

**ROLE OF NEUTROPHIL MATRIX METALLOPROTEINASE-9 (MMP-9) AND
TISSUE INHIBITOR OF METALLOPROTEASES-1 (TIMP-1)
IN THE KILLING OF MICROORGANISMS**

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**“THE FREEDOM THAT WE ARE ENJOYING TODAY IS
THE RESULT OF THOSE FIGHTERS THAT HAD
FORGOTTEN THEIR LIFE DURING CERTAIN PERIOD
OF TIME”**

SAWA, 1997

To my Mouminah Ibrahim

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from February 2002 to July 2003, under the supervision of Dr. Edith Elliott.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

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ABSTRACT

Microorganisms may evade killing by neutrophils (PMNs) by altering signal transduction and hence phagosome maturation. Secreted, active matrix metalloproteinases (MMPs) appear to be required for PMN killing of pseudomonas microorganisms, via an MMP- and complement-dependent, but otherwise unknown mechanism. This also depends on the absence of the inhibitor of MMPs, tissue inhibitor of metalloproteinases-1 (TIMP-1). By altering their particular complement opsonin and hence the PMN complement receptor bound, microorganism may evade killing, as not all PMN complement receptors trigger phagosome maturation and hence killing of microorganisms. C1 inhibitor of the classical complement cascade, required for the exposure of C1q and further assembly of complement factors on the bacterial surface and hence binding to specific PMN receptors, is MMP sensitive. MMP secretion may, therefore, not only facilitate the killing of microorganisms, but inappropriate secretion, induced by pathogens, may prevent complement assembly and killing via complement-mediated pathways. It was, therefore, decided to assess MMP-9 and TIMP-1 secretion in the presence of C1q-opsonized polystyrene beads and subsequently upon stimulation with pseudomonas organisms, and explore the relationship between secretion of PMN MMPs (specifically MMP-9) and TIMP-1 and phagocytic uptake and maturation of the PMN phagosome into a killing body.

MMP-9 and TIMP-1 secretion was seen to occur at low levels under most conditions. However, in the presence of serum, and hence complement, MMP-9 secretion was found to be upregulated during uptake of C1q-coated beads. MMP-9 possibly inactivates C1 inhibitor at this stage, causing local tissue swelling (normally associated with the inactivation of C1-inhibitor), entry of various white blood cells and further complement into the area of infection, assisting in the extracellular killing of microorganisms. MMP-secretion may simultaneously down-regulate the activation of further PMNs via inactivation of C1q assembly and hence phagocytic uptake and activation of PMNs.

Unlike MMP-9, secretion of TIMP-1 was not upregulated by C1q receptor binding, implying that any secreted MMP-9 may, therefore, be in excess and hence uninhibited by TIMP-1. A distinct regulatory mechanism seems to be responsible for the release of TIMP-1, though TIMP-1 secretion was upregulated by extracellular calcium levels, partially contradicting previous findings which suggested that TIMP-1 was not calcium regulated. It seems unlikely that extracellular calcium levels would be the only mechanism by which TIMP-1 is regulated, however, and further surface receptor-mediated agonists should be explored. Levels of MMP-9 and TIMP-1 secretion in the presence of pseudomonas microorganisms now need to be assessed to see whether these secretion patterns are altered to favour the evasion of opsonization by C1q. Uptake of C1q-opsonized beads was also increased by the presence of serum, possibly due to presence of complement. MMP-9 and TIMP-1 secretion patterns still need to be correlated with phagosomal uptake and killing of microorganisms, before their role in killing of microorganisms becomes fully evident.

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LIST OF ABBREVIATIONS USED

| | |
|---------------------------------|---|
| A ₂₈₀ | absorbance at 280 nm |
| APMA | aminophenylmercuric acetate |
| BCIP | 5-bromo-4-chloro-3-indolyl-phosphate |
| BPI | bacterial permeability inducing protein |
| BSA | bovine serum albumin |
| C | complement component |
| C1q | the first component of complement |
| C1qRO ₂ ⁻ | C1q receptor that mediate respiratory burst |
| C1qRp | C1q receptor that mediate phagocytosis |
| CLR | collagen like region |
| CMC | critical micelle concentration |
| CR | complement receptor |
| CRP | C-reactive protein |
| DAF | decay accelerating factor |
| DAG | diacylglycerol |
| DDSA | dodecenylsuccinic anhydride |
| DMP | (2,4,6-[(tri (dimethylaminoethyl) phenol)]) |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribose nucleic acid |
| ECL | enhanced chemiluminescence |
| ECM | extracellular matrix |
| EDTA | ethylene diamine tetra-acetic acid |
| EGF | epidermal growth factor |
| ELISA | enzyme linked immunosorbent assay |
| FAD | flavine adenine dinucleotide |
| FCγR | constant fragment receptor of IgG |
| FGF | fibroblast growth factor |
| fMLP | formyl L-methionyl-leucyl-phenylalanine |

| | |
|-------------------------------|--|
| FPR | formyl peptide receptor |
| GTP | guanosine tri-phosphate |
| H ₂ O ₂ | hydrogen peroxide |
| HOCl | hypochlorous acid |
| HRPO | horseradish peroxidase |
| ICAM | intracellular adhesion molecule |
| IgG | immunoglobulin G |
| IL-8 | interleukin 8 |
| IP ₃ | inositol triphosphate |
| LF | lactoferrin |
| LFA-1 | leukocyte function associated molecule 1 |
| LPS | lipopolysaccharide |
| LTB ₄ | leukotrene B ₄ |
| Mac-1 | leukocyte adhesion molecule |
| MASP | mannan-binding lectin-associated serine protease |
| MBP | mannose binding lectin protein |
| MCP | membrane cofactor of proteolysis |
| MMP | matrix metalloproteinase |
| MPO | myeloperoxidase |
| MT-MMP | membrane type matrix metalloproteinase |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NBT | nitroblue tetrazolium |
| NGAL | neutrophil granule associated lipocalin |
| NO | nitric oxide |
| OB | oligosaccharide/oligonucleotide binding protein |
| OPZ | opsonized zymosan |
| PA | phosphatidic acid |
| PAF | platelet activation factor |
| PCMB | p-chloromercuribenzoate |
| PCR | polymerase chain reaction |
| PHMB | p-(hydroxymercuri)-benzoate |

| | |
|------------------|--|
| PIP ₂ | phosphoinositide phospholipase 2 |
| PI-PLC | phosphoinositide phospholipase C |
| PK | protein kinase |
| PKC | protein kinase C |
| PLA ₂ | phospholipase A ₂ |
| PLC | phospholipase C |
| PLD | phospholipase D |
| PMA | phorbol myristate acetate |
| PMC | phenol-mercuric-chloride |
| PMN | polymorphonuclear leukocytes |
| PMSF | phenyl methyl sulfonyl fluoride |
| PS | physiological saline |
| PUMP-1 | putative membrane protein-1 |
| RBC | red blood cells |
| SDS PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SDS | sodium dodecyl sulfate |
| SERPIN | serine proteinase inhibitor |
| SPA | pulmonary surfactant A |
| SPRAC | secreted protein acidic and rich in cysteine |
| TEMED | N-N-N'-N'-tetramethyl ethylenediamine |
| TIMP | tissue inhibitor of matrix metalloproteinase |
| TMB | tetramethyl benzidine |
| TNF- α | tumor necrosis factor α |
| TPA | 12-O-tetradecanylphorbol-13-acetate |
| TPP | three phase partitioning |
| WBC | white blood cells |

CHAPTER ONE

INTRODUCTION

1.1 Neutrophils

Neutrophils or polymorphonuclear leukocytes (PMNs) are the first phagocytes recruited to sites of infection, injury or inflammation (Suchard *et al.*, 1997). Like other granulocytic leukocytes these cells differentiate from bone marrow precursor cells in response to haematopoietic factors. On maturation, however, they are distinguished from other members of the granulocytic series by their final content of granules (Borregaard and Cowland, 1997).

The primary function of PMNs is to phagocytose and destroy potentially pathogenic microbes. PMNs remain circulating in blood vessels until they are induced to cross into tissues in response to chemoattractants such as tissue breakdown products or bacterial peptides (Rabonovich, 1995). During crawling towards the source of the chemoattractant, a process known as diapedesis, they express L-selectins which bind to complementary selectins or sulfated carbohydrates present on the activated endothelium in the post capillary venules (Evans *et al.*, 1999; Sengeløv, 1996). Complementary ligands in the endothelium, P-selectins and E-selectins (Smollen *et al.*, 2000; Sengeløv, 1996), may be bound without stimulation (Figure 1.1) but stimulation with chemoattractants result in upregulation of integrin and chemoattractant receptors on the surface of PMNs (Sengeløv, 1996) (Figure 1.1). Even though PMNs have $\beta 2$ -integrins such as LFA-1 and p150, 95 which bind to the complementary receptors (ICAM-1) on the endothelium, firm attachment is facilitated by expression of Mac-1 (Sengeløv, 1996) (Table 1.1, Figure 1.1).

When PMNs reach the site of infection they attempt to kill the microorganisms in one of two ways. They may either phagocytose and digest them intracellularly, by secreting

reactive oxygen metabolites (respiratory burst products), bactericidal substances and enzymes into a digestive body known as the phagosome, or may release these at the site of infection (Figure 1.1).

Table 1.1 Endothelial adhesion receptors and their counter receptor on leukocytes
(adapted from Hellewell and Williams, 1994).

| Endothelium | Counter Receptor | Leukocyte |
|----------------------------------|---------------------|----------------|
| ICAM-1 | LFA-1 + Mac-1 | All leukocytes |
| ICAM-2 | LFA-1 | All leukocytes |
| VCAM-1 | VLA-4 | EO+LO+MO+BO |
| PECAM | PECAM +? | All leukocytes |
| E-Selectin | Sle ^x | All leukocytes |
| P-Selectin | Sle ^x | All leukocytes |
| Sle ^x ? (Glycan 1) | L-Selectin | All leukocytes |

LO, lymphocyte; MO, monocyte; BO, basophil; EO, eosonophil.

Due to their complement of proteases, active against components of the extracellular matrix (ECM), PMNs may also be involved in inflammatory tissue breakdown (Kjeldsen *et al.*, 1992) and cleavage of humoral factors such as complement (Knäuper *et al.*, 1991; Reboul *et al.*, 1987; Pemberton *et al.*, 1989). This may occur due to over-activation of membrane receptors during overwhelming infections or the implantation of large foreign bodies such as grafts. Both may cause PMNs to undergo a process of uncontrolled degranulation and respiratory burst with enzymes such as the matrix metalloproteinases (MMPs), MMP-8 and MMP-9 most highly active against the ECM causing tissue destruction and inflammatory diseases. During the normal protease-assisted functions of PMNs, such as tissue digestion and movement of PMNs through the ECM, the activity of the MMPs may be controlled by the release of a newly discovered granule population containing one of the MMP inhibitors, tissue inhibitor of metalloproteinase-1 (TIMP-1) (Price *et al.*, 2000). The release of MMPs and other enzymes without the release of their TIMP inhibitors may result in inflammatory disease. The content of granules present in

the PMN, their role, regulation of release and specificity is, therefore, important for the normal and pathological function of PMNs.

In the context of PMNs being able to fight infection, Osiewicz *et al.* (1999) showed that TIMP-1-deficient mice had a complement-dependent improved resistance to pseudomonas infections. As this hyper-resistance was lost when synthetic MMP inhibitors were added to the cell/bacteria mix (Osiewicz *et al.*, 1999), extracellular active MMPs and complement seem to be required for such resistance. Since TIMP-1 release may inhibit extracellular MMP activity we hypothesized that pseudomonas microorganisms may trigger the release of TIMP-1 and hence interfere in some way with subsequent MMP and complement-dependent uptake and killing of microorganisms. One way this may occur is if the protease: inhibitor balance is disturbed and hence the assembly of complement opsonin coating bacteria and hence the types of receptors bound and activated by the opsonized bacteria is altered. Altered, receptor-mediated messages transferred into the PMN, via distinct receptor signal transduction pathways may prevent phagocytosis and respiratory burst (Goodman and Tenner, 1992) as some receptor-mediated messages do not result in uptake of opsonized bacteria (Tenner and Cooper, 1982) or the maturation of the phagosome into a bactericidal body (Botelho *et al.*, 1999) (Table 1.5). Many pathogenic microorganisms such as *Mycobacterium tuberculosis* bacteria, escape killing in the phagosome by, in some manner, interfering with this process (Chastellier and Thilo, 1997). Before a mechanism by which TIMP-1 and MMP-9 may be involved in the killing of microorganisms or the mechanism by which bacteria may escape killing in the phagosome may be proposed, it was important to first review how uptake and extracellular or phagosomal killing usually take place and to explain which of the various PMN granule populations are usually involved in the killing of microorganisms.

