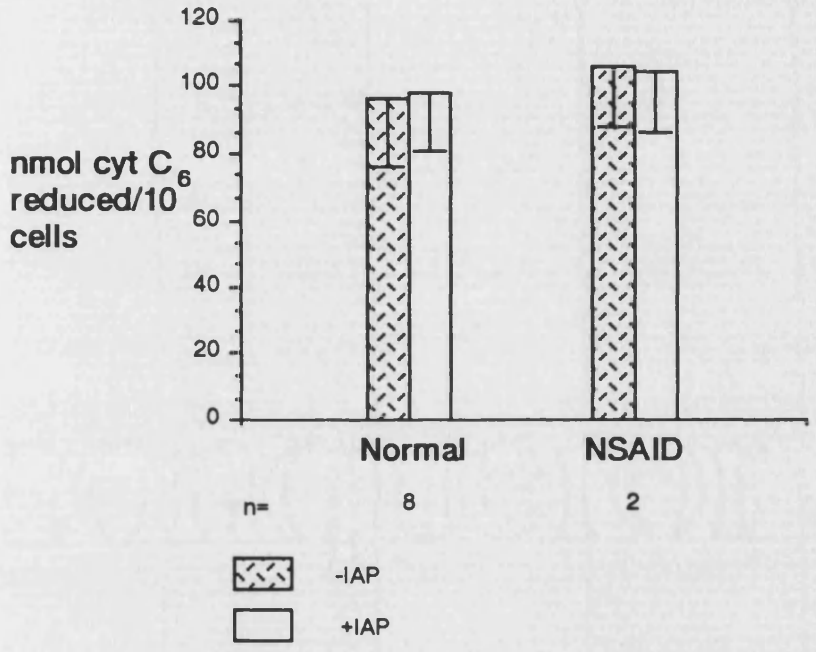


Figure 46

Effect of IAP Preincubation on Superoxide Production by Fluoride-Stimulated MNC.

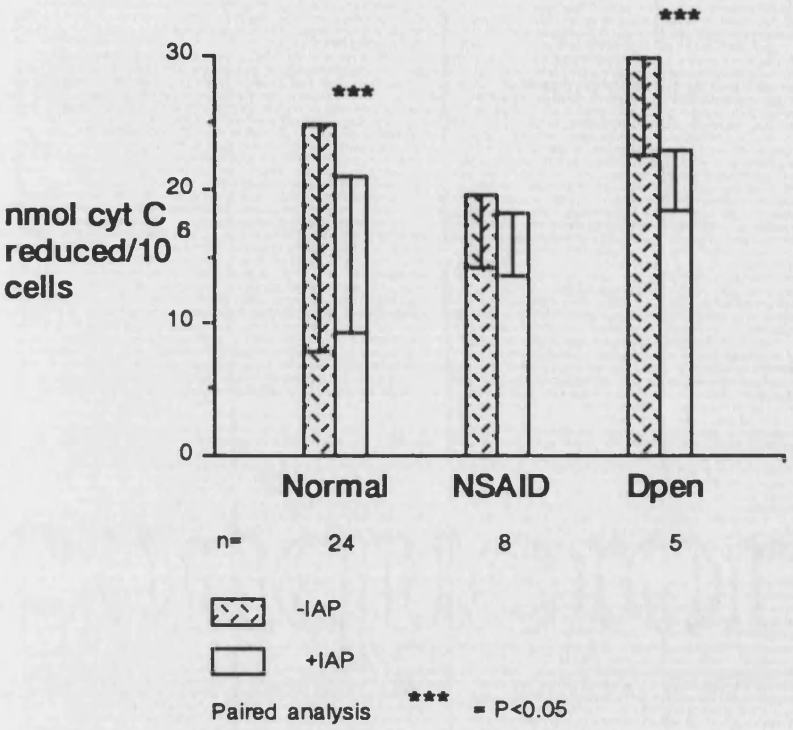


Figure 47

C. PHMPSA and IAP Co-preincubation.

The effects of preincubating PMN with PHMPSA and IAP were investigated in normal cells to see if there was any interaction between their sites of action. The results (Figure 18) showed that in the case of fluoride-stimulated cells there was a significant difference in the effect of PHMPSA and IAP preincubation on the cells ($P<0.003$) but no significant difference between those cells and the ones preincubated with both reagents at once. The FMLP results showed no significant difference in any of the three preincubation groups.

Percentage Activity Remaining from Normal PMN After Preincubation with PHMPSA, IAP or Both.

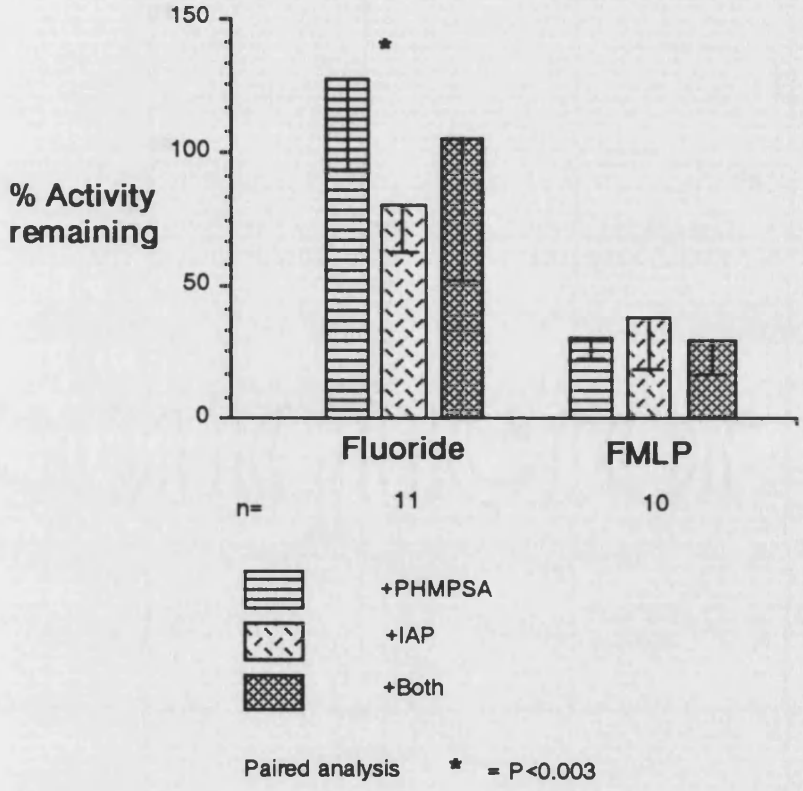


Figure 48

It was decided to find out if the pathways activated by the chosen ligands and the effect of PHMPSA could be further defined by monitoring the internal calcium flux.

SECTION 4.

CALCIUM FLUX ASSAY.

Indo-1 AM requires intracellular de-esterification of its acetomethoxy group to fluoresce in the presence of calcium. Adding calcium to the cell suspension does not raise fluorescence unless calcium is internalized - or unless metabolised, calcium-bound Indo-1 leaks out.

The release of intracellular calcium by normal PMN is presented below in tabular form and results are expressed as nMol calcium per 2.6 x 10^6 cells, with the basal levels deducted from the stimulated responses.

Results from this assay have been expressed both as the ratio of basal level of calcium to stimulated level (Table 4A), and as the absolute amounts of calcium released (Table 4B).

Table 4A.

Calcium Release expressed as P/B Ratio.

	n	-PHMPSA	+PHMPSA	%Activity Remaining
			(s.d.) (s.d.)	
Calcium	6	3.1	2.0	65
		(0.7)	(0.6)	
FMLP	3	10.3	1.3	13
		(8.5)	(0.3)	
FMLP+Ca	4	63.2	5.1	8
		(51.3)	(2.3)	
FMLP+Ca+cyt B	2	75.9	12.3	16
		(29.4)	(13.9)	
FMLP+cyt B	2	2.5	1.5	60
		(0.1)	(0.1)	
$FMLP_2$ +Ca	4	62	4.2	7
		(49.5)	(1.5)	
$HAGG_1$	1	2.7	2	74
$HAGG_1$+Ca+cyt B	2	22.6	5.6	25
		(4.6)	(1.8)	
$HAGG_1$+Ca	3	39.6	6.9	17
		(31.8)	(2.6)	
$HAGG_2$+Ca	1	7.8	2.5	32
$HAGG_3$+Ca	1	186	4.3	2
Fluoride $peak_1$	2	2.7	0	0
		(1.9)	(0)	
" $peak_2$		3.2	2.3	72
		(0.6)	(1.6)	

Table 4B.