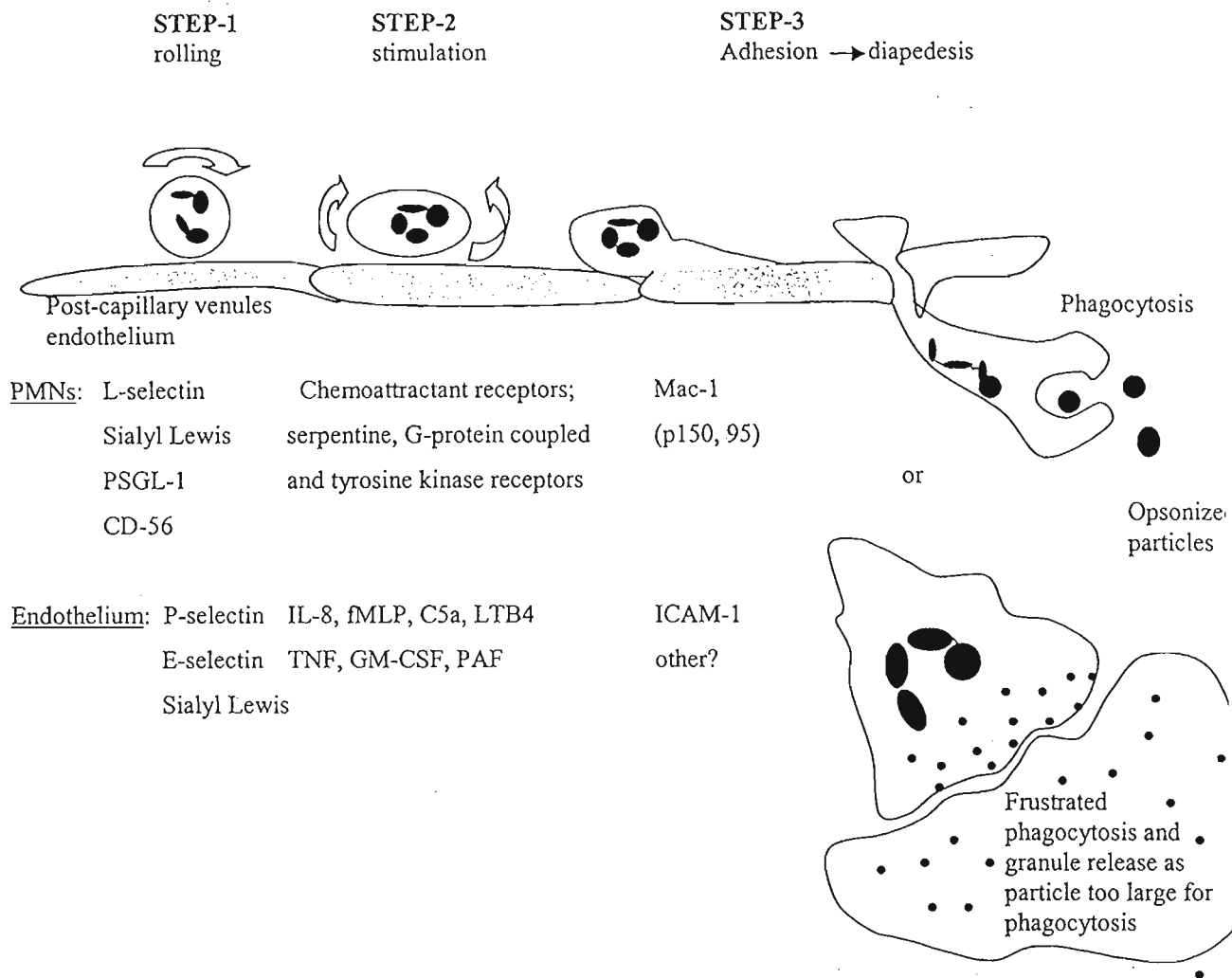


Figure 1.1 PMN migration and role in fighting infections (modified from Sengeløv, 1996).

1.2 PMN granule populations

PMN granule proteins are formed throughout the maturation period of PMNs. i.e. from the myeloblast to the segmented mature stage. During maturation, granule proteins are synthesized in the endoplasmic reticulum packaged into coated vesicles in the Golgi apparatus and aggregated by homotypic fusion with larger granules (Gullberg *et al.*, 1999) before being transported and fused with their respective granules. Thus granules

formed at any given stage of maturation contain granule proteins synthesized at that particular stage of maturation of PMNs (Borregaard and Cowland, 1997) (Table 1.2).

Exocytosis of PMN granule proteins is facilitated by the fusion proteins SNAP and SNARE. Syntaxin-4 (t-SNARE) occurs in the plasma membrane of PMNs, whereas the v-SNARE, VAMP-2 is present on the membrane of most granules (Brumell *et al.*, 1995). Vesicle docking is achieved when vesicles associated with the high specific fusion protein (SNAP), are fused with membrane associated with t-SNAREs.

The order of exocytosis of granules and granule proteins, therefore, may depend on the presence and concentration of these fusion proteins. For example, the VAMP-2 is found at the highest density on the membrane of the most highly mobilizable granules, the secretory vesicles followed by gelatinase granules and specific granules. VAMP-2, however, is not detected on azurophil granules (Brumell *et al.*, 1995). Since these granules are secreted last, exocytosis seems to be affected by the presence and density of VAMP-2 on the membrane of the various granule populations (Borregaard and Cowland, 1997).

PMN granules are classified on the basis of their size, morphology, electron density, and their content of a given marker protein (Bainton *et al.*, 1971) (Table 1.2). The initial classification of two major types of granules was based on their content of myeloperoxidase (MPO). The first granule population that appears during myeloblastic development is called “azurophil” or “primary” granules as these granules stain red/purple with azure dyes and are first expressed in the myeloblast stage. The granules are spherical in shape and have a unique marker known as myeloperoxidase (Gullberg *et al.*, 1995) (Table 1.2). These granules are also found in large numbers in the promyelocyte stage of cell development. At the end of this stage, another additional granule population becomes evident, the specific (secondary) granule. These granules have irregular and elongated shapes (Borregaard and Cowland, 1997) and have a specific marker called lactoferrin (LF). In metamyelocytes, band cells and segmented cells, specific granules are twice as abundant as azurophilic granules (Edwards, 1994).

The “gelatinase” (tertiary) and “secretory” granules start to be synthesized at these stage (Gullberg *et al.*, 1995). These peroxidase negative granules are known as specific and gelatinase granules and have overlapping morphological features and it is difficult to distinguish between them. Borregaard *et al.* (1995) showed that, of these granules, 15% contain lactoferrin and about 60% contain both lactoferrin and gelatinase (MMP-9). Both of these granule subsets were classified as “specific” or “secondary granules” (Table 1.2). However, 25% of the peroxidase negative granules contain only gelatinase (MMP-9) but no lactoferrin and are now termed as “gelatinase” granules or “tertiary” granules (Borregaard *et al.* 1995) (Table 1.2).

The last granules to be synthesized during the maturation of the PMNs are known as the secretory vesicles. These are characterized by their alkaline phosphatase marker enzyme (Gullberg *et al.*, 1995) whereas, MMP-8, is found in the lumen of specific granules, cytochrome b₅₅₈ and the NADPH oxidase enzyme, components of the respiratory burst complex, are found on the membrane of specific granules and the secretory vesicles, respectively (Table 1.2). The discovery of new granule populations is ongoing (Schettler *et al.*, 1991). Recently a TIMP-1-containing granule was discovered and described by Price *et al.* (2000). Due to its pleomorphic shape and the overlap in marker proteins seen in a sub-population of TIMP-1 granules, this granule seems to be synthesized late in the maturation of the PMNs, after the synthesis of the specific granule but before the synthesis of the secretory vesicles (Price *et al.*, 2000) (Table 1.2).

The order of biosynthesis of granules, azurophil, specific, gelatinase and secretory vesicles (Table 1.2) (Gullberg *et al.*, 1999), is also reported to be opposite to the order of calcium-induced secretion (Sengeløv *et al.*, 1993; 1995; Borregaard *et al.*, 1992). The release of TIMP-1 granules would, therefore, be anticipated to occur just after the release of secretory vesicles.

Table 1.2 Granules of the human PMNs (modified from Hellewell and Williams, 1994).

| | Granules ^a | | | | |
|--|--|--|---|--|--|
| | Primary | Secondary | Tertiary ^b | Phosphasomes | Calciosomes |
| Synonyms | Azuropilic, non-specific, basophilic | Specific, eosinophilic, acidophilic, adhesomes ¹ | C-particles ² Secretory vesicles ³ TIMP-1 ¹⁴ | Secretory vesicles ⁴ TIMP-1 ¹⁴ | |
| Size/shape | Mostly spheres, some elipsoid, approx. 500 nm diameter | Spherical (approx. 200 nm diameter) or rod-shaped (130 x 1000 nm) ⁵ | | | Variable shape, (spherical to pleomorphic) 50-200 nm diameter ⁶ |
| Lysosomal acid hydrolases | β -glucuronidase, acid phosphatase, cathepsin B, cathepsin D, etc. | | | | |
| Neutral serine proteases | Elastase, Cathepsin G, Proteinase 3, etc. | Plasminogen activator | | | |
| Neutral metalloproteinases | | Collagenase (MMP-8) | Gelatinase (MMP-9) | | |
| Anti- microbial factors | Myeloperoxidase, defensins, cationic proteins | Lysozyme, Cytochrome b ₅₅₈ ⁷ | | NADPH oxidase ⁸ | |
| Adhesion molecules/ chemotactic factor receptors | | Receptors for laminin, fibrinogen, vitronectin, ⁹ fMet-Leu-Phe receptor, CR3 (Mac-1) ¹⁰ , p150, 95 ¹⁰ | | Fc γ RIII (speculative) ¹¹ | |
| Miscellaneous | | Vitamin B12-binding protein, lactoferrin | | Alkaline Phosphatase, ^{11,12} Plasma proteins ¹³ , Decay accelerating factor (speculative) ¹¹ | Calsequestrin ⁶ [?] TIMP-1 ¹⁴ |

^a Unless otherwise indicated the data are taken from Bagginolini (1980)

^b The existence of tertiary granules as a distinct population is disputed.

References: ¹Singer *et al.* (1989); ²Dewald *et al.* (1982); ³Lew *et al.* (1986); ⁴ Borregaard *et al.* (1990); ⁵Bainton (1988); ⁶Volpe *et al.* (1988); ⁷Borregaard *et al.* (1983); ⁸Sengeløv *et al.* (1992); ⁹Singer *et al.* (1989); ¹⁰Bainton *et al.* (1987); ¹¹Kobayashi and Robinson (1991); ¹²Smith *et al.* (1985); ¹³Borregaard *et al.* (1992); Price *et al.* (2000)¹⁴.

1.2.1 Role of various granule population

PMN granules contain a variety of anti-microbial substances. These include lysozyme, which acts on the cell wall of gram positive microorganisms (Table 1.3), several cationic

proteins, such as the bacteriocidal permeability increasing protein (BPI), which are pore-forming proteins and create pores in the cell walls of various bacteria, and a family of broad spectrum anti-microbicidal peptides called defensins which kill micro-organisms by sequential permeabilization of the outer and inner membranes (Selsted and Ouellette, 1995). Lactoferrin is also included as an anti-bacterial protein as, once released extracellularly or into the phagosome, it complexes with free iron making it unavailable for the growth of microorganisms (Table 1.2).

PMNs have the potential to secrete these granules in a regulated manner either towards an extracellular target or towards a pathogen contained within the phagosome, the digestive body of the PMN (Tapper, 1996), as previously mentioned. Some granules have to be secreted to up-regulate certain receptors e.g. several receptors are associated with specific granule types. Since acid hydrolases and neutral serine proteases are packaged into the same granule, though they are active at different pHs (Table 1.2), it would seem that such granules are released into a closed internal digestive body formed upon uptake of bacteria or large particles, as such a body, the pH may be varied using proton pumps and other mechanisms, enabling first a neutral and subsequently the acid proteases/ hydrolases to become active. Assembly of the respiratory burst components by a granule fusion process may also be most effectively achieved in such a body. Such a process may, however, occur during phagocytosis, in the body known as the phagosome, or on the plasma membrane (Regier *et al.*, 2000).

1.3 Phagocytosis

Phagocytosis is the process by which cells, such as PMNs, and macrophages, fibroblasts and other cells responsible for fighting infection or tissue remodelling, take up relatively large particles ($>0.5 \mu\text{m}$) into the phagosomes and usually requires actin polymerisation (Rabonovitch, 1995). There are two types of phagocytosis. One is non-specific and is not aided by receptors and is seen when tissue debris is being removed. The other is a highly specific and ligand-dependent. In this case phagocytosis is

triggered when certain ligand-specific receptors bind to their corresponding opsonins or structural receptor molecules on the surface of a particle or a bacterium. This may be followed by actin polymerisation membrane ruffling and internalisation of the particle (Tjelle, 2000). Once internalized, the phagosome may undergo sequential fusion with various granule types to give rise to different environments aimed at destruction and digestion of the endocytosed bacterium or particle (Botelho *et al.*, 2000) (Table 1.3 and Figure 1.2).

Table 1.3 Ingestion of antibody-coated bacteria triggers production or release of many bacteriocidal agents in phagocytic cells (Janeway *et al.*, 1999).

| Class of mechanism | Specific products |
|-------------------------------|---|
| Acidification | pH≈3.5-4.0, bacteriostatic or bactericidal |
| Toxic oxygen-derived products | Superoxide O_2^- , hydrogen peroxide H_2O_2 , singlet oxygen 1O_2 , hydroxyl radical OH; hypochlorite OCl. |
| Toxic nitrogen oxides | Nitric oxide NO |
| Anti-microbial peptides | Defensins and cationic proteins |
| Enzymes | Lysozyme dissolves cell walls of some gram-positive bacteria. Acid hydrolases further digest Bacteria |
| Competitor proteins | Lactoferrin (binds Fe^{2+}) and vitamin B ₁₂ binding protein |

When the phagosome becomes a killing body, budding of vesicles from the phagosome compensate for vesicle fusion. These maintain the surface area of the phagosome approximately constant but eventually the membranes and the constituent enzymes and proteins of the phagosome are changed in a budding and fusion process known as maturation (Botelho *et al.*, 2000). In such a process, the pH of the phagosome is initially neutral. However, it subsequently increases despite the influx of acidic granule contents

to the phagosome. This is because H^+ ions produced from granules are consumed during the conversion (O_2^- and O_2^{2-}) of the NADPH oxidase products into H_2O_2 . NADPH oxidase, however, causes the influx of electrons to the phagosome which is also compensated by the K^+ influx. The influx of the K^+ elevates the pH and the ionic strength of the vacuole which results in the release of the cationic granular proteins, including elastase and cathepsin G, from the anionic sulphated proteoglycan matrix, which assist in packaging such proteases in an inactive form (Reeves *et al.*, 2002). When the phagosome is matured, microbial killing is facilitated by a highly acidic pH (pH 3.5 – 4.0 resulting from H^+ ATPase activity) and acid hydrolases (Botelho *et al.*, 2000) (Table 1.3 and Figure 1.2).

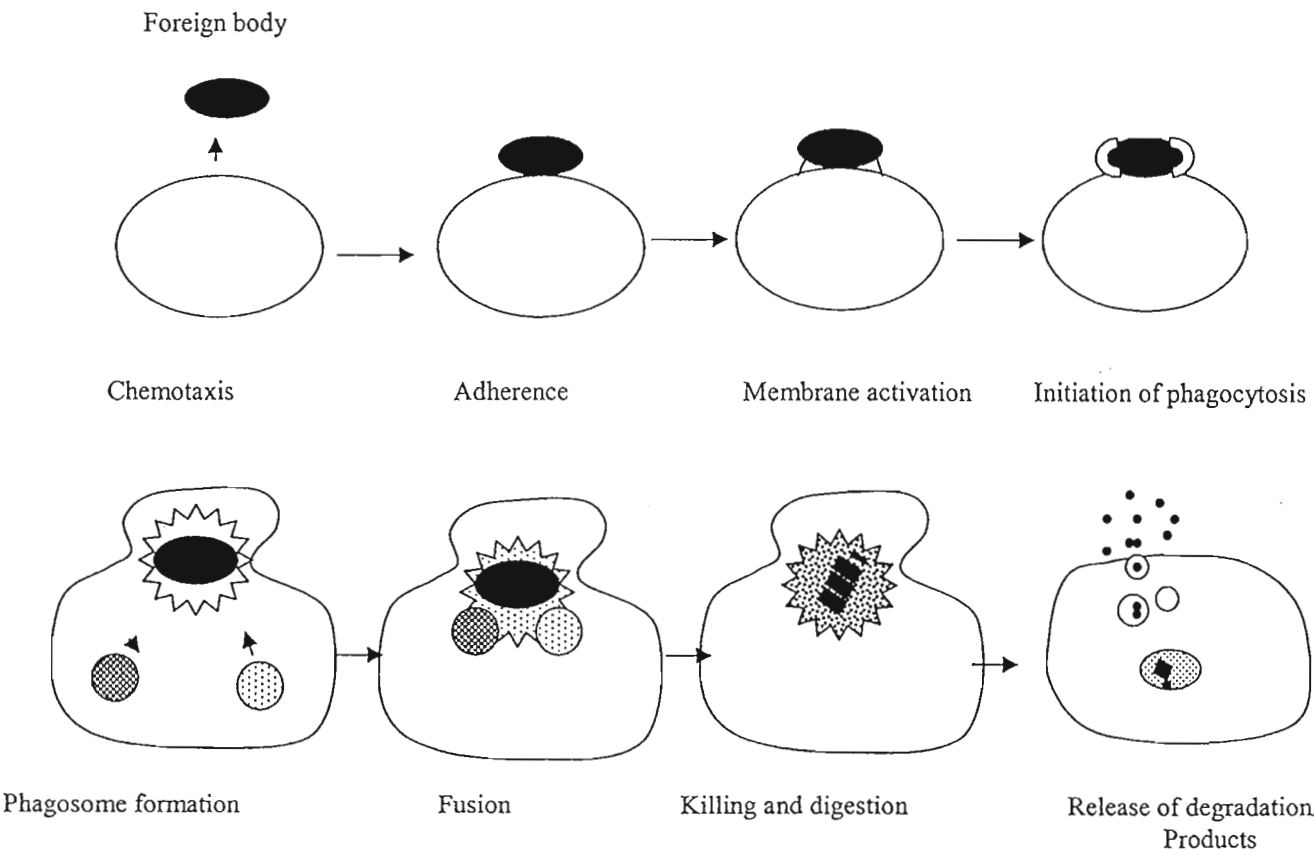


Figure 1.2 Stages of phagocytosis and destruction of foreign bodies (adapted from Roitt, 1999).

Entry of a particle or bacterium into a cell via phagocytosis requires activation of the actin-based cytoskeleton underlying the region of plasma membrane. F-actin assembly in this region is initiated by signals arising from the interaction of ligand-activated

receptors on the cell surface. Interaction occurs either directly, utilizing structural determinants present on the surface of the targets (non-opsonic phagocytosis), or indirectly by recognizing opsonins (complement or IgG) found in host plasma (opsonin-dependent phagocytosis) (Greenberg, 1995). The type of opsonization or ligand recognized and bound by a specific receptor determines the signal transmitted into the cell, the internalisation or non-internalisation of the receptor-ligand complex and the type and extent of granule fusion with the phagosome, which in turn determines the degree of respiratory burst and hence success of killing of microorganisms (Zhou and Brown, 1994; Chastellier and Thilo, 1997). There is also evidence that microorganisms may influence this process (Osiewicz *et al.*, 1999). In order to explore how this process may occur, complement and IgG opsonization and resultant receptor binding will first be described.

1.3.1 Oxygen-dependent and peroxidase-independent microbicidal activity

During peroxidase-independent, oxygen-dependent respiratory burst, phagocytic cells produce superoxide anions, precursors of anti-bacterial oxygen radicals, upon activation of the PMN NADPH oxidase complex (Figure 1.2). This multi-component enzyme complex consists of both cytosolic (Rac-GTP, phosphoproteins p47^{phox}, p40^{phox}, and p67^{phox}) and membrane-bound components (p22^{phox} and gp91^{phox}) which form the heterodimeric flavocytochrome b₅₅₈ complex and contains a putative NADPH binding site, FAD, and two hemes (Regier *et al.*, 2000). This complex possesses all the enzymatic and other machineries required to transfer two electrons from NADPH to two molecules of oxygen (Hampton *et al.*, 1998). PMN activation leads to the phosphorylation and translocation of the cytosolic NADPH oxidase components to the plasma membrane or the membrane of the phagosome. The NADPH oxidase components subsequently interact with the cytochrome b₅₅₈ components brought into the phagosome by fusion of specific granules and the resulting electron flow gives rise to respiratory burst. The activation of the NADPH oxidase complex may possibly be

regulated by a conformational changes in the cytochrome b_{558} component (Regier *et al.*, 2000).

The NADPH oxidase complex catalyses the following reaction:



The superoxide anion is the primary anti-microbial product but may be dismutated into hydrogen peroxide by an enzyme called superoxide dismutase or dissociated into water and oxygen by an enzyme called catalase. These enzymes can be produced by both the host and some microorganisms, thus, microorganisms that possess such enzymes can survive such killing mechanisms.

Peroxidase-dependent microbicidal activity, on the other hand, depends on the activity of the myeloperoxidase enzyme contained in the azurophil granules. Fusion of such a granule with the phagosome or extracellular release, allows the myeloperoxidase enzyme to catalyse the conversion of hydrogen peroxide to bacteriocidal hypochlorate (HOCl) (Andrews and Krinsky, 1986) and chloramines (Hampton *et al.*, 1998; Badwey and Karnovsky, 1980). On the other hand there are many humoral proteins of the innate immune system which also have bacteriocidal functions.

1.3.2 Complement and PMN complement receptors

The complement system is part of the innate immune system and consists of 30 plasma proteins which opsonize and/or kill extracellular bacteria. In the presence or absence of phagocytosis these form part of an important humoral defence system (Osiewicz *et al.*, 1999).

There are three pathways by which complement activation and opsonization of bacteria may occur. The “classical pathway” which is mainly triggered by the binding of C1 and

conversion of C1 to C1q on the surface of foreign particles. Binding is mainly triggered by IgG or IgM bound to pathogen surface (Duncan and Winter, 1998; Kishore and Reid, 2000), but may also be triggered by other factors including immune complexes, DNA, lipid A, certain viruses, and bacterial and parasitic components (Bobak *et al.*, 1987), i.e. charged surfaces or some sugar residues (Tenner *et al.*, 1981; Butko *et al.*, 1999; Guan *et al.*, 1991). The “manan-binding lectin” pathway (MB-Lectin), on the other hand, is initiated by the binding of a serum lectin, the mannan-binding lectin, to mannose-containing carbohydrates on the surface of microorganisms (Tan *et al.*, 1996) (Figure 1.3) (Table 1.4). The last pathway, the “alternative pathway”, is initiated when free C3b in serum spontaneously binds to the surfaces of foreign particles including endotoxins, zymosan or aggregated IgA (Figure 1.3) (Nielsen *et al.*, 1997).

In the classical complement pathway C1q, C1r and C1s are complexed to form C1. Binding of C1, to pathogen surface activates the serine protease C1r which subsequently cleaves the other serine protease C1s (Tenner and Cooper, 1982; Eggleton *et al.*, 1998; Ruiz *et al.*, 1999). The cleavage of C1s results in the dissociation of the C1 and exposure of C1q, a process regulated by a serum protein called C1-inhibitor (Reboul *et al.*, 1987). The dissociation of C1r and C1s from C1q exposes the collagen-like domain of C1q, which has an effector role in binding to one of the three types of C1q receptors on PMNs (Ruiz *et al.*, 1999; Nicholson-Weller and Klickstein, 1999; Guan *et al.*, 1991, 1994) (Table 1.5).

The binding of C1q to receptor C1qRp initiates phagocytosis while binding to the C1qRO₂⁻ receptor generates superoxide production (Ruiz *et al.*, 1999; Kishore and Reid, 2000; Eggleton *et al.*, 1998) (Table 1.5).