Change in Internal Calcium Concentration with and without PHMPSA Preincubation.

		nMol Calcium.		
Stimulus	n	-PHMPSA (SD)	+PHMPSA (SD)	%Activity Remaining
1. None (basal level)	7	175 (73)	284 (90)	162
2. Calcium	6	235 (35)	158 (49)	67
3. $FMLP_1$	3	4997 (4543)	125 (138)	2
4. $FMLP_1$ + calcium	4	7468 (4040)	546 (172)	7
5. $FMLP_1$ +ca+cyt B	2	9139 (4827)	1303 (1364)	14
6. $FMLP_1$ + cyt B	2	364 (19)	192 (83)	53
7. $FMLP_2$ +calcium	4	8809 (8649)	759 (390)	9
8. $HAGG_1$	1	305	231	76
9. $HAGG_1$+ca+cyt B	2	1942 (936)	493 (148)	25
10. $HAGG_1$ + ca	3	4330 (3423)	1115 (743)	26
11. $HAGG_2$ + ca	1	852	1333	156
12. $HAGG_3$ + ca	1	16660	2500	15
13. Fluoride peak1	2	379 (445)	0 (0)	0
14. " peak2		487 (182)	283 (334)	58

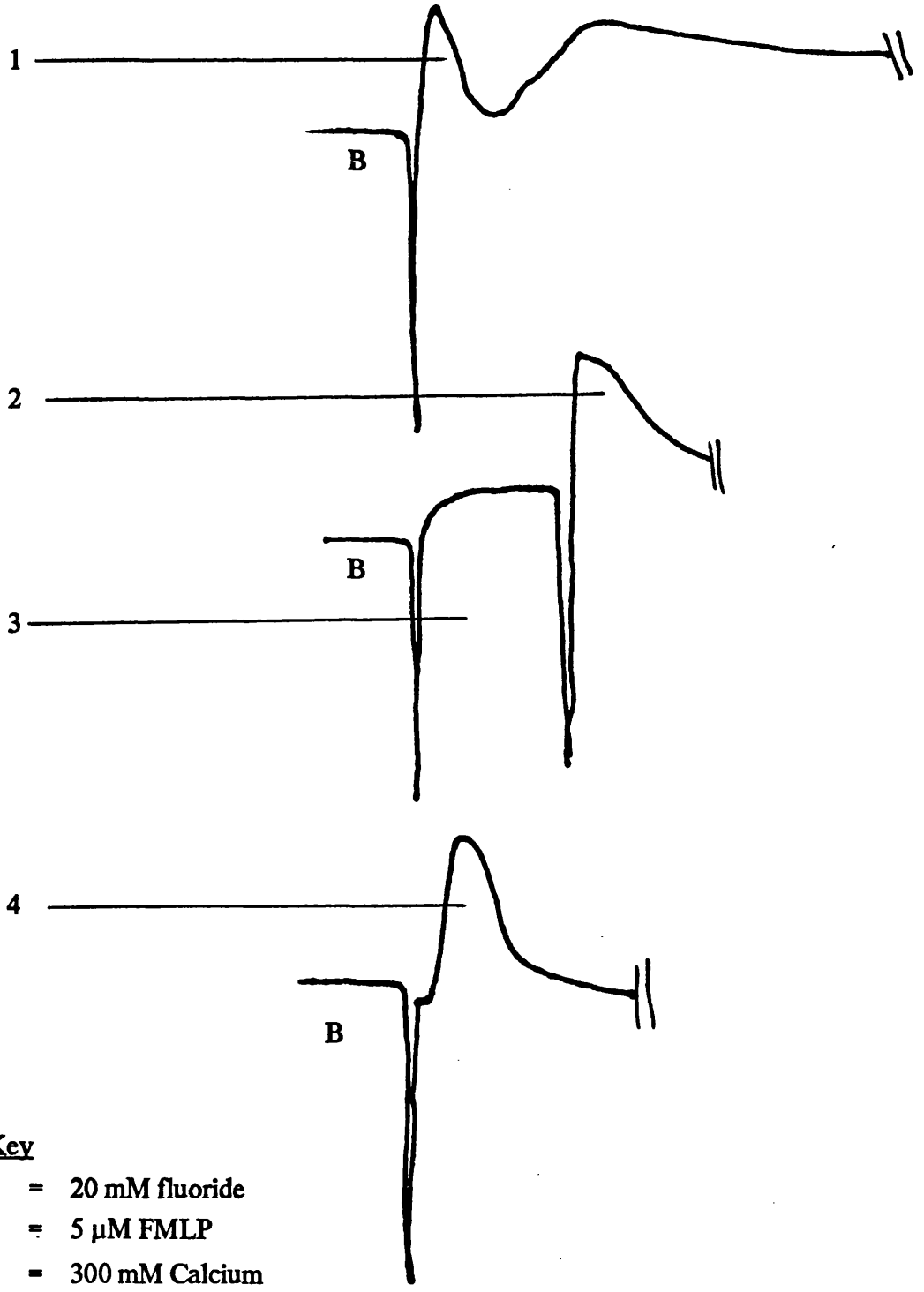
key: $FMLP_1$ = [5uM] $FMLP_2$ = [100nM]

$HAGG_1$ = [500ug/ml] $HAGG_2$ = [250ug/ml] $HAGG_3$ = [1mg/ml]

Fluoride = [20mM]

Note: the responses for fluoride in the table are those found in experiments carried out in Hanks/hepes buffer.

Figure 51 Curves obtained with the various stimuli.



Key

1 = 20 mM fluoride
2 = 5 μM FMLP
3 = 300 mM Calcium
4 = 500 μg/ml HAGG
B = Basal level

i. Basal

Overall basal levels of stimulation were seen to be enhanced to 162% by the PHMPSA preincubation:

Note: The decrease in the FMLP and HAGG responses is greater than can be accounted for by the increase in the basal level of response after PHMPSA preincubation.

ii FMLP

In a comparison of the response from FMLP-stimulated PMN with no calcium added and those similarly stimulated but in the presence of 300μM exogenous calcium ($calcium_e$), deduction of the response due to the calcium itself revealed that the FMLP response is nearly doubled by the presence of $calcium_e$, an effect which was still seen in the small response of the PHMPSA-preincubated cells. Thus $calcium_e$ increased the level of the FMLP-induced response, but it did not affect the inhibition seen by PHMPSA.

The inhibitory effect of PHMPSA on the FMLP-stimulated response was almost total and was greater than that seen with the O_2^- and peroxide responses.

The effect of FMLP stimulation was almost immediate, having reached a maximum by 5 seconds with no lag period. This rapid response was not maintained, the peak being followed by a sharp decline as the calcium was sequestered back into internal stores. The decline from the maximal response was not as rapid in the PHMPSA-preincubated cells. PHMPSA was as inhibitory for cells stimulated with 5uM FMLP as for those stimulated with 100nM FMLP.

iii. Cytochalasin B

Cytochalasin B, whilst not eliciting a response itself (not shown) enhanced the FMLP response in the presence of $calcium_e$.

iv. HAGG.

In the presence of 300uM calcium$_e$, the response to HAGG was not as large as that induced by FMLP, and whereas with the FMLP response only 7% activity remained after PHMPSA, there still remained 26% of the HAGG response.

v. PMA.

PMA did not elicit a response (not shown).

vi. Calcium$_e$.

Calcium$_e$ induced a slight stimulatory response, which was PHMPSA sensitive, 67% activity remaining after PHMPSA preincubation.

vii. Fluoride.