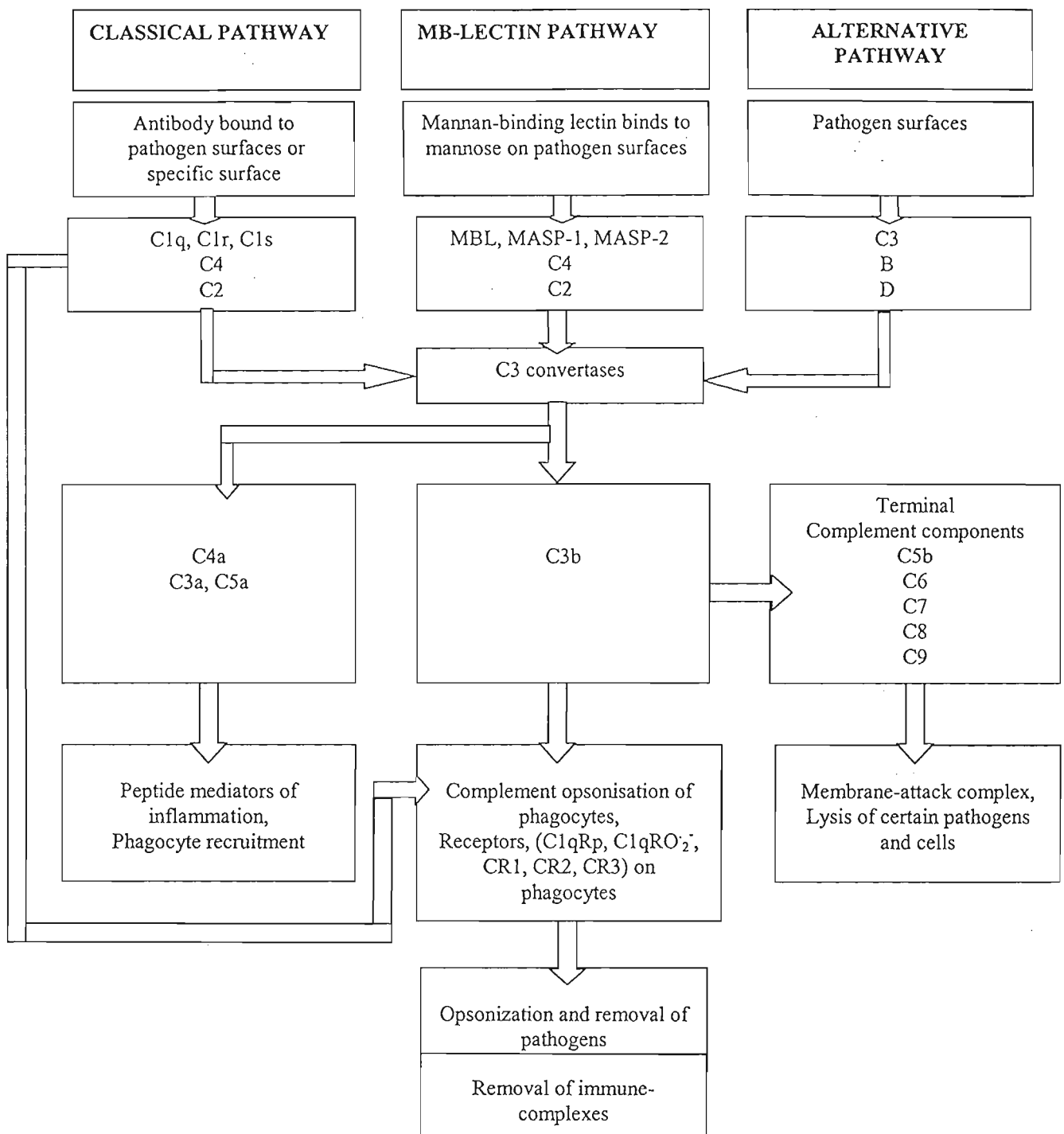


Figure 1.3 Overview of the main components and effector actions of complement. MBL- Mannan-binding lectin; MASP-1 mannan-binding lectin-associated serine protease-1; MASP-2- mannan-binding lectin-associated serine protease-2 (modified from Janeway *et al.*, 2001).

Alternatively, if C1q receptors on PMNs are not bound, C1s may cleave C4 and C2 proenzymes to form C3 convertase complex (Harrison, 1983) (Figure 1.3). This cleaves

C3 into C3b and inactive iC3b. C3b and C4b may coat particles to make them palatable for phagocytosis via the CR-1 receptor (which may also bind C1q) (Eggleton *et al.*, 1998; Nicholson-Weller and Klickstein, 1999; Kishore and Reid, 2000) (Table 1.5). The complement cascade may also continue in the amplification loop of C3 with activated serine enzymes cleaving the next serine proenzyme giving rise to C-9 which inserts into the membrane of the bacteria giving rise to pore formation causing bacterial lysis (Esser and Sodets, 1988) (Figure 1.3). The whole proteolytic cascade liberates anaphylotoxins such as C3a, C4a and C5a. C3a, C4a and C5a, which are cleavage products of C3, C4 and C5, respectively, and are potent chemotactic factors (Figure 1.3) (Janatova, 1988; Särndahl *et al.*, 1996).

Table 1.4 Functional protein classes in the complement system (modified from Janeway *et al*, 2001).

| Functional protein classes in the complement system | | | |
|--|--------------------------------------|-----------------------------------|---|
| Function | Domain | Function | Domain |
| Binding to antigen: antibody complexes and IgG opsonized pathogen surface. | C1q | Peptide mediators of inflammation | C3a, C4a, C5a |
| Binding to mannose on bacteria | MBL | Membrane attack proteins | C5b, C6, C7, C8, C9 |
| Activating enzymes | C1r, C1s, C2b, Bb, D, MASP-1, MASP-2 | Complement regulatory proteins | C1INH, C4BP, CR1, MCP, DAF, Factor H, I, P, CD59. |
| Membrane-binding proteins and opsonins | C4b, C3b | | |

MASP- (1,2), mannan-binding lectin-associated serine protease; C1INH, C1- inhibitor; C4BP,C4 binding protein; MBL, Manan binding lectin; MCP, membrane cofactor protein; DAF, decay accelerating factor.

The CR3 complement receptor, the most abundant receptor on the surface of PMNs is a complement receptor and is also an important integrin of PMN adhesion. The CR1 receptor binds C3b with an affinity of $5 \times 10^7 \text{ M}^{-1}$ while the CR3 receptor binds to monomeric iC3b with a very low affinity, but binds strongly to particles coated with many iC3b, molecules, with an affinity of $10^3\text{-}10^5 \text{ M}^{-1}$ (Edwards, 1994). The CR2 receptor is absent in PMNs. CR1, CR3 and CR4, therefore, may trigger phagocytosis but may induce respiratory burst synergistically with other ligands and receptors (Table 1.5) (Zhou *et al.*, 1994).

Table 1.5 **Distribution and function of receptors for complement proteins on the surface of PMNs** (modified from Janeway *et al.*, 2001; Eggleton *et al.*, 1998; Zhou *et al.*, 1994; Fällman *et al.*, 1993).

| Receptor | Specificity | Functions |
|------------------------------------|-----------------------------|--|
| C1qRp | C1q collagen like Domain | Stimulates phagocytosis |
| C1qRO ₂ ⁻ | C1q collagen like Domain | Stimulates respiratory burst |
| CR1 (CD35) | C3b, C4b, iC3b, C1q | Promotes C3b and C4b decay Stimulates phagocytosis |
| CR3 (CD11b/CD18) | iC3b | Stimulate phagocytosis (Respiratory burst with FcγRIII) |
| CR4 (gp150, 95) (CD11c/CD18) | iC3b | Stimulate phagocytosis |

Although both CR1, CR3 and CR4 receptors are involved in phagocytosis, the CR1 receptor primarily promotes the adhesion of particles and uptake is mainly mediated via the CR3 and CR4 receptors (Fällman *et al.*, 1993). Receptor binding to either CR3 or CR4 results in activation of phospholipase D and production of phosphatidic acid followed by initiation of phagocytosis (Figure 1.4) (Fällman *et al.*, 1993). CR3-mediated

phagocytosis, requires subsequent activation with inflammatory mediators and extracellular matrix proteins (Greenberg, 1995) or requires cooperation or co-ligation of an IgG-Fc receptor (FcγR) in order to give rise to phagocytosis and respiratory burst. The C1qRO₂⁻ receptor, however, initiates respiratory burst without synergy but requires the engagement of an intracellular domain of the CR3 receptor. It is, therefore, an important receptor as the triggering of respiratory burst via this receptor gives rise to maturation of the phagosome into bacteriocidal organelle. In this respect, therefore, C1q and the C1qRO₂⁻ receptor may be the most important complement factors and receptors for triggering maturation of the phagosome via the complement opsonin-receptor system. Hence they are possibly the most important factor for triggering phagosomal killing of microorganisms in a non-immune host.

In a primed host IgG and IgM are, however, also important opsonins triggering phagocytosis and killing of microorganisms, depending on which Fc receptor is bound.

1.3.3 Fc receptors and effect of receptor binding

PMNs do not have receptors for opsonizing IgM. There are, however, many inhibiting and activating types of Fc receptors in PMNs, interacting with the Fc domain of various classes of IgG molecules, the main activating receptors being represented in Table 1.6. All of these bind IgG₁ with the highest affinity. Free IgG molecules do not trigger PMN responses, however. Only when IgG molecules are aggregated and multimerized on the surface of opsonized particle may binding to the Fcγ receptors on the surface of PMNs, trigger phagocytosis and/or respiratory burst (Table 1.6). This prevents tissue damage due to spontaneous activation of PMNs in the blood (Janeway *et al.*, 1999).

The FcγRI receptor, has a high binding affinity for monomeric IgG but is not normally found on PMNs unless PMNs are exposed with INF-γ, whereas, the FcγRII receptor has a low affinity for monomeric and dimeric IgG₁ and is involved in both IgG-mediated phagocytosis and respiratory burst (Williams *et al.*, 2000) (Table 1.6). The FcγRIII

receptor has the lowest affinity to IgG₁ but is also involved in phagocytosis. In PMNs FcγRII, which have the highest affinity for the most abundant immunoglobulin subclass in serum (IgG₁), are most numerous.

Though, the main function of PMN Fcγ receptors would be anticipated to be to facilitate phagocytosis and respiratory burst, they do not necessarily initiate these responses. The FcγRI receptor neither initiates respiratory burst (Walker, *et al.*, 1991) nor phagocytosis (Gabriela and Carlos, 1998) unless tyrosine residues on the receptor are phosphorylated. This, however, cannot occur unassisted by other receptors, as such residues are not part of an associated cytoplasmic motif of this receptor. The FcγRII receptors, which have the tyrosine residues in an immunoreceptor tyrosine activation motif (ITAM), however, can directly initiate respiratory burst by activation of their own associated tyrosine kinase or other tyrosine kinases (Greenberg, 1995; Zhou *et al.*, 1994). This activation is pertussis toxin sensitive indicating its dependence on G-proteins (Zhou *et al.*, 1994) (Figure 1.4). The FcγRIII receptor also does not contain the ITAM motif and is unable to initiate respiratory burst on its own. However, the synergistic activation of the FcγRIII and the CR3 receptors may induce activation of tyrosine kinases and actin depolymerization leading to initiation of respiratory burst in a G-protein independent pathway (Zhou *et al.*, 1994). The FcγRII receptor is, therefore, the most effective FcγR for triggering phagocytosis and respiratory burst.

Table 1.6 Leukocyte Fcγ receptors and their characteristics (modified from Janeway *et al*, 2001).

| Receptor | FcγRI (CD-64) | FcγRII a (CD-32) | FcγRIII b(CD-16) |
|--|---|---|--|
| MW | 72 kDa | 40 kDa | 50-70 kDa |
| Binding | IgG ₁ 10 ⁸ M ⁻¹ | IgG ₁ 2 x 10 ⁶ M ⁻¹ | IgG ₁ 5 x 10 ⁵ M ⁻¹ |
| Order of affinity | IgG ₁ =IgG ₃ > IgG ₄ >>IgG ₂ | IgG ₁ >IgG ₃ IgG ₃ = IgG ₂ | IgG ₁ = IgG ₃ |
| Cell type | Macrophages PMNs Eosinophils Dendritic cells | Macrophages PMNs Eosinophils Platelets Langerhans cells | Macrophages PMNs Eosinophils NK cells Mast cells |
| Effect of ligation of PMN receptors | Binding only | Phagocytosis Respiratory burst | Phagocytosis |

During the initial period of infection, however, only complement components and perhaps IgM may circulate in the blood. Though PMNs have no receptors for IgM, IgM bound to the surface of target bacteria is very effective in triggering the binding of C1q components and generating PMN respiratory burst and phagocytosis via the C1qRO₂ receptor. As complement-deficient mice are unable to mediate immune response to several bacterial pathogens (a process requiring phagocytosis and antigen presentation) and antibodies are usually only produced later in an infection, complement opsonization and the triggering of phagocytosis and respiratory burst seems more important in the control of pathogens during early infection than the presence of antibodies and hence seems more important than the adaptive immune system in the early stages of infection (Ravetch and Clynes 1998). For this reason the current study focussed on complement-

mediated PMN responses such as phagocytic digestion (including respiratory burst) even before the arrival of the macrophage. The primary interest in this study, therefore, is the PMNs and their role in phagocytic killing of microorganisms, and the observation of Osiewicz *et al.* (1999) which suggest that active MMP-9 is involved in this process i.e. that uninhibited MMP or lack of TIMP-1 secretion is required. During such early events, exposure of the complement factor C1q and binding to its receptors also seem to be most important in the phagocytosis and respiratory burst killing of microorganisms. Since certain factors of the classical complement pathway, C1-inhibitor (Knäuper *et al.*, 1991; Pemberton *et al.*, 1989) and C1q (Ruiz *et al.*, 1995; 1999), are sensitive to cleavage by PMN MMPs this research will focus on TIMP-1 and MMP release from PMNs under various circumstances i.e. the TIMP-1 and MMP-9 ratios seen, their possible effect on C1q opsonization and the C1q receptor triggering of phagocytosis and respiratory burst. Before such a study could be undertaken some knowledge of how granule mobilization and release and respiratory burst may occur was required and whether it was known how release of TIMP-1 and MMP-9 may be regulated.

1.4 Granule mobilization and release and respiratory burst.

The PMN responds to external stimulation in a variety of ways and how many and the nature of the pathways that are triggered still remains unclear. The most studied molecule for triggering granule release is fMet-Leu-Phe (fMLP). Formyl methionine-labelled proteins expressed by bacteria are easily distinguished from the host proteins (Hellewell and Williams, 1994). Binding of the fMLP receptor is followed by the activation of G-proteins which sequentially trigger three phospholipases, phospholipase D (PLD), phosphoinositide-specific phospholipase C (PI-PLC), and phospholipase A₂ (PLA₂). Occupancy of fMLP chemoattractant receptor induces dissociation of α sub-units from the β and γ sub-units of the G protein and the α sub-unit is activated by binding to GTP (Sengeløv, 1996). The activated α subunit lowers the calcium concentration required to stimulate a membrane-associated PLC, which in turn hydrolyzes phosphatidylinositol biphosphate (PIP₂) to diacylglycerol (DAG) and

inositol triphosphate (IP₃). Diacyl glycerol is an activator of protein kinase C (PKC) which in turn phosphorylates a variety of serine and threonine residues, and IP₃ induces release of calcium from internal cellular stores (Figure 1.4). Release of calcium increases the cytosolic calcium level and results in differential granule fusion with the plasma membrane in the order opposite to the order in which the granules are synthesized, in a calcium concentration-dependent manner, the secretory vesicles being released by the lowest level of calcium (Sengeløv *et al.*, 1993).

Schettler *et al.* (1991) showed that during stimulation with formyl peptides and phagocytosis of IgG- and complement-opsonized zymosan, only collagenase (MMP-8) and gelatinase (MMP-9) were secreted to any significant extent. Less than 6% of elastase, myeloperoxidase and lactoferrin-containing granules were released with either of these agents. Secretion was microtubule-dependent and disruption of microtubules with colcemid inhibited release of all granules. Thus, granules seem to be differentially associated with the cytoskeleton. Disruption of microfilaments by cytochalasin B, however, resulted in release of all granule contents under formyl peptide stimulation. These results show that some granule types are more easily released than others, possibly because they have an extracellular function while other granules are not easily mobilized and possibly have a largely intra-phagosomal function.

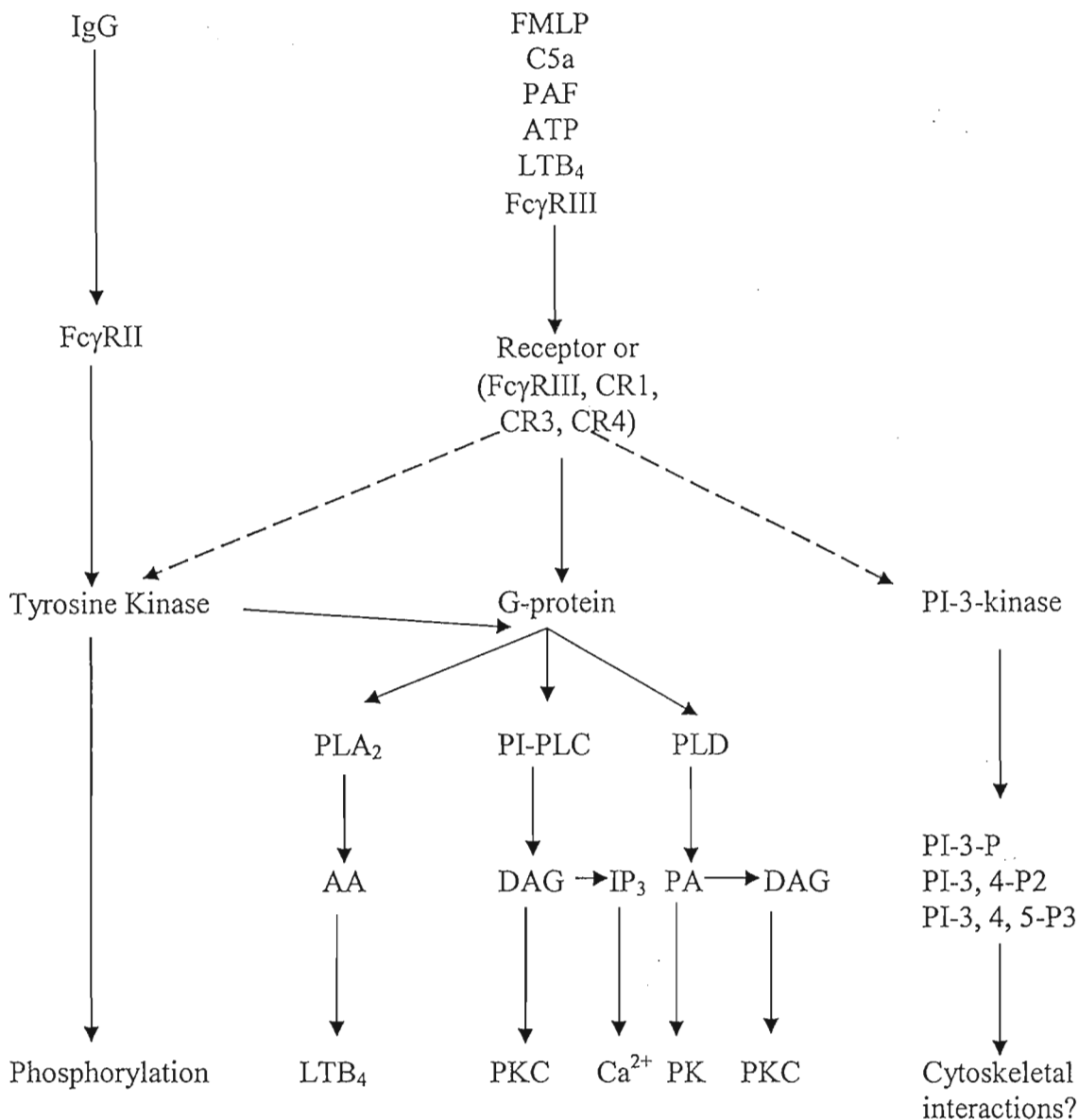


Figure 1.4 Multiple second messengers derived from signalling pathways triggered by the G-protein-coupled receptors, Fc and complement receptors. Rapid activation of three phospholipases as well as the activation of tyrosine phosphorylation and phosphatidylinositol-3-kinase is known to be regulated by occupied receptors. The products of the individual reactions which function as second messengers are indicated as well as the downstream events that they regulate. Dotted lines indicate alternative pathway of activation. (IgG, immunoglobulin G; FMLP, formyl methionyl leucyl phenylalanine; C5a, complement fragment 5a; PAF, platelet aggregation factor; ATP, adenosine triphosphate; LTB₄, leukotriene B₄; PLA₂ phospholipase A₂; PI-PLC phosphoinositide phospholipase C; PLD, phospholipase D; AA, arachidonic acid; DAG, diacyl glycerol; IP₃, inositol triphosphate; PA, phosphatidic acid; DAG, diacyl glycerol; PK, protein kinase; PKC, protein kinase C; PI3-P phosphoinositide phosphate, PI-3, 4-P2, phosphoinositide diphosphate; PI-3, 4,5-P3, phosphoinositide triphosphate, FcγRII, FcγRIII, Fc receptors, CR3, complement receptor 3) (modified from Hellewell and Williams, 1994).

How TIMP-1 release is regulated was, however, unknown. Preliminary unpublished results of Price *et al.* (2000) indicated that, unlike other granules, the TIMP-1 vesicle did not seem to be released via a calcium-mediated mechanism. Other investigators indirectly indicated that the TIMP-1 vesicle may be possibly differentially associated with the cytoskeletal elements and that a different non-calcium-dependent discharge mechanism may be involved in granule release (Schettler *et al.*, 1991). As knowledge of how TIMP-1 is released may help in understanding and controlling many MMP-induced pathologies and may be involved in the evasion of respiratory burst by microorganisms, the regulation of release of TIMP-1 formed a preliminary sub-investigation of this study. It was decided to explore various opsonins coatings in a latex bead model system and use such a system to check the effect of activating various receptors, especially those for complement in causing the release of MMP-9 and TIMP-1.

It was known that, activation of fMLP receptor results in the activation of PLD and phosphatidic acid (PA) is subsequently produced. Phosphorylation of $p22^{phox}$ follows activation of a protein kinase (PK) by PA and gives rise to assembly of the respiratory burst complex (Figures 1.4 and 1.5). Alternately, PMA may directly activate PKC which subsequently phosphorylates $p22^{phox}$ resulting in respiratory burst (Regier *et al.*, 2000) (Figure 1.5). IgG- and complement opsonization of zymosan (OPZ) i.e. activation of the FcγRIII and CR3 receptors, gives rise to phagocytosis and respiratory burst mediated by both PLD- and PLC-dependent $p22^{phox}$ phosphorylation (Regier *et al.*, 2000) (Figure 1.5). This gives rise to a strong respiratory burst which would not be achieved by activation of the CR3 or FcγRIII receptors separately, potentially explaining why IgG/complement opsonization and activation of the FcγRIII and CR3 receptors together gives effective maturation of the phagosome and hence killing of microorganisms (Zhou *et al.*, 1994), an effect enhanced by fMLP (Regier *et al.*, 2000).

Low concentration of free C1q molecules do not induce respiratory burst. However, in the absence of antibodies, C1q-opsonized particles or C1q in high concentration can induce respiratory burst in a manner which is different from the pathway used by other

complement factors and their receptors. It was shown that C1q mediated superoxide production is Ca^{2+} flux-and actin polymerization-dependent but PKC independent (Goodman and Tenner, 1992). Even though, C1q triggers respiratory burst via the C1qRO_2^- receptor, respiratory burst is initiated by binding of the C1qRO_2^- receptor to the intracellular domain of CR3 (CD11b/CD18) receptor (Goodman *et al.*, 1995). Thus, there is interdependence between the complement receptors.

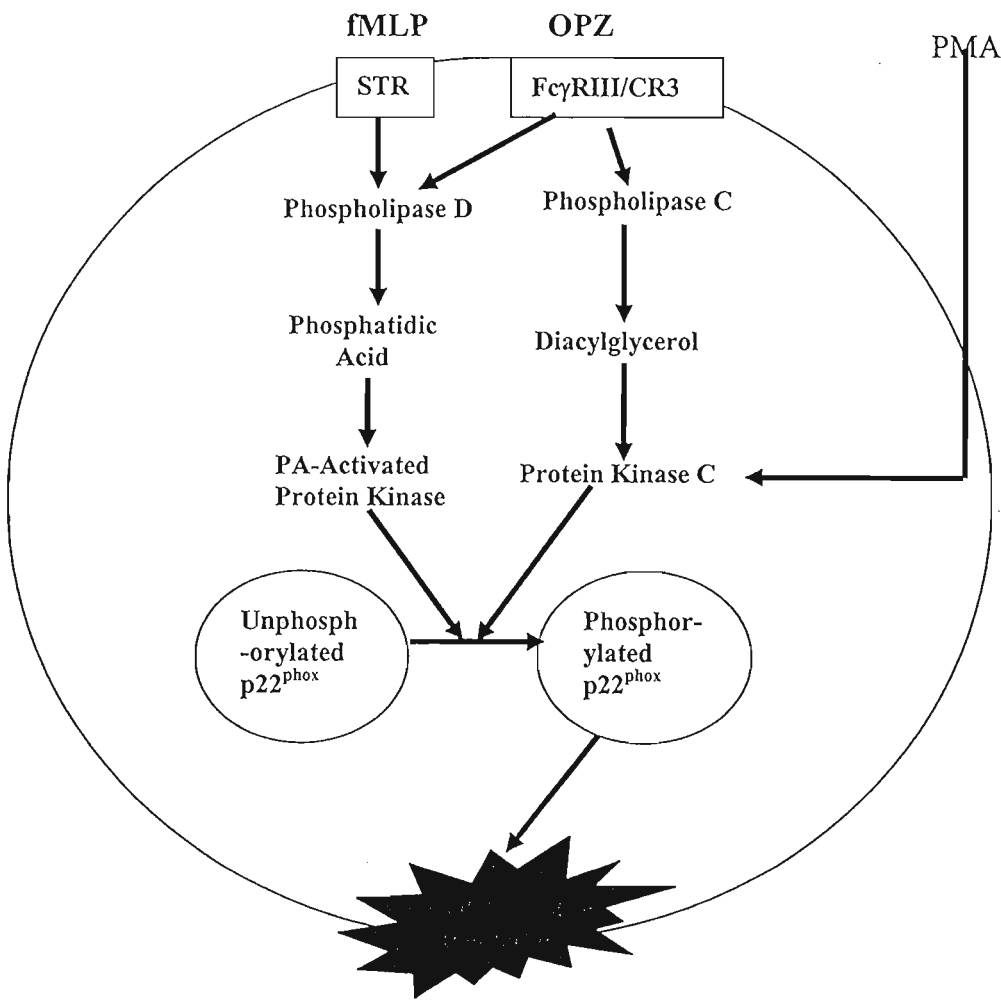


Figure 1.5 Model of p22^{phox} phosphorylation in PMNs. STR (seven transmembrane receptors), OPZ (opsonized zymosan) (modified from Regier *et al.*, 2000; Löfgren *et al.*, 1999; Zhou *et al.*, 1994).