Fluoride uniquely induced two peaks of calcium release from PMN, one almost immediate and, after a return to basal level, a second more gradual one. This biphasic response was more marked when the buffer was Hanks/hepes (calcium [1.5mM]) rather than PBSG with added calcium ([300μM]) (not shown). No fluoride response was seen in the absence of calcium$_e$. A dose response curve in Hanks/hepes buffer demonstrated that the two peaks were of the same magnitude, differing only in the timecourse of the response, and that it demonstrated the same range as that found for the O_2^- response, both peaks showing maximal response at [20mM] and decreasing again with greater concentrations. The peaks were both affected by PHMPSA preincubation of cells, the first peak being totally abolished and the second much diminished with the timecourse to the second peak being greatly extended.

viii. Time

(See Table 5)

a. HAGG.

PHMPSA preincubation almost doubled both the lag time seen in the HAGG response, and the time taken for the response to reach maximum.

b. FMLP.

The FMLP response had no measurable lag time, with or without PHMPSA, but after PHMPSA the time taken for response to return to a plateau was considerably increased (-PHMPSA 13sec; +PHMPSA >60sec).

c. Calcium.

The calcium-induced response had no lag period, and the response was a steady increase which, on reaching a plateau did not fall off in the 3 minutes allowed before termination of the experiment. PHMPSA did not affect the timecourse of the response.

d. Fluoride.

The initial peak was abolished by PHMPSA and the time taken to reach a plateau in the second response was nearly doubled.

Table 5.

Timing of Calcium Flux (seconds).

Stimulus.	-PHMPSA (s.d.)		+PHMPSA (s.d.)	
	Lag.	To max. response	Lag.	To max. response
$HAGG_1$:	17 (10)	69 (35)	29 (10)	114 (30)
(n=3)				
FMLP	0	13 (10)	0	96 (75)*
(n=6)				
Fluoride				
(n=2)				
peak one:	0	8	-	-
peak two:		107		185

Note: * This represents only those experiments in which the response was not completely abolished.

ix. Pilot studies were carried out using more than one stimulus in the same experiment which suggested the following:

1. The first fluoride-induced peak was abolished if the cells were first stimulated by another ligand, such as FMLP, PMA, or cross-linked monoclonal antibody 1V.3 (directed against FcR11), whilst the second peak still occurred.

2. An initial stimulation with fluoride abolished the FMLP response.

3. Prior stimulation with PMA did not affect the FMLP response.

4. PMA added to cells before FMLP had no effect on the level of stimulation induced by the FMLP, nor did it affect the inhibition due to PHMPSA preincubation. However the recovery of the FMLP response to a plateau was quicker.

5. When monoclonal antibody 1V.3 was used PHMPSA was found to cause an inhibition of the same order of magnitude as for HAGG.

6. Use of monoclonal antibodies directed against FcRs 1, 11 and 111, in experiments with PMN or purified monocytes showed that occupancy of any of the three receptor subtypes did not trigger release of internal calcium until they were cross-linked. Also, cross-linked IgG_1 activated FcR1 on purified monocytes was effective at inducing release of calcium, and this was the same IgG as was used to make HAGG for PMN and MNC stimulation in the rest of this project.

SECTION 5.

MYELOPEROXIDASE ASSAY.

It was decided to measure another parameter of PMN activation, namely azurophilic degranulation. Cells were preincubated with PHMPSA in PBSG or in PBSG alone, for 30 minutes at 37°c, and their supernatants assayed for myeloperoxidase (MPO). The results in Table 6a demonstrate that unlike the O_2^- response and the calcium flux, MPO release stimulated by FMLP was enhanced by the SH blockade. Cytochalasin B induced a significant stimulation in its own right, which was also enhanced by PHMPSA. Either PMA or fluoride, neither of which released MPO, caused a slight repression of spontaneous MPO release.

PHMPSA reduced the total MPO released by Triton X100 lysis. This effect required the whole cells or the cell lysate to be in contact with the PHMPSA for at least 30 minutes and was not seen if PHMPSA was added to the cell lysate directly before assay. PHMPSA had no effect on the cell free reagents (not shown).

Table 6a shows the percentage MPO released by various stimuli, where 100% is the amount of MPO released by triton X100 lysis. In these results the spontaneous level of release has not been deducted from the stimulated levels. In Table 6b is shown the effect of PHMPSA on total MPO release, expressed as the change in absorbance at 560 nm.

Three experiments were carried out using PMA and fluoride, although they were not expected to induce MPO release. From the table it can be seen that fluoride, PMA and a combination of fluoride and PMA reduced the release compared with that from the unstimulated cells, an effect overidden by PHMPSA except when both agents were used together.

Table 6a.

MPO Release Expressed as The Percentage Total Release Obtained from Triton X100-Lysed PMN.

n=5	-PHMPSA	+PHMPSA
Unstimulated	13	25
5uM FMLP	18	39
n=4		
Cytochalasin B	12	30
" + FMLP	54	92
n=3		
Unstimulated	12	23
PMA	9	35
Fluoride	9	21
" + PMA	8	11

Table 6b. Total MPO Release by Triton X100 Lysis.

Change in absorbance at 560nm.

	-PHMPSA	+PHMPSA
n=3	0.340	0.191
s.d.	0.051	0.025

RESULTS SUMMARY

Use of receptor-mediated ligands and of PMA in the O_2^- peroxide and MPO assays allowed the conclusion that the PHMPSA blockade occurred after ligand binding but before PKC activation, and fluoride further confined the likelihood of the blockade to its occurring before G-protein interaction. Cytochalasin B-induced activity raised the possibility that the relevant SH involvement could be on a site other than the receptor or G-protein molecules themselves, the MPO enhancement by PHMPSA further suggesting no inhibitory modulation of the receptor itself, at least in the case of FMLP.

Use of autologous PMN and MNC in the same assays showed that these cells were differentially affected by PHMPSA; the PMA response demonstrating an enhancement of PMN O_2^- release and not affecting monocytes, and the fluoride response only being inhibited in monocytes.

Use of cells from RA patients on NSAID and on SH-containing drugs did not indicate any obvious effect of the SH-containing drugs on receptor-mediated responses, and indications of SH group modulation of spontaneous release of reactive oxygen species were inconclusive. Increased spontaneous peroxide release by PMN and MNC was significantly higher in cells from patients, effects not seen in the absence of Ca^{2+}_e, which may indicate that disease modifies calcium regulation.

These conclusions will be discussed with supporting evidence in the following discussion.

Chapter four.

DISCUSSION

The project's overall aim, of determining the involvement of SH groups in phagocyte activation and of further defining the mechanism of activation by specific ligands, met with some success. Two pathways were expected to contribute to activation, namely the PLA_2 and PIP_2-specific PLC pathways, with possible modification by the activity of adenylate cyclase. The project has compared the activation of PMN and of MNC by a number of ligands. The initial discussion will be of FMLP-receptor-mediated signal transduction and its modulation by PHMPSA and IAP and this will necessarily include calcium and cyt B. From this will procede the events involving HAGG, PMA and fluoride, followed by the RA study.

In this project, the neutrophils and monocytes from normal peripheral blood were each a microheterogeneous population with respect to maturation, and in the RA cells to both maturation and activation. The normal cells were not distinguishable in this respect. The isolation procedure based on buoyant density could result in significant contamination of the mononuclear fraction by activated (or primed) PMN, and occasionally contamination (of >10%) resulted in the cells being abandoned. The use of a mixed mononuclear fraction had an advantage over monocytes which have been further purified in that the whole monocyte population present in the blood was represented. It also allowed the comparison of autologous PMN and MNC in concurrent experiments. It is still possible that the normal and patient groups represent an unbalanced analysis. In RA the cell population isolated from whole blood may be different from normals in that chronic activation may result in more adherent cells and so a specifically less adherent population being examined. In this project it was noted that cell yield varied, but this variation in number was not restricted to the patient groups, and no analysis of sub-populations of monocytes or PMN was

undertaken (such a may be carried out by buoyant density analysis (Froebel et al 1984).