1.5 Summary of objective of the current study

The presence of active extracellular MMPs and complement has been shown to be necessary for the killing of microorganisms by PMNs (Osiewicz *et al.*, 1999). Since MMPs bind and are inhibited by TIMP-1 in a 1:1 ratio, the activity of MMPs seen extracellularly may depend on the relative amount of MMPs and TIMP-1 present. Whether MMPs and TIMP-1 are released individually, together or at all upon stimulation of PMN complement receptors by opsonizing complement and hence whether the extracellular MMP:TIMP-1 ratio may influence the outcome of an infection, is unknown. The current study will focus on one possible mechanism by which the outcome may be influenced by the MMP:TIMP-1 ratio. This in essence is a mechanism by which TIMP-1 release may be envisaged to influence complement assembly, and hence opsonization, the PMN receptors bound and hence phagocytosis, respiratory burst and killing of microorganisms.

Attention was focused on the first complement component to bind to the surface of antibody-coated or charged residues on microorganisms, the C1-complex, the lectin component of which is C1q. The C1 complex does not bind to any of the phagocyte receptors (Tenner and Cooper, 1980), however, normal dissociation of C1r and C1s, due to the presence of C1-inhibitor, may lead to binding of C1q to PMN C1q receptors, stimulation of reactive oxygen radical production, phagocytosis, and killing of microorganisms (Tenner and Cooper, 1980). C1-inhibitor, has been shown to be cleaved and inactivated by PMN MMPs (MMP-8 and -9) (Knäuper *et al.*, 1991) and by PMN elastase (Pemberton *et al.*, 1989; Gigli and Tausk, 1988). Release of MMP-9 and not MMP-8 and elastase is likely to occur during early phases of infection (Goodman and Tenner, 1992; Kishore and Reid, 2000), however. The effect of MMP-9 (and TIMP-1) release was, therefore, to be monitored and correlated with respiratory burst or killing of microorganisms. It was reasoned that secretion of MMP-9 and possibly TIMP-1, may be constitutive as MMPs are not usually processed in the other cells and are usually released constitutively in their pro-form. Pro-MMP-9 is also the only pro-MMP which is able to bind to both the MMP-binding and the inhibitory domain of TIMP-1. It was,

therefore, hypothesized that any simultaneously released TIMP-1 may stabilize pro-MMP-9, facilitate activation, and protect MMP-9 against degradation, possibly allowing limited activity after activation, before preventing large scale degradation of ECM components and MMP-sensitive factors such as complement C1-inhibitor (Price *et al.*, 2000). Therefore, it was our hypothesis that 1) MMP-9 was probably secreted at a low level facilitating cell movement 2) in a case where phagocytosis was not seen, C1-inhibitor may possibly be cleaved by PMN MMP-9, preventing the dissociation of C1q-C1r-C1s, complex, the binding of C1q receptors (C1qRp and C1qRO₂⁻), respiratory burst and phagocytosis. We hypothesized that this may be due to the prevention of TIMP-1 release by signalling molecules released by microorganisms which have evolved to evade phagocytosis and complement-mediated killing by PMNs.

Therefore, prior to monitoring respiratory burst, in order to investigate whether MMP-9 and TIMP-1 secretion normally occurs when complement is present and whether complement and especially C1q may form an opsonin, polystyrene beads with surfaces which would favour the binding of C1q were incubated with complement and the release of MMP-9 and TIMP-1 was monitored. However, before such experiments were performed, methods for the semi-fractionation and detection of MMP-9 (Chapter 3) and TIMP-1 (Chapter 4) were optimized. After this methods for detection of latex bead-complement opsonins were assessed using either pre-coating of latex beads or incubation in the presence of PMNs and complement. The secretion of MMP-9 and TIMP-1 by phagocytosing PMNs was, therefore, assessed (Chapter 5). The future course of action is discussed in Chapter 6. To assist the flow of subsequent chapters, methods which were recurrently used in the thesis are described in Chapter 2.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

This chapter contains materials and methods that were routinely used throughout the project. These are included here to increase the readability of subsequent chapters and allow the use of this thesis as a method reference.

2.1 Materials

All reagents were of analytical grade.

Tris (2-amino-2- (hydroxy methyl)-1,3-propanediol), bovine serum albumin (fraction V), nitro blue tetrazolium [2,2'- di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-(3,3'-dimethoxy-4, 4'-diphenylene) di-tetrazolium chloride], [5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt] were from Roche Diagnostics, Mannheim, Germany. Acrylamide, NN'-methylene bisacrylamide, calcium chloride-2-hydrate, glycine, ethylenediamine tetraacetic acid (EDTA), ammonium peroxodisulfate, ammonium sulfate, sodium dihydrogen ortho-phosphate, sodium chloride, EDTA.Na₂, 2-methylpropan-2-ol (tert-butanol), sodium azide, ethanol (99%), Triton X-100, were from BDH Laboratory Supplies Poole, England. Brij-35, rat IgG, anti-chicken IgG alkaline phosphatase conjugated, molecular weight markers (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, myoglobin, lysozyme), gelatin (porcine skin), levimsole (L [-]-2,3,5,6-tetrahydro-6-phenylimidazo [2,1- β] thiazole), trypan blue, 3,3'-diaminobenzidine tetrachloride, phorbol 12-myristate 13-acetate (PMA), citrate phosphate dextrose solution, Percoll, tricine, rabbit anti-TIMP-1 antibody (T-8687), mouse anti-TIMP-1 monoclonal antibody (T-8187), rabbit anti-chicken antibody alkaline phosphatase tagged (A-9171) and goat anti-rabbit antibody alkaline phosphatase tagged (A-3687) were from

Sigma Diagnostics, St. Louise, MO, USA. Hybond blotting paper, ECL western blotting analysis detection kit, lyophilized low molecular weight markers were from Amersham, Pharmacia Biotech, UK. Film developer (multicontrast developer) and fixer were from Agfa Corporation, Germany. Tween 20, 2-mercaptoethanol, polyethylene glycol (6 000), polyethylene glycol (20 000), magnesium chloride-6-hydrate, N-N-N'-N'-tetramethyl ethylenediamine (TEMED), di-sodium hydrogen phosphate dodecahydrate and potassium carbonate were from Merck, South Africa. Potassium chloride, Coomassie brilliant blue R250, potassium dihydrogen orthophosphate were from Saarchem, South Africa. Sodium dodecylsulfate (SDS) and phenylmethylsulfonylfluoride (PMSF) were from Boehringer Mannheim, Mannheim, Germany. Hydrogen peroxide 35% (v/v) was from Riedel-de Haën, Germany. Ponceau S was from GURR, Searle Diagnostic, Inghamshire, UK. Carboxylated latex beads (1 μ m) were from Bangs Laboratories Inc, Indianapolis, U.S.A. The chicken anti-TIMP-1, chicken anti-MMP-9 and chicken anti-molecular weight marker antibodies were raised by Dr. Brendon Price from the Department of Biochemistry, University of Natal, South Africa.

2.2 Protein quantitation assays

The simplest and most widely used method for protein determination is a spectrophotometer method measuring the absorbance of a protein at 280 nm (A_{280}) and calculating protein concentration using the extinction coefficient of that protein. Most proteins show maximal absorption at 280 nm due to the presence of aromatic amino acids, such as tyrosine and tryptophan. Different proteins have different ratios of aromatic amino acids, therefore, in order to use absorbance at 280 nm, proteins must be pure and of known extinction (absorption) coefficients. For IgG and IgY antibodies their extinction coefficients are 1.43 and 1.25 ml/mg/cm, respectively (Dennison, 2003).

For pure protein preparations with known extinction coefficient, the concentration using a cuvette with a 1 cm path length was calculated according the following formula.

$$A = E \times C$$

Where A = Absorbance at 280 nm.

E = Extinction coefficient (ml/mg/cm) i.e. (1 mg/ml of 0.1% solution) of the test protein in a 1 cm cuvette.

C = protein concentration in mg/ml

2.2.1 Bradford dye binding assay

As it is impossible to determine the concentration of a protein mixture using an extinction coefficient, Bradford (1976), therefore, developed a method which determines the total protein concentration using a dye-binding assay in which the binding of a dye to a protein causes a shift in the absorption maximum. For Coomassie brilliant blue G-250 the shift is from 465 nm to 595 nm allowing monitoring of absorption shift at 595 nm. This method for quantitation of proteins is still the best for protein measurements. Many authors have come up with slight modifications but the Bradford assay remains widely used because of its ease of performance, rapidity, relative sensitivity, and specificity for proteins (Zor and Selinger, 1996).

Bradford's Coomassie brilliant blue G-250 protein-binding dye exists in three forms: red (cationic), green (neutral), and blue (anionic) which absorbs at 470 nm, 650 nm and 590 nm, respectively. As the binding of dye to protein ratio increases the blue colour increases and the red colour decreases. Due to the overlapping absorption characteristics of the red and blue dye forms the method gives a slight non-linearity (Bradford, 1976). To avoid this non-linearity Zor and Selinger (1996), recommended reading the absorbance at 590/450 nm so that protein concentration may be established by measurement of the increasing dye absorption at 590 nm and decreasing dye absorption at 450 nm. This ratio was found to be directly proportional to the concentration of protein present and gave a more linear curve than Bradford's method, increasing

sensitivity 10-fold and allowing quantitation of down to 50 ng of a standard protein. Measuring at 590/450 nm also decreased the interference due to competition of low amount of SDS with the dye.

Another modification was introduced by Read and Northcote (1981). These researchers observed that increasing the amount of dye in assay solution or decreasing the amount of phosphoric acid and replacing Coomassie brilliant blue R-250 with Serva Blue G-250 increases sensitivity and also adjusts the colour range achieved with proteins to values close to that seen for equivalent concentration of the standard protein BSA. BSA is a commonly used standard protein in the Bradford assay as it has high colour yield unlike other standards which almost have the same absorbance as the proteins for analysis (Zor and Selinger, 1996). The modification of Zor and Selinger (1996) and Coomassie brilliant blue was replaced with Serva Blue in the study as the increased amount of dye used in the Read and Northcote (1981) method has previously shown to have a precipitating effect.

2.2.1.1 Reagents

Bradford dye reagent. Dye reagent was prepared according to Bradford (1976) with the exception that Serva Blue G-250 (50 mg) was dissolved in 25 ml 95% ethanol and used instead of Coomassie blue. A volume of 50 ml phosphoric acid [85% (w/v)] was added and the solution was diluted to 500 ml with double de-ionized water (ddH₂O). The solution was stirred for 30 min and filtered twice. The final concentration of the reagent was 0.01% (w/v) Serva Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Standard BSA solution (1 mg/ml). BSA (Fraction V) (10 mg) was dissolved in 10 ml ddH₂O to make a 1 mg/ml solution.

2.2.1.2 Procedure

Stock bovine serum albumin (BSA), prepared as in the above, was used to prepare standard curves for the microgram and nanogram range of protein. Five replicates of 40 μ ls of the diluted sample were dissolved in 160 μ ls of ddH₂O each. Each sample (200 μ ls) was mixed with 800 μ ls of Bradford reagent in 1.5 ml Eppendorf tubes to make up 1 ml. The tubes were gently vortexed and transferred to clean plastic cuvettes. [Only plastic cuvettes were used in the experiment as Coomassie brilliant blue dye binds to glass cuvettes and gives erroneous readings (Bradford, 1976)]. Dye-protein mixtures were allowed to react for two min and absorbance of each sample was measured at 590 nm and 450 nm within one hour. Double de-ionized water (ddH₂O) served as a blank instead of dye reagent as in the conventional Bradford's assay because the free dye is also measured at 450 nm thus, no free dye control is needed. The ratio of $A_{590/450}$ was calculated and a standard curve was plotted against the amount of protein applied. A graph was constructed in Microsoft Office Excel software and a linear regression equation was calculated to determine the concentration of protein.

2.3 Protein sample concentration

Protein samples which were too dilute may need to be concentrated for various purposes. Proteins can be concentrated in different ways such as by changing the properties of the solvents by addition of high concentration of certain salts or miscible organic solvents. Some of the precipitation procedures such as the use of certain organic solvents and changes of pH and temperatures denature proteins and are used only for certain analytical purposes (Deutscher, 1990).

2.3.1 Salt precipitation

Very dilute proteins for SDS-PAGE analysis may need to be concentrated for detection due to the limited capacity of wells in the gel where protein samples are loaded. Some of the precipitation methods which may be used are the KCl, NaCl, Na₂SO₄ or (NH₄)₂SO₄ methods (England and Seifter, 1990).

(NH₄)₂SO₄ precipitation is important method of protein concentration. In the absence of (NH₄)₂SO₄, proteins remain as open structured, more soluble and less stable forms. Upon addition of increasing (NH₄)₂SO₄, however, they start to become compact in structure, less soluble and more stable. This occurs as the (NH₄)₂SO₄ sequesters more water (the SO₄²⁻ binds to 14 H₂O molecules) and causes dehydration and crowding of the proteins. This eventually causes certain proteins to precipitate as they reach their solubility limit in a process called salting out (Dennison and Loverein, 1997). The use of (NH₄)₂SO₄ is chosen because next to citrate, sulphate is the second strongest Hofmeister Cosmotrope which is characterized by its high effectiveness in salting out proteins and stabilizing protein structure (Dennison and Lovrein, 1997). Ammonium sulfate, however, needs to be removed by dialysis before electrophoresis may be used. Therefore, this not is not a convenient method for small volumes.