Investigation of the intracellular events proceeding from receptor-mediated activation by others has included monitoring phospholipid metabolism, calcium mobilization and changes in phosphorylation profiles. Much work is dependent on the use of specific inhibitors such as the PKC inhibitor H7 (Hidaka et al 1984), the PLA_2 inhibitors p-bromophenacyl bromide (Duque et al 1986), deazoadenosine (Pick and Mizel 1982) or mepacrine (Bokoch and Gilman 1984), and the PLC inhibitor neomycin (Nakashima et al 1988). A wealth of different cell-types has been used, sometimes as whole cells, sometimes as permeabilized cells and sometimes as isolated fractions. When comparing one system to another each may only indicate what to investigate in the other. For instance, repression of GTP-gamma-S-induced secretion with H7 (Smolen 1989) by permeabilized PMN suggested that PKC activation did not promote degranulation. These workers found that PMA induced beta-glucuronidase release and this is not the case in whole cells. Also, effects of $calcium_e$ on inositol phospholipid metabolism differ in whole and in permeabilized human PMN (Bradford and Rubin 1986). The fine regulation of G-protein-mediated systems may be tissue and species specific at the transcriptional and translational level (Milligan 1989) which may alter with the developmental stage of the cell (Gierschik et al 1986; Luetje et al 1987) and with age (Milligan 1987b). Monoclonal antibodies to alpha-subunits form more specific probes than the fluoride used in this project. However the use of fluoride has made a general contribution to recognising G-protein involvement. This project for instance, has demonstrated that there is a difference in the mechanism by which PMN and MNC are activated in that only in MNC is there an SH group accessible to modification by PHMPSA. This thiol is either not present or is inaccessible on PMN. The effect of PHMPSA on the activity of G-proteins may

be indirect because of their cytoplasmic siting either in the plasma membrane or situated proximally to it. PHMPSA does not enter the cell and some of its effects are not the same as permeant SH-modifying agents such as N-ethylmaleimide (NEM). For instance, PCMBS, another impermeant SH reagent did not affect phagocytosis (Tsan et al 1976a) or PMN adhesiveness (Giordano et al 1973) whereas NEM inhibited both of these events, and NEM totally abolished O_2^- activity by all stimuli (Akard et al 1988). In rat myoblasts (D'Amore and Lo 1986) cell surface SH blockade was found to inhibit hexose uptake, and this could have been a contributing factor to the deterioration of the cells in the presence of PHMPSA as seen by LDH release in this project. Cytoskeletal involvement in the FMLP activation process is suggested in this project to be modified by PHMPSA and to be critical in determining the activation events which follow stimulation, as witnessed by the differential effect of PHMPSA on the release of reactive oxygen species and of MPO from azurophil granules in response to FMLP + cyt B. A difficulty of determining the effect of cyt B is that it is so generally disruptive. However, the response to FMLP + cyt B was just enhanced by cyt B, not prolonged; so the activation step benefitted from the lack of microfilamentous control, but the cessation of response was not affected. Downey et al (1989) have found that FMLP-induced microfilament assembly was 60% inhibited by IAP in intact cells (human PMN). Fluoride also induced actin polymerization, demonstrating that G-proteins are cytoskeletally associated. In this project limited use of was made of cyt B, always in a direct comparison with cells without it and only intact cells were used. Cyt B was considered to alter FMLP signalling in a manner which it was not initially intended to investigate and so it was usually omitted from experiments. Cyt B prevents actin polymerization. It has no known effect on actin-linking proteins e.g. spectrin (Carraway and Carraway 1989) by which the cytoskeletal network is formed and bound to the plasma membrane and to intracellular structures such as granules. Although the cytoskeleton has been

described as independent of NADPH oxidase (Naccache et al 1989) this is not the case indirectly as the signalling of O_2^- release is activated by cytoskeletal disruption and NADPH oxidase has been found to co-sediment with an actin-fodrin-rich cytoskeletal membrane vesicle fraction (Jesaitis 1988). The same group has reported FMLP receptors in membrane vesicles as cytoskeletally-related and disassociated from their regulatory G-proteins; receptor activation in whole PMN being dependent on their being cytoskeletally unassociated. Potentiation of FMLP activity by cyt B may include increased mobility of granules bearing subunits of the oxidase, and expression of new FMLP receptors at the cell surface from granule membranes.

Other work on PMN activation by FMLP is a mixture of data obtained from permeabilized cells, which may not be directly comparable with work on intact cells, and those which have or have not been preincubated with cyt B. In the use of cyt B it is important to note when the events seen do <u>not</u> occur in cells with an intact cytoskeleton. Organisation of the cytoskeleton is important. It is controlled by a number of cross-linking and membrane-associated molecules which may be rearranged on activation. For instance, Roos et al (1985) localized gold-labelled glycoproteins on the plasma membrane to interconnected regions of the filamentous network of the cytoskeleton. Secretory granules have binding sites for the actin cross-linking protein, fodrin, in chromaffin cells, the proteins in the cytoskeleton being cell-type specific (Aunis and Bader 1988) and thus the cytoskeleton forms the means of regulating selective granule release and other interactions such as those with G-proteins, with enzyme substrate, and with interactions with other ligand-binding as in priming. In chromaffin cells, G-protein-mediated secretion is accompanied by a decrease in cytoskeletal actin, both events being inhibited by neomycin. The decrease in cytoskeletal actin can be mimicked by PKC stimulation (Burgoyne et al 1989). The assumption that events may be similar in phagocytes may end here, unless it is

assumed that secretion thereby is of discrete populations of granules which are differentially controlled because, as found in this project, PKC (PMA) does not induce azurophil granule release. Calcium may be the key to normal reorganization of the cytoskeleton, for cross linking, for instance. Others have found that on ligand-binding a transient and calmodulin-dependent association of the FMLP receptor occurs which could account for some if not all of the calcium requirement of FMLP-mediated activation (Jesaitis et al 1988). A theory put forward here is that FMLP may normally induce a respiratory burst, NADPH oxidase activity and release of specific granules. In the absence of a priming stimulus no release of azurophilic granules occurs. The differential release of granules is dependent on the nature of a priming agent and the cytoskeleton is the link co-ordinating the events which follow the two signals. Such differential regulation may involve for instance a protein which is required for 5-lipoxygenase activity and which must be involved in the receptor-mediated activation of LTB_4 synthesis (Dixon et al 1989; Miller et al 1989). TNF-alpha priming allows FMLP stimulation of O_2^- in a manner independent of exogenous calcium but has no effect on FMLP-induced actin polymerisation or MPO release with or without cyt B, but does enhance lysozyme release (Berkow et al 1987). It is possible that the modification of FMLP-induced activity by PHMPSA is cytoskeletally related, the membrane attachment sites of the cytoskeleton being modified so that the FMLP signal is compromised. This would leave the receptor itself intact, overcoming the problem of explaining major modification of the protein (by PHMPSA) without affecting binding, and would also leave the G-protein similarly unmodified as would be expected of a cytoplasmically sited protein. Differential regulation by PHMPSA of pathways activated by FMLP, that is of selective degranulation and NADPH oxidase activation, could thus be explained, reflecting their degree of dependence on the cytoskeleton. SH modulation would be likely to cause a conformational change and alter the function of the molecule to which it bound, and it may be

significant therefore that after PHMPSA preincubation, FMLP receptor binding was found to be intact by the MPO assay, G-protein function was unimpaired in fluoride-stimulated PMN, and NADPH oxidase and the proton pump were still activated by PMA stimulation. The order which can be assumed to be imposed on the plasma membrane by cytoplasmic linkage could be altered by PHMPSA binding. FMLP has been found to activate both PLA_2 and PLC (Duque et al 1986; Cockcroft et al 1985b) and whereas these pathways may act in concert, they do seem able to be activated mutually exclusively. It has been beyond the scope of this project to investigate this thoroughly, but it is thought that calcium may influence the activation which follows FMLP binding. Adding calcium to the reaction mixture potentiated the effect of FMLP and the effect of FMLP + cyt B. The stimulation of a calcium flux by calcium alone was also blocked by PHMPSA and had no modulating effect on the PHMPSA inhibition of the peroxide response to FMLP. The role of calcium is not clear but it is not necessary for actin polymerisation (Bengtsson et al 1986). Although FMLP induces a calcium flux with little delay, it may be that more immediate availability of calcium is necessary for a process which must occur in a calcium-dependent manner before activation of the IP_3-induced calcium release. $Calcium_e$ may be necessary for PLA_2 activation. It may be required for maintaining the functional integrity of another element in the activation process such as external enzymes or transport proteins required for a normal energy supply, or for a step in which activation of PKC and non-calcium-releasing isomers of IP_3 is inappropriate. Others have found that blocking cytosolic calcium release with TMB-8 blocked FMLP-induced arachidonic acid release (Ohta et al 1985). FMLP can induce O_2^- production without added calcium (Okajima and Ui 1984), as also found in this project, but it is much lower than in its presence. There was a noticeable enhancing effect induced by the addition of 1.5mM calcium in the peroxide experiments. In both PMN and monocytes two small proteins have been found (P8 and P14) of which P14 is

phosphorylated when $calcium_e$ enters, but not by PKC, and which is thought to be concerned with motility (Edgeworth et al 1989). Dose-dependent effects of FMLP-induced PLA_2 activity have been shown to be species-specific. The calcium-dependent, biphasic response of rabbit PMN PLA_2 to FMLP (Bormann et al 1984) suggests that at the doses used in this project FMLP might not have activated PLA_2 at all, maximal responsiveness being found at $10^{-9}M$ (which also corresponded to their maximal chemotactic activity). The chemotactic dose responsiveness of human cells is in the same range (Dillon et al 1987). However FMLP-induced activation of PLA_2 in rat PMN has been found at $10^{-6}M$ (Duque et al 1986), a dose at which rabbit PMN PLA_2 is inactive. If PLA_2 activity is a primary and descrete requirement in the sequence of events activated by FMLP, either the elevation of cAMP or the elevation of calcium induced by $calcium_e$ entry might be required for PLA_2 regulation. Its indirect activation after PLC activation could be different, in that PKC would be active also. Activation of cAMP- or calcium-requiring kinases may be necessary for activation of PLA_2, if it is repressed by unphosphorylated lipocortin 1 for instance (Hirata et al 1981;1984). The PHMPSA inhibition of the PMN calcium flux induced by $calcium_e$ may suggest that the entry of $calcium_e$ is unrelated to the ability of the cells to produce reactive oxygen species, as its presence or absence did not alter the effect of PHMPSA on the FMLP or HAGG responses.

FMLP-binding leads to receptor-cytoskeletal association, and dissociation from G-proteins (Jesaitis 1988). Blocking this by using cyt B should lead to inhibition as associated G-proteins would not undergo the normal cycle and nor would GTPase activity ensue. This would not necessarily be the case if there was also a G-protein-independent pathway activated by FMLP. In the experiments using IAP or PHMPSA there was a small but persistent residual release which was affected by neither blocker. This activity indicated that the FMLP receptor protein itself activated internal events independently of SH-

dependent mechanisms and G-proteins. Could this be the primary, receptor-mediated event required for raising intracellular calcium? The small release of O_2^- could be induced by the partial disturbance (rather than activation per se) of the system, a full response being dependent upon activation of the SH- and IAP-dependent pathway(s). It has been suggested (Cockcroft 1987) that calcium release by IP_3 can account for the initial peak in internal calcium release found in response to stimulation of PIP_2-PLC and that a subsequent further, more prolonged response is a function of the metabolism of IP_3, including the isomers which do not themselves induce calcium release. Just as some isomers do not induce calcium release (Spat et al 1986), so also the production of specific isomers may be a function of $calcium_i$ concentration at the time of PLC activation (Dillon 1987).

The costimulation experiments with clonidine and salbutamol in conjuction with FMLP, PMA or fluoride were rather general, in that only a single dose each of clonidine and salbutamol was used and numbers were low. No significant effects were seen either by blocking cAMP or by raising it, suggesting that the brief rise seen with FMLP stimulation may be independent of peroxide production. There was no effect on the responses by PHMPSA preincubation. Futher investigation of cAMP regulation was considered inappropriate in this project but it would be interesting to see if the enhancement by salbutamol of the fluoride response reached significance with increased numbers (P=0.1 in this project) and to see if this was seen also using salbutamol in conjunction with FMLP or HAGG.

In the MPO assay, PHMPSA affected the responses by enhancement. This could be a significant indicator of the SH interaction being an effect on an element which controls the predominant production pathway by a given stimulus, rather than the receptor itself. The cytoskeleton seems a logical target in this case. Neither PMA nor fluoride was expected to induce release of MPO,

but neither were they expected to lower the basal amount released. This effect was overcome by PHMPSA, except when the two agents had been added together. Without being able to contribute any evidence why these responses should occur, the effect of PMA in particular must support a lack of a direct effect of PHMPSA on either receptor proteins in the plasma membrane or on G-proteins.

Activation of both PLA_2 and PLC is affected by IAP, after FMLP stimulation, this may be because both enzymes are controlled directly by G_i-like G-proteins, or because one is and it indirectly controls activation of the other, or because they are both controlled by different subunits of the same G-protein. No definite evidence has been reported so far that both the G-alpha subunit and the beta-gamma moiety of the same G-protein complex have regulatory activity, but in independent, descrete systems each has been found to be active (Jelsema and Axelrod 1987; Okajima et al 1987); the idea of both elements from the same G-protein being active at once has been suggested elsewhere as being worthy of further consideration (Bourne 1987). The IAP inhibition of the FMLP-stimulated O_2^- production by PMN and MNC in this project implicates a G_i-like G-protein in this signal transduction pathway. Others have found PMN responses to FMLP to be IAP-inhibited including IP_3 production and lysozyme release (Bradford and Rubin 1985b), release of calcium from internal stores (Ohta et al 1985), arachidonic acid release (Bokoch and Gilman 1984) and actin polymerisation (Bengtsson et al 1986). The proton pump has been found to be inactivated during IAP inhibition of FMLP-stimulated activity, however this was indirect as PMA activated the pump under the same conditions (Volpi et al 1985). In this project the pump was also only indirectly affected by PHMPSA as determined by the continued activity of NADPH oxidase stimulated by PMA after PHMPSA preincubation. Although the proton pump activity was not

measured directly it has been found by others to be essential for NADPH oxidase to function (Henderson et al 1988).

PHMPSA has been found in this project to modulate the effects of $calcium_e$ on internal calcium flux and not to affect NADPH oxidase or the proton pump nor to affect all G-protein signalling; to leave the FMLP receptor still capable of signalling azurophil granule release but to prevent activation of NADPH oxidase by either FMLP or HAGG. This suggests that the effects may be multiple but that they are specific.

Whilst demonstrating a link between SH and activation, use of HAGG as a gross stimulus of Fc receptors had the disadvantage of stimulating two different receptor types both on PMN and on MNC. This may be significant in the case of PMN in particular, where the FcR11 and FcR111 expressed are known to have different membrane linkage; FcR111 being covalently attached to a phosphatidyl inositol in the outer side of the plasma membrane bilayer, and FcR11 being a transmembrane protein. Although both MNC FcR receptors are transmembrane proteins, it is notable that the level of inhibition by PHMPSA of the HAGG response is of the same order in both cell types and considerably less than the inhibition of the FMLP response. The results from HAGG-mediated activation are therefore a more general indication of events than those from FMLP; carrying out the peroxide, O_2^- or the MPO assay using monoclonal antibodies would be more specific and reveal any receptor sub-type-specific differences. Recent work following the cessation of the PhD project has monitored the internal calcium flux in response to monoclonal antibodies against the separate FcR on PMN and on MNC, but this has not yet been done including PHMPSA blockade. In the case of each receptor type, release of internal calcium was found in response to cross-linking the appropriate monoclonal antibody, and also MNC were found to release internal calcium in response to cross-linking human IgG_1 bound to FcR1 - the IgG_1

being from the same source as that used for the HAGG experiments in this project.