A slightly modified method, SDS/KCl precipitation was used for most proteins which were needed to be concentrated for electrophoresis. This method is unsuitable for general protein concentration as proteins would be denatured by SDS.

2.3.1.1 Reagents

5% (m/v) SDS. SDS (0.5 g) was dissolved in 10 ml of ddH₂O.

3M KCl. KCl (2.24 g) was dissolved in 10 ml of ddH₂O.

2.3.1.2 Procedure

SDS [5% (v/v), 10 μ l] was added to the sample (100 μ l) in a 1.5 ml Eppendorf tube. The solution was mixed by inverting the tube and 3 M KCl (10 μ l) was added. The mixture was again inverted and centrifuged (12 000 x g, 2 min, RT) and the supernatant was discarded. For SDS-PAGE the precipitate may be dissolved in stacking gel buffer (10 μ l) and reducing treatment buffer (10 μ l) (Section 2.6.1.1).

2.3.2 Concentration by dialysis

Dialysis uses the principle of osmosis to desalt or effect a buffer change or may be used to concentrate a sample. Dissolved molecules and dialysing solutions are separated by semi-permeable membrane and movement of water and ions is effected from the region of low ion concentration to the higher ion concentration. The selective sieving of the membrane due to its defined pore size also allows the movement of protein molecules of a certain size. The size at which molecules are retained by the membrane is called molecular cut off. When dialysis is used for desalting, distilled water or buffer with low ionic concentration may be used as the dialysis solution. This may cause influx of water and efflux of salts from the protein causing the membrane to swell. If the ionic strength of the buffer used is low or ddH₂O is used to dialyse a solution a phenomenon called Donnan membrane effect may occur. The large size of proteins and the fact that they cannot pass through the pores causes an overall build-up of charge associated with the overall charge on the protein. To compensate this there is an influx of opposite charged ions. This may result in extremes of pH, either caused by influx of H⁺ protons (acid pH) or OH⁻ ions (alkaline pH). For this reason proteins should be dialysed against a buffer where possible as buffer ion dissociation and movement would compensate for ion inequalities across the membranes, correcting the pH to the desired value.

Maximal diffusion of solute and hence exchange of ions and H₂O can be achieved only by frequently changing the dialysis buffer or H₂O or dialyzing against large volumes. The number of changes is less important than the total volume of dialysis buffer as large volumes maintain maximal concentration differences across the dialyzing membrane for longer (England and Seifter, 1990).

When used for concentration of highly diluted samples, the dialysis bag may be surrounded by solution of high concentration or with compounds with high affinity for H₂O such as granular sucrose or PEG 20 000. Under these conditions water flows out from the membrane and dissolves in the granular or high concentration of the solute. Sucrose may enter the bag contaminating the concentrated protein sample, whereas PEG 20 000 would not be able to do so due to its size.

2.4 Three phase partitioning (TPP) semi-fractionation of proteins.

Three-phase partitioning is a method of protein fractionation in which proteins are harvested in a layer between an upper t-butanol layer and lower aqueous (ammonium sulfate) layer. Using this method about 25 enzymes and proteins from different sources has been harvested (Dennison and Lovrein, 1997).

When ammonium sulphate is added to an aqueous solution containing a co-solvent t-butanol (which is totally H₂O miscible), the solution separates into an upper t-butanol and lower aqueous layer. As different proteins become more butanolated and progressively dehydrated, due to the progressive complexing of water by ammonium sulfate, they reach their solubility limit and come out of solution (Dennison and Lovrein, 1997).

t-Butanol is unique among other common organic solvents because it does not denature proteins. This may be due to its larger size or due to binding to surface hydrophobic patches on proteins which constitutes 50% of their surface area. Instead of gaining

access to the interior of the protein and causing distortion of their structures (Dennison *et al.*, 2000). Hydrated sulphate ions also become too large, due to aggregation of large amount of water molecules on their surfaces, to enter into interior of proteins and cause denaturation. Other organic solvents unlike t-butanol and salts unlike ammonium sulphate, however, have smaller sizes which allow them to enter into the interior domain of proteins and denature them. When t-butanol is used at a concentration of 30% (v/v), it also inhibits enzyme activities and hence protein denaturation that may occur during homogenization (Dennison *et al.*, 2000).

TPP has additional advantages over conventional salting out because, in TPP the proteins float and are desalted, in the mid layer, between the t-butanol and solvent/ammonium sulphate layers. In conventional salting out precipitation of proteins, becomes increasingly difficult with increasing ammonium sulfate concentrations (Dennison *et al.*, 2000). Conventional salting out results in high salt content in the precipitated proteins and, therefore, is usually followed by desalting before further processing (Dennison *et al.*, 2000). Therefore, TPP was a method of choice for fractionation of MMP-9 from sputum.

2.4.1 Procedure

Sputum (20 ml) was collected into a sterile tube from a normal individual fasted for 12 hours. The sputum was clarified (10 000 x g, 4°C, 10 min), the pellet removed the supernatant was measured and pre-warmed t-butanol [30% (v/v) calculated as indicated in Equation 1 below] and the required amount of ammonium sulfate (see Equation 2 below) added. The ammonium sulfate was dissolved by inversion, the solution centrifuged (6 000 x g, 10 min, 20°C) and the protein precipitate was harvested from the layer formed between the t-butanol and aqueous layer.

The amount of t-butanol added:

$$V_{t-but} = 0.3/0.7 \times V_s \quad \text{.....} \quad (1)$$

Where V_{t-but} = Volume of t-butanol

V_s = Volume of the sample

The amount of ammonium sulphate added:

$$m(NH_4)_2SO_4 = P [V_s + V_{t-but}] \quad \text{.....} \quad (2)$$

Where $m(NH_4)_2SO_4$ = mass of ammonium sulphate to be added.

P = the decimal percentage required.

V_s = Volume of the sample solution.

For the subsequent percentage increase of $(NH_4)_2SO_4$, addition was calculated as follows

$$m(NH_4)_2SO_4 \text{ added} = P [V_s + (0.3/0.7 \times V_s)] - \sum_{mass} \quad \text{.....} \quad (3)$$

Where \sum_{mass} = the summation of all previously added $(NH_4)_2SO_4$.

The procedure was repeated and the subsequent percentage increase of ammonium sulphate was calculated as in Equation 3. The protein precipitate was removed by filtration in Whatman No. 1 filter paper and was immediately dissolved in cold MMP-9 buffer [50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, 2 mM PMSF, pH 7.5] before it was quickly frozen and stored at $-20^\circ C$.

2.5 Fractionation of IgY from chicken egg yolk

Since some of the antibodies used such as anti-TIMP-1 and anti-MMP-9 were raised in chickens, fractionation of preimmune egg yolk antibodies (IgY) from uninoculated chickens was prepared for use as pre-immune control IgY preparations for immunolabelling experiments.

2.5.1 Reagents

Sodium phosphate buffer (100 mM sodium phosphate, 0.02% (m/v) NaN_3 , pH 7.6). $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml of purified water, titrated to pH 7.6 using NaOH, and made up to 1 liter.

2.5.2 Procedure

Egg yolks were separated from the egg white and carefully washed under running water to remove all traces of albumin. The yolk sac was punctured and the yolk volume determined in a measuring cylinder. Two volumes of 100 mM Na-phosphate buffer, pH 7.6, were added and mixed in thoroughly. Solid PEG (M_r 6 000) was added to 3.5% (m/v) and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420 x g, 30 min, RT) and the supernatant fluid was filtered through non-absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% [i.e. 8.5% (m/v) was added], the solution was mixed thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the pellet was dissolved in 100 mM Na-phosphate buffer, pH 7.6, in a volume equal to the volume obtained after filtration. PEG [12% (m/v)] was again added and the solution was stirred thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant fluid was discarded and the final antibody pellet was dissolved in 1/6 of the original egg yolk volume, using 100 mM Na-phosphate buffer, pH 7.6, and stored at 4°C.

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separation is the most widely used method to determine the approximate number of proteins in a crude extract and identity of proteins in a mixed solution. This method uses the rate of migration of charged molecules in an electric field from one pole to the other, to aid in the separation of proteins. Macromolecules in an electric field will

accelerate until the magnitude of frictional force is equal to their accelerating force due to charge. The resulting steady state mobility of free macromolecules is, therefore, proportional to the net charge and inversely proportional to the frictional coefficient (Goldberg *et al.*, 1984). The rate of migration would then be rapid for those macromolecules which have a high charge density.

Migration, however, takes place in gels formed by polymerization of acrylamide and bis-acrylamide co-monomers which form a meshwork of pores that have sieving effects on proteins. Large proteins, therefore, would move slowly while small molecules would move more rapidly. Separation of proteins according to molecular weight and charge density, therefore, would create complications during interpretation of results.

A modification was, therefore, made to the sample preparation method to give proteins the same charge, so that separation is based only on their sizes (M_r). This modification simplified determination of molecular weights as only one parameter (size), would now be the basis of separation. Before loading of protein samples into gels, they were, therefore, treated with an ionic detergent sodium dodecyl sulphate (SDS). The SDS found in this treatment buffer subsequently binds to all regions of the protein by disruption of most non-covalent inter-molecular and intra-molecular protein interactions and produces a long negatively charged polypeptide chain (Switzer and Garrity, 1999). Excess amount of soluble thiol (2-mercaptoethanol or dithiotreitol) is also added to the protein samples and all disulfide bonds are reduced. This helps the SDS to gain access in binding to all reduced polypeptides.

Under these conditions the same amount of SDS (1.4 g SDS/g polypeptide) binds to all reduced polypeptides. This results in total unfolding (denaturation) of the proteins in the sample and, yielding unfolded, highly anionic polypeptide chains. This causes the polypeptides to form rods of negative charges with equal charge densities or charge per unit length (charge to mass ratio). Subsequent migration, therefore, depends only on one parameter, that is the molecular weight. Such a process as electrophoresis may be carried out using two different gel/buffer systems, the Laemmli and Tris-tricine systems.

2.6.1 Laemmli system

Most of the higher molecular weight proteins and samples for zymography and reverse zymography in this study were separated using the Laemmli system (Laemmli, 1970) using a modification of the original Laemmli system described in Ornstein and Davis (1964). This is known as a discontinuous SDS system and is now the most widely used electrophoretic system. In this method proteins are concentrated in the stacking gel phase at one pH and separated in the running gel phase at another pH. The stacking gel has a non-restrictive large pore size gel, and is layered on top of a sieving, smaller pore size separating gel. Concentration of proteins in the stacking gel before entering into the running gel, allows the application of diluted protein samples. When electrophoresis of the sample is initiated in the stacking gel at pH 6.8, glycine in the tank buffer carries no negative charge, however, at pH 8.3 of the running gel buffer it has an average charge of about -0.1 per molecule. In contrast, the SDS coated protein in the system carries a high negative charge that is essentially independent of the pH of the system (Switzer and Garrity, 1999). Chloride ions contained in the stacking gel, therefore, migrate fastest than the proteins and glycine due to their small size and full negative charge, followed by the proteins, which slowed by their frictional force, and lastly by the glycine (Switzer and Garrity, 1999).

Upon entering the running gel at higher pH, however, the frictional force caused due to decreased pore size of the gel slows the migration of proteins, therefore, the chloride ions pass the proteins and move fastest. Chloride ions are followed by the glycine and leave proteins behind to be separated according to their molecular weight (Switzer and Garrity, 1999).

This method has been used for many years using the formulation of acrylamide/bis-acrylamide monomer by Hjerten, (1962) and the method of Laemmli, (1970). Electrophoretic separation of proteins is accompanied by molecular weight markers of known molecular weight. Upon subsequent staining of the gel the separated proteins

appear as blue bands. A plot of relative mobility of each of the standard molecular weight marker proteins as a logarithm of its molecular weight is plotted (Weber and Osborn, 1969) and molecular weight determined by the linear regression using Microsoft Office Excel software.

2.6.1.1 Laemmli system reagents

Acrylamide/bisacrylamide monomer solution [30 % (m/v) T, 2.6% (m/v) C]. Acrylamide (58.44 g) and *N, N'*-methylenebisacrylamide (1.56 g) were dissolved and made up to 200 ml with distilled water and stored in an amber coloured bottle at 4°C. The solution was filtered through Whatman No.1 filter paper before use.

4 x Running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (36.3 g) was dissolved in approximately 170 ml of distilled water, adjusted to pH 8.8 with HCl and made up to 200 ml. The solution was filtered through Whatman No.1 filter paper before use.