The response of cells in vivo to FMLP or to immune complexes (HAGG in vitro) can be expected to be different. FMLP must induce a sequence of events designed to locate and destroy a bacterial invasion. Immune complex contact must involve the phagocyte in an already active humoral response, for instance altering adherence of both PMN and MNC (Appelboom et al 1988) and stimulating ADCC (Petroni et al 1988). Chemotaxis and degranulation of bactericides may therefore have a higher priority in the order of events in response to FMLP. Both ligands appear to activate an interaction between PLA_2 and PLC pathways, and therefore the fine regulation required to meet the different responses needs to be considered. In this project FMLP stimulation of O_2^- has been found to be IAP-sensitive in both PMN and MNC. Only one experiment was carried out blocking PMN with IAP and using HAGG stimulation; no effect was seen on O_2^- release. A regulatory difference via G-protein linkage is thus revealed in the FMLP and HAGG-mediated control of the two enzyme pathways in PMN. This could be either from a lack of G-protein mediation of FcR stimulation or from the mediation of this by a G-protein which is not ADP-ribosylated by IAP. The Fc receptors do not have the structural characteristics which are associated with other receptors known to be G-protein-mediated (Dohlman 1987). Partial inhibition of the O_2^- response to FcR stimulation has been found using PKC inhibition, with PLA_2 blockade causing more complete inhibition (Sakata et al 1987). However this work was carried out on guinea pig peritoneal macrophages and PMN, preincubated with cyt B. As cyt B inhibits phagocytosis (Roos et al 1976), and must totally disorganise the subplasmalemma, the normal sequence of events cannot be determined. The effect of IAP on IgG-induced O_2^- release by PMN has been found to inhibit in some studies (Feister et al 1988) but not in others (Blackburn

and Heck 1989), whereas there is agreement by the same authors on the lack of effect on degranulation. As with FMLP, activation of more than one pathway is indicated. Similarly also, cyt B has been found to enhance some Fc-gamma-R-mediated effects such as lysozyme release and azurophil granule release (Ferreri et al 1986) and O_2^- production (Roos et al 1976). More parallels can be drawn between FMLP- and Fc-gamma-R-mediated pathways; PMA inhibits azurophil degranulation induced by either stimulus in PMN (Blackburn and Heck 1989) and there is co-operation with other receptor-mediated stimuli; FMLP with TNF-alpha (Berkow et al 1987) and IgG with C3b (Malbran et al 1987) for instance. In this project both FMLP- and HAGG-stimulated systems have been subject to SH blockade which inhibited peroxide and O_2^- production. Other workers have found parallel activation and effects of cyt B. An element which may differ particularly between activation by the two systems is the necessity for $calcium_e$; different methods of activating PLA_2 could be a reason for this. It was suggested earlier that $calcium_e$ could be a requirement for discrete release of a PLA_2 repressor without activation of other functions of FMLP stimulation. Fc-gamma-R may have intrinsic PLA_2 activity (Suzuki et al 1980) which would negate the need for $calcium_e$.

In this project, lack of PHMPSA effect on the PMA response seen in normal PMN demonstrated that the SH involvement in receptor-mediated responses occurred prior to PKC activation, and also demonstrated that neither the NADPH oxidase complex itself, nor the Na^+/H^+ proton pump were affected directly by the external SH blockade. A comparison between the effect of PMA with and without calcium present was not carried out with PMN. The fact that the MNC response to PMA was slightly inhibited in the absence of $calcium_e$, but not in its presence, may indicate a relationship between SH, PKC and $calcium_e$ in MNC.

The project did not set out to investigate any effects of calcium in particular, and so from the outset calcium was not specifically excluded (by the addition of EGTA to washing buffers for instance), and as previously mentioned it was probably evident at all times as a contaminant. However despite the levels being only in the micromolar range even when added, it appeared to have effects in its own right. Extracellular calcium is normally in the millimolar range, perhaps suggesting that it is the difference between extra- and intracellular levels or a localized change in the vicinity of the cell which is relevant, rather than the absolute amount. Here, $calcium_e$ was been found to cause an elevation in the internal basal level of calcium which was SH-dependent, and the addition of $calcium_e$ brought about a release of MPO which was also inhibited by PHMPSA (not shown). No calcium flux experiments were carried out on cells from RA patients, but a comparison of the $calcium_e$-induced flux in patients on NSAID with that from those on Dpen might make a useful avenue to investigate.

In contrast to the major blockade of FMLP-induced activity by IAP or PHMPSA the PMN fluoride-induced O_2^- response was not inhibited. That of MNC was. From the FMLP experiments it is clear that a G_i -like G-protein does mediate O_2^- production and can be inhibited by IAP in both PMN and MNC. Fluoride must in PMN, be assumed to activate a second pathway to O_2^- production mediated by an IAP-insensitive G-protein, the <u>in vivo</u> activator of which is unknown. It could be HAGG. Others (Bokoch and Gilman 1984) have found (calcium-dependent) fluoride stimulation of arachidonic acid was inhibited by IAP, suggesting that the fluoride pathway to NADPH oxidase activation in this project was not mediated by arachidonic acid. Also Bokoch and Gilman found the arachidonic acid released by fluoride was subject to a lag time of 5 minutes with O_2^- release not occurring for another 5 minutes. The timecourses of PMN and MNC stimulation in this project also showed a long

lag time before O_2^- production, although production was near maximal by 10 minutes.

As MNC activation by fluoride was only partly inhibited by IAP this could have been because the G-protein was not all available for ADP-ribosylation, but as the FMLP-stimulated G-protein was significantly affected in the hour-long preincubation period it is more likely that it was accessible but was a poorer substrate for IAP. More than one G_i-like G-protein has been identified in PMN (Kanaho et al 1989) and IAP- and cholera toxin-insensitive G in other tissues by others (Higashijima et al 1987;Evans et al 1986). It is likely that there is a cell-type specific discrepancy between those found in PMN and MNC, especially as PMN are terminally differentiated and MNC are not. It is also possible that fluoride activated another GTP-dependent system which is not subject to ADP-ribosylation by IAP. In permeabilized PMN GTP-gamma-S has been found to have another site of action which stimulated secretion in an IAP-independent manner (Barrowman et al 1986).

The calcium flux induced by fluoride in this project was induced under two sets of conditions. Firstly it was induced from PMN in PBSG containing 300uM calcium. In the second series of experiments the PMN were in Hanks/hepes buffer which contained 1.4 mM calcium. Under either of these conditions an initial, immediate peak of calcium release was seen which terminated rapidly, calcium returning to the basal level, followed by a lag then a second curve of long (slow) duration, which at its peak released the same amount of calcium again. The dose response mimicked the range for O_2^- release. It is possible that the first peak was caused by precipitated calcium fluoride acting as a non-specific particulate stimulus. Previously calcium had been kept low (300uM) to prevent such precipitation. Whether particulate or more specific, this peak showed a dose dependence in magnitude, and was PHMPSA inhibitable. The second peak was not inhibited by PHMPSA, but the time taken for it to reach

maximum was nearly doubled and the release was enhanced. Some pilot experiments were carried out (not shown) in which fluoride stimulation of the calcium flux was combined with other stimuli. The initial peak was abolished by prestimulation of cells with either FMLP or with PMA. The FMLP response had returned to basal before the addition of fluoride, and PMA did not stimulate calcium release at all. There is no reason for an effect of either FMLP or PMA to be the prevention of calcium fluoride precipitation, if such there was. Another explanation could be that the FMLP stimulus resequestered internal calcium into pools which were not then subject to release by the mechanism seen in the first fluoride-induced calcium peak, and the effect of the PMA may have been to bring about a modulation (phosphorylation) which made the calcium pool(s) inaccessible to release by fluoride. This in turn may indicate that there are not two discrete pools, but one pool, which is modified by activation to resist a second stimulus. The effect of fluoride as the first stimulus and FMLP as the second one showed a complete abolition of the FMLP response which supports the theory that the calcium released by FMLP and the first fluoride peak is from the same pool, which then becomes inaccessible after stimulation. The PMA effect suggested that PKC had a role in this; however PMA did not affect the calcium flux induced by FMLP. Other workers have found that PKC may negatively feedback on PLC to modulate the release of calcium by IP_3 (Muldoon et al 1987), and have found that PMA inhibited fluoride-induced PIP_2 hydrolysis and IP_3 accumulation (Blackmore and Exton 1986). The lack of effect by PMA found here on the FMLP induced flux may indicate that either FMLP does not induce the calcium flux by activation of PIP_2 hydrolysis, which is not likely in view of the other evidence of activation of this pathway previously mentioned, or that the PKC substrate which regulates the response is not available until after FMLP-induced activation; which would apply, for instance, to G-alpha subunits and this would accommodate the results of Blackmore and Exton. Hypotheses on the regulation of calcium by interacting

pools have been illustrated by Gill et al (1988) and by Berridge and Irvine (1989). The inability of PMA to stimulate azurophilic degranulation and its ability to suppress both FMLP-induced degranulation and calcium release (Smith et al 1988) may suggest that the calcium could be in a granule fraction itself, as opposed to in endoplasmic reticulum for instance. The complex series of events in FMLP stimulation involve activation of PIP_2-specific PLC and subsequent activation of PKC and the respiratory burst, but inhibition of PKC does not necessarily inhibit O_2^- production (Balazovich et al 1986). However using PMA to activate PKC only monitors the phosphorylation events associated with PKC activation, which in vivo may be quite different as calcium would be released at the same time by IP_3; this may alter the substrate preference for the PKC dictating whether or not phosphorylation by the membrane bound or cytosolic fraction predominates. It seems that there is a balance operating in internal calcium regulation and release by PKC and IP_3. Potentiation of FMLP O_2^- release by PMA preincubation suggests that phosphorylation by PKC may selectively depress aspects of the FMLP response in favour of a O_2^- generating pathway (Smith et al 1988); the calcium flux induced by FMLP was depressed by PMA priming. The calcium flux induced by FMLP as seen in this project is both rapid and rapidly resolved. The method for monitoring the O_2^- response delineates a plateau in the timecourse which is also reached rapidly. The sequestration of calcium back into internal stores may only be delayed for instance by the time taken for PKC to translocate, if it controls this process.

Recent work with monoclonal antibody stimulation of FcR has shown that it is possible to stimulate two receptor sub-types on monocytes and induce a calcium flux with each in turn. Calcium may be regulated differently after IgG stimulation. The stimulation of PMN seen with HAGG which has been described in the results section here, as well as the monoclonal stimulation of

monocytes, demonstrated that after stimulation calcium was not taken up again rapidly as in FMLP-stimulated cells, and although the level of calcium did go down, it did not return to the original basal level. It is not possible that the lack of FMLP-induced response after fluoride was due to phagocytosis of the FMLP receptors, as fluoride inhibits phagocytosis (Roos and Balm 1978). These results suggest that fluoride will make a useful tool for further investigation of regulation of internal calcium by different activating ligands. For instance a particular study could involve activation through FcR by IgG or monoclonal antibodies (as appropriate), in which the significance of cross-linking could be determined with respect to the order of activation events and the interaction with G-proteins. It may also be possible to use the inhibitory properties which fluoride has with respect to phagocytosis and degranulation to investigate receptor tunover using a fluorescence-activated cell sorter. The multiple properties of fluoride may have consequences overlooked in its use as a G-protein agonist.

PMA was seen in this project to interact with the fluoride stimulated production of O_2^- in PMN, the response to both ligands added together being greater than that found on addition of fluoride alone but considerably less than the response to PMA alone. The timecourse experiments demonstrated that the lag period of the PMA/fluoride-stimulated cells was reduced by the PMA compared to the lag seen with fluoride alone, and that the cessation of a response coincided with the end of the lag phase for the fluoride response. It could be that if the cells had been exposed to fluoride first, for a period exceeding the lag phase, the PMA response may have been totally inhibited. As well as inhibiting degranulation, fluoride could be inhibiting the translocation of cytosolic enzymes to the plasma membrane, and if this is its effect on PKC, fluoride stimulation of O_2^- would suggest that NADPH oxidase activation can be an event totally independent of PKC activation. There is evidence that one of the proteins found to be

phosphorylated by PKC activation may be part of the NADPH oxidase complex (Heyworth et al 1989). If this is so then it may be a negative feedback event (Nishizuka 1984). G-alpha subunits are also substrates for PKC (Matsumoto et al 1986). It has been found that fluoride does not induce phosphorylation of any protein found to be phosphorylated by all other activators of NADPH oxidase but in common with these (FMLP included) it does induce dephosphorylation of a particular protein band seen on polyacrylamide gel electrophoresis (Andrews and Babior 1983). Geny et al (1989) found that PMA inhibited fluoride-stimulated PLC activity in permeabilised HL60 cells (a human leukaemic cell line). Evidence from platelets in which PMA did not affect the fluoride-stimulated release of IP_3 (Fuse and Tai 1987) suggests cell-specific differences. The effect of fluoride need not be related to its activity with G-proteins, but could be connected to another function effected with phosphate group substitution by AlF_4. There are small molecular weight G-proteins with unknown functions which are GTP-binding (Haldar et al 1989). Some have been found to be associated with cytochrome B in PMN and so may be concerned with NADPH oxidase activity (Quinn et al 1989). The slight but significant enhancement of O_2^- induced by PMA from PMN indicated a difference in SH availability on PMN compared with MNC, in which no effect of PHMPSA was seen, in normal cells. Nothing investigated in this project indicates what might be affected on the plasma membrane which could affect PKC activity. Translocation of the enzyme to the cytosolic surface is coincident with PKC activity but not essential for O_2^- production following its stimulation with PMA (in which translocation is found) or TPA (in which it is not) although the pattern of phosphorylation differs in each case (Balazovich et al 1986), and an indirect effect on either its binding site (phosphatidylserine) or substrate availability could occur.

Production of peroxide in response to HAGG and to FMLP in normal MNC demonstrated an almost twofold increase in stimulation in the presence of calcium, PMA stimulation being much less affected in this respect. Other workers have noted an increase O_2^- produced by RA MNC stimulated with opsonized zymosan in the presence of $calcium_e$ (Bell et al 1986a). In this project no comparison was made with MNC from patients. However the significant inhibition of the MNC PMA response by PHMPSA was lost in the presence of $calcium_e$ which had no effect on the lack of inhibition seen in PMA stimulated PMN (Figures 13,15 and 30). The conclusions drawn from these findings were that regulation of PKC activity in MNC and PMN differs, and that regulation in MNC may be related to SH availability in a calcium-dependent manner.

The absolute amounts of reactive oxygen species from PMN and MNC shown in the results section have been presented as the amount produced by a million cells in thirty minutes. In the case of the MNC, this can be assumed to represent production by fivefold fewer monocytes than PMN. Scrutiny of the peroxide dose response curves shows that the monocyte production in response to HAGG and FMLP is comparable. This may be a function of receptor number required for activation or of duration of response. From the O_2^- timecourse curves it can be seen that the timecourses are similar in each cell type. Also that the FMLP-induced release by MNC can be multiplied by five to give a very similar result. This comparison cannot be made using the PMA results as the response was not found to be linear with respect to cell number (not shown). The plateau reached by the PMA dose response curves may represent either negative feedback of the response at the given doses or the exhaustion of substrate in the reaction. The latter is unlikely as there is a one to one relationship with cytochrome c reduction and production of O_2^-, and the cytochrome C concentration was 150μM.

A preliminary investigation of membrane fluidity in this project may suggest that it is increased by PHMPSA preincubation of PMN, although this is suggested only tentatively as the conditions of the assay fell short of the ideal, temperature regulation in particular being less controlled than necessary. The results from the three experiments carried out (see below) are inconclusive, and it is suggested that further investigation of the effect of PHMPSA on fluidity would be useful.

	- PHMPSA	+PHMPSA
	Fluidity unit.	
PMN 1	0.1433	0.1356
PMN 2	0.1589	0.1537
PMN 3	0.1595	0.1567

Rheumatoid arthritis patients on Dpen or Aur were compared with those on NSAID alone to examine the possible effects of second line drugs on cell responses. Patients on second line therapy were all also taking NSAID. No distinction was made between the different NSAID because they do not affect the overall disease process and it was not their function which was under study. Also the numbers of patients would have been too low in each group to allow any useful analysis as such a wide range of drugs is taken. The inclusion of the psoriatic arthritis group in a limited number of experiments allowed comparison of another inflammatory condition, all of these patients also being on NSAID.

RA patients were included in the study of the spontaneous release of peroxide with and without calcium. The PMN and MNC from patients with treatment including SH-containing drugs showed a marked elevation of spontaneous peroxide release compared with those on NSAID alone. This may suggest that Dpen and Aur alter the entry of $calcium_e$ as the difference in release was dependent on $calcium_e$. Alternatively it could be a function of the disease process, those patients on the second line drugs being representative of a more severely affected group than those on NSAID alone, their cells being in a chronically activated state. Other effects due to cellular localization of the drugs could be contributing to the increase. As spontaneous O_2^- release did not differ significantly between the control and disease groups in either PMN or MNC it may be that Dpen and Aur have an effect on the transduction pathway resulting in more peroxidation, unrelated to O_2^- production. Auranofin was found to decrease the level of O_2^- released by PMA stimulation or by FMLP stimulation of normal PMN at concentrations thought to be attained in vivo(Hurst et al 1989). This suggests that the increase seen in the spontaneous peroxide release in this project by PMN from patients on Aur may be a function of the disease process.

There was no significant difference in the inhibition by PHMPSA between any PMN or MNC group peroxide release (not shown). This (less sensitive) assay indicated that the SH available for modification by PHMPSA were equally subject to blockade in either cell type and regardless of disease or drug regimen. The spontaneous release of O_2^- by MNC was not found to be significantly inhibited by PHMPSA in the NSAID group whereas it was in the normal and Dpen groups. Dpen may make more SH available for blockade by PHMPSA by disulphide exchange reactions, compared with the NSAID treatment.

The inhibition of the spontaneous O_2^- by IAP was not seen in Dpen PMN. The statistics were carried out on the absolute values for O_2^- release in which there was a wide range of values (vis. s.d.), but when comparing the percentages of activity remaining IAP had no significantly different inhibitory effect on any group.

Fluoride stimulation of normal MNC and PMN was sensitive to IAP inhibition, whereas the cells from the patients groups were less so. Dpen appeared to restore RA MNC sensitivity to IAP. Previously the effect of fluoride stimulation with and without PHMPSA revealed a difference in the SH blockade on normal PMN and MNC G-proteins, and these results with IAP also demonstrate that there is a further difference, between MNC from normals and RA groups. RA G-proteins could be altered in their availability but alternatively could have a change in the structure of the G-proteins themselves. The cell-specificity of the defect mitigates against it being an overall effect of reactive oxygen species on plasma membranes. It is considered here that despite the evidence being based on a small sample number, these results warrant further investigation.

FMLP-induced levels of peroxide and O_2^- were inhibited by either IAP or PHMPSA in RA cells as in normals. Also the effect of PHMPSA on PMA-stimulated PMN (a slight enhancement) and of IAP (no effect) was the same in

RA as in normal cells. PMA stimulation of MNC only included normal and NSAID groups. The NSAID MNC responded to PMA with a significantly greater production of O_2^-, both with and without PHMPSA, with no effect of either PHMPSA or IAP on the stimulation. Thus inhibition of cyclooxygenase activity by NSAID could be an example here of preferential pathway activation. The comparison of PMA stimulation was not made with Dpen MNC, and it would have been interesting to see if the indicated increase in PKC activity was affected by second line treatment bearing in mind that such patients would also be taking NSAID. Aur has been found to increase PLC activity in vitro (Snyder et al 1986) others finding a biphasic, dose-dependent effect of Aur on PMA stimulation of O_2^- (Hurst et al 1989), suggesting that the effect seen might be an even greater enhancement.

The response to fluoride stimulation indicated again that G-proteins may be involved in the RA disease process. In normal PMN it was found that only cells from male donors reacted with an enhanced O_2^- response after PHMPSA preincubation. All PMN from both the NSAID and Dpen groups showed this enhancement. The effect of IAP on the PMN fluoride response was also different from normal, neither NSAID nor Dpen groups showing the inhibition found in normal PMN. The significance of both the difference in PHMPSA/fluoride response of PMN from male and female donors and of the finding that this difference was lost in RA PMN is entirely open to speculation. There has been no report of any sex-related differences in G-proteins in any species. It is possible that hormonal regulation alters cell membranes in cells from males and females however, and this may include a difference in the number of G-proteins of a particular sub-type. Support for this hypothesis is to be found in the lack of IAP inhibition of the RA PMN fluoride response. These could be G_0 G-proteins rather than defective G_i-like G-proteins.

The fluoride response of MNC from all sources was inhibited by PHMPSA, although the degree of significance was largest in the normal group, less so in the NSAID group and least in the Dpen group. Analysis of absolute amounts of O_2^- released showed no significant difference between the groups. Whereas normal and Dpen MNC were inhibited by IAP, NSAID MNC were not, suggesting that Dpen modified the fluoride-G interaction. As there is a cysteine at the site of ADP-ribosylation in G-alpha subunits (Milligan 1989) this site could be deficient (oxidized) in RA.

In the presence of $calcium_e$ the level of peroxide stimulation by FMLP was significantly increased in the NSAID group compared with the control, and although numbers were too low for statistical analysis it can be seen from the histogram (Figure 11b) that the Dpen stimulated levels fell into the same range as the NSAID group. It was not decided to add calcium until after the HAGG experiments ceased and so the comparison cannot be made using this stimulus and as with the FMLP data, no difference was seen in the levels of stimulation between the groups without $calcium_e$. This has revealed that the increase in levels of stimulation are both in the spontaneous and stimulated production of peroxide, and that they are dependent on $calcium_e$. An increased number of experiments could determine if this was as SH-dependent in RA groups as in controls.

PMN peroxide release induced by FMLP or HAGG stimulation was inhibited by PHMPSA equally in all groups, which suggests that the affected SH are not instrumental in the disease process with respect to activation by these ligands. There was no statistically significant difference in the O_2^- release by FMLP stimulation of normal, Dpen or NSAID PMN but there was significantly more peroxide released in response to FMLP by NSAID and Dpen cells. Similar

comparison cannot be made with MNC as the peroxide experiments did not all include $calcium_e$.

The field for investigation of pathways to cell activation has been broadened by the effects seen using PHMPSA or IAP preincubation and by the actions and interactions of the different stimuli used in this project.

G-proteins have been found to differ in PMN and MNC in both their susceptibility to PHMPSA and to IAP, in pathways activated by the same ligand. PMN G-proteins may differ in men and women and these may be altered in RA. MNC G-proteins may be affected by the disease process in RA in a manner modified by Dpen.

Calcium decreased normal cellular sensitivity to SH blockade and spontaneous and stimulated activity of PMN and MNC was seen to be enhanced by RA in a calcium-dependent manner.

PHMPSA preincubation showed that a SH group in receptor-mediated activation which is modifiable by impermeant agent is likely to be located at a site in or on the plasma membrane other than the receptor protein itself or its regulatory G-protein.

Conclusions.

PHMPSA preincubation demonstrated differential effects on PMN and MNC and on some responses of cells from Rheumatoid Arthritis patients compared with those from normal donors.

IAP preincubation demonstrated a difference in PMN and MNC G-protein activation and a modifying effect of Dpen on MNC.

1. PHMPSA inhibited receptor-mediated peroxide and O_2^- production in both PMN and MNC and internal calcium release in PMN.

2. PHMPSA enhanced receptor-mediated MPO release from PMN.

3. PHMPSA differentially affected fluoride stimulated O_2^- production. The stimulatory effect on PMN was evident in RA donor cells of both sexes but absent in PMN from normal females.

4. PHMPSA caused a slight enhancement of the PMN O_2^- response to PMA and did not affect the MNC response.

5. IAP inhibited spontaneous and receptor-mediated O_2^- release by PMN and MNC.

6. IAP did not affect PMA release of O_2^-.

7. IAP inhibited fluoride-induced O_2^- release by PMN and MNC. The effect was reduced in PMN from both NSAID and Dpen groups but only the NSAID MNC group.

8. $Calcium_e$ modulated spontaneous peroxide release by PMN and MNC.

9. $Calcium_e$ overcame PHMPSA blockade of PMA-stimulated peroxide release from MNC.

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