4 x Stacking gel buffer [500 mM Tris-HCl, pH 6.8]. Tris (6.05 g) was dissolved in 80 ml of distilled water, adjusted with HCl to pH 6.8 and made up to 100 ml. The solution was filtered through Whatman No.1 filter paper before use and made freshly every week.

10% (m/v) SDS. SDS (10 g) was dissolved and made up to 100 ml with distilled water.

Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was dissolved in 1 ml of distilled water just before use.

Tank buffer [250 mM Tris-HCl, 192 mM glycine, 0.1 % (m/v) SDS, pH 8.3]. Tris (151.2 g) and glycine (72 g) were dissolved and made up to 5 litres with distilled water. Prior to use, 2.5 ml of SDS stock was added to 250 ml for use in the Mighty Small II apparatus.

Reducing treatment buffer [125 mM, Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Stacking gel buffer (2.5 ml), 10% SDS (4 ml), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with distilled water.

Non-reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, pH 6.8]. Stacking gel buffer (2.5 ml), 10% (m/v) SDS (4 ml) and glycerol (2 ml) were made up to 10 ml with distilled water.

Stain stock solution [1% (m/v) Coomassie brilliant blue R-250]. Coomassie brilliant blue R-250 (1 g) was dissolved in 100 ml of distilled water by magnetic stirring for 1 h at room temperature. The solution was filtered through Whatman number 1 filter paper.

Staining solution [0.125% (m/v) Coomassie brilliant blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml with distilled water.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with distilled water.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml), and made up to 1 litre with distilled water.

Table 2.1 Preparation of Laemmli running and stacking gels of different acrylamide concentrations.

| Reagent | Volume (ml) | | | | | | |
|--|-----------------|--------|--------|--------|--------|------------------|-------|
| | Running gel (%) | | | | | Stacking gel (%) | |
| | 15.0 | 12.5 | 10.0 | 7.5 | 5.0 | 4.0 | 3.0 |
| Acrylamide/ bisacrylamide monomer solution (ml) | 7.5 | 6.25 | 4.99 | 3.75 | 2.5 | 0.67 | 0.5 |
| Running gel buffer (ml) | 3.75 | 3.75 | 3.75 | 3.75 | 3.75 | 0 | 0 |
| Stacking gel buffer (ml) | 0 | 0 | 0 | 0 | 0 | 1.25 | 1.25 |
| 10% SDS (ml) | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.05 | 0.05 |
| Initiator (ml) | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 | 0.025 | 0.025 |
| Dist. water (ml) | 3.5 | 4.75 | 5.98 | 7.25 | 8.5 | 3.0 | 3.17 |
| TEMED (ml) | 0.0075 | 0.0075 | 0.0075 | 0.0075 | 0.0075 | 0.005 | 0.005 |

2.6.1.2 Procedure

The SDS-PAGE electrophoresis unit (Hoefer Mighty Small II[®]) was assembled in accordance to the manufacturer's instructions. All the accessories were cleaned either with 1M KOH in ethanol or 99% ethanol. Gel buffers and monomer solutions were added in to a clean glass beakers and polymerization was initiated by the addition of

initiator and TEMED as per Table 1. The solution was mixed by aspiration into 10 ml syringe and loaded between a glass and aluminium plate to within 3 mms of the top of the glass plate. Bubbles were dislodged and the gel overlaid with purified water to allow uniform polymerisation. The gel was allowed to set (1 h, RT). After polymerization the excess water was removed and stacking gel was cast according to Table 1 and either 10 or 15 well combs were inserted (30 min) until the gel polymerized to form wells into which samples were loaded.

After the gel was prepared it was transferred in to Hoefer Mighty Small II[®] gel electrophoresis unit. The instrument was connected to a power pack at maximum voltage and 18 mA per gel. The Laemmli tank buffer was added between the glass and aluminium plates and into the tank. Samples were mixed with an equal amount of reducing treatment buffer and boiled (1.5 min), or half their volume of non-reducing treatment buffer and left unboiled. The samples were loaded in the wells and samples were subjected to electrophoretic separation for a period depending on the monomer concentration, while the equipment cooled by continuous circulation of ice-cold water. After the completion of the running time gels were stained with staining solution or processed for zymography, or reverse zymography or blotted into nitrocellulose filters.

2.6.2 Tricine system

Proteins with molecular weight below 14 kDa are not well resolved by standard SDS-PAGE using a Laemmli discontinuous buffer system. The Tricine system developed by Schagger and Von Jagow, (1987) uses Tricine as the trailing ion. This system allows a resolution of small proteins especially in the range of 1 and 100 kDa, and uses acrylamide concentration as low as 10% thus low cross linking. The stacking of small peptides in the presence of SDS is difficult because small peptides form complexes with proteins and detergents of the same size and charge as the SDS. Separation of proteins, therefore, becomes a problem. Glycine (pK 9.6) of the Laemmli system and tricine (pK 8.15) behaves quite differently in the stacking of proteins (Schagger and Von Jagow,

1987). Glycine leads to stacking even of very large proteins, because it migrates very slowly in the acidic stacking gel, leaving proteins below 20 kDa unseparated from the bulk of SDS. In a tricine system the pH of stacking and running gels is the same (Schägger and Von Jagow, 1987). At the usual pH values between 6.8 and 8.8, Tricine migrates much faster than glycine in stacking gel, despite its higher molecular mass, because much more Tricine is in the migrating, anionic form. As a consequence, the stacking limit is shifted to the low-molecular-mass range of 30 kDa and this facilitates the separation of small peptides from SDS micelles (Schägger and Von Jagow, 1987). The stacking of the proteins of interest can be improved by tailoring the stacking band as narrow as possible. This is best for the resolution of the small proteins as the proteins above 30 kDa are already separated from the stack of the smaller proteins before reaching the separating gel. The stacking and destacking of proteins therefore may be achieved at lower acrylamide concentrations than those in the glycine systems (Schägger and Von Jagow, 1987).

2.6.2.1 Tricine system reagents

Reducing and non-reducing buffers and stains were prepared as described in Section 2.6.1.1.

Gel buffer: [2.4 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) was dissolved in approximately 180 ml of distilled water, SDS [6 ml of 10% (m/v) solution] added, the pH was adjusted to pH 8.45 with HCl, and made up to 250 ml. The buffer was filtered with Whatman No.1 filter paper and stored at 4°C.

Acrylamide/bisacrylamide monomer stock solution [51 % (m/v) T, 5.88 % (m/v) C]. Acrylamide (48 g) and bis-acrylamide (3 g) were dissolved and made up to 100 ml with distilled water. The buffer was filtered with Whatman No.1 filter paper and stored in an amber coloured bottle at 4°C.

Initiator [10% (m/v) ammonium per-sulphate]. Ammonium persulfate (0.1 g) was made up to 1 ml with distilled water prior to use.

Anode buffer [200 mM Tris-HCl, pH 8.9]. Tris (24.2 g) was dissolved in approximately 950 ml of distilled water, adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [100 mM Tris-HCl, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.25]. Tris (12.1 g) and tricine (17.9 g) were dissolved in approximately (950 ml) of distilled water. SDS [10 ml of a 10% (w/v) stock solution] was added, the pH adjusted to 8.25 with HCl, and made up to 1 litre. The buffer was stored at 4°C.

Table 2.2 Preparation of Tricine running and stacking gels

| Type of gel | Solution | | | | | |
|-------------|----------|-----------------|--------------------|-------------------|---------------|--------------------------------|
| | % Gel | Monomer (ml) | Gel buffer (ml) | Initiator (ml) | TEMED (ml) | Dist. H ₂ O (ml) |
| Stacking | 10.0% | 3.6 | 6.0 | 0.06 | 0.006 | 8.4 |
| | 7.5% | 2.66 | 6.0 | 0.06 | 0.006 | 9.34 |
| | 5% | 1.77 | 6.0 | 0.06 | 0.006 | 10.23 |
| Running | 4% | 0.5 | 1.5 | 0.05 | 0.012 | 4.0 |
| | 3% | 0.375 | 1.5 | 0.05 | 0.012 | 4.12 |

2.6.2.2 Procedure

The same procedure as described in Section 2.6.1.2 was followed (using reagents as described in Table 2.2). The electrophoresis apparatus was subsequently run at 80 volts and maximum current until the bands pass through stacking gel before the voltage was increased to 100 volts.

2.7 Western blotting

Electrophoretic separation of proteins does not allow definite identification of a protein from a mixture as there may be proteins of similar molecular weight. Unless separation is followed by Western blotting and proteins are recognised using specific antibodies an enzyme conjugated specific (secondary) antibody and a substrate which gives a coloured reaction. Western blotting has the advantage over ELISA as it gives information about the molecular weight or the purity of antigen for example if the protein is degraded.

Western blotting was first described by Towbin *et al.* (1979). The procedure assisted detection of proteins down to 100 ng. Separation and blotting of polypeptides depends on the pore size of the polyacrylamide gel, the molecular weight, the net charge of the peptide and the electric current applied (Dunbar, 1994). When proteins and peptides are separated by SDS-PAGE, they are completely surrounded by SDS and are negatively charged. SDS decreases binding of protein and peptides to nitrocellulose. Thus if there is too much SDS bound to the protein or peptides, these migrate quickly from the gel but may not bind well to the nitrocellulose (Dunbar, 1994). Methanol in the blotting buffer, however, removes SDS and exposes the hydrophobic groups of the polypeptides and proteins and assists binding to the nitrocellulose (Towbin *et al.*, 1979). On the other hand methanol decreases the pore sizes of the gel and may precipitate proteins.

A modification of western blotting by Gershoni and Palade, (1982), exploits the charge of SDS and omits methanol from the blotting buffer. This is called Zeta binding and involves transfer of negatively charged proteins to the positive nylon-66-based membrane. This method facilitates better transfer of polypeptides and subsequent overlay of ligands. However, its high affinity to proteins creates high background and makes quenching difficult. To overcome this problem the membrane must be blocked with high concentration of a blocking protein for extended period of time, thus the procedure by Towbin *et al.* (1979) is preferred.

2.7.1 Detection of blotted proteins

Detection of blotted proteins using Western blotting usually employs either enzymatic detection systems or autoradiography. Detection by autoradiography gives sharp bands but is not convenient due to the danger of exposure to radioactivity (Dunbar, 1994). There are many detection systems using different enzymes and substrates. One of the most commonly used systems is the alkaline phosphatase enzyme conjugated to the reporter antibody and the use of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates at pH 9.5. When BCIP a substrate for alkaline phosphatase is catalysed it liberates phosphate group and a 5-bromo-4-chloro-3-indoxyl group will be formed. The 5-bromo-4-chloro-3-indoxyl group is spontaneously oxidized in the presence of molecular oxygen to give an insoluble indigo dye. To enhance detection NBT and BCIP are mixed together. Thus, instead of an oxidizing agent, NBT is used as electron acceptor. The 5-bromo-4-chloro-3-indoxyl group which is a product of the reaction of BCIP and alkaline phosphatase is oxidized by NBT to give an insoluble indigo dye. NBT is reduced in the reaction and give a blue colour.

The secondary antibody can also be conjugated to horseradish peroxidase and can be detected by addition of 4-chloro-1-naphthol, diaminobenzidine or tetra-methylbenzidine (TMB) but generally less sharper and fainter bands are produced (Dunbar, 1994). The various reagents used with different detection systems are given below.

2.7.2 Reagents

Towbin blotting buffer [25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, 0.01% (w/v) SDS, pH 8.3]. Tris (3.02 g) and glycine (14.4 g) was dissolved in 777 ml of purified water and SDS [1 ml of the 10% (w/v)] was added. After mixing the solution, \pm 200 ml of methanol was added to make the volume up to 1 litre. Usually no pH adjustment is required.

Ponceau S [0.1% (w/v) in 1% (v/v) acetic acid]. Ponceau S (0.1 g) and acetic acid (1 ml) were added and made up to 100 ml with distilled water.

Tris buffer saline (TBS) [20 mM Tris, 200 mM NaCl, pH 7.4]. Tris (2.42 g) and NaCl (11.69 g) was dissolved in approximately 950 ml of distilled water and adjusted to pH 7.4. The solution was made up to 1 litre with distilled water.

Blocking solution [5% (w/v) low fat milk powder/TBS]. Low fat milk powder (5 g) was added into 100 ml of TBS prepared as in the above. Detergents [(0.3% (v/v) Brij-35, Tween-20 or Triton X-100] were added where necessary.

Alkaline phosphatase substrate buffer [50 mM Tris, 5 mM MgCl_2 , pH 9.5]. Tris (0.605 g) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g) were dissolved in 90 ml of distilled water and adjusted to pH 9.5. The solution was made up to 100 ml with distilled water.

Endogenous alkaline phosphatase quenching solution [2 mM levamisole]. Levamisole (0.05 g) was added to blocking solution (100 ml) just before use.

Substrate for alkaline phosphatase. BCIP (4-toluidine salt) (1.5 mg dissolved in 30 μl DMF) and NBT (3.0 mg dissolved in 30 μl of 70% (v/v) DMF) were mixed separately and added to substrate buffer (10 ml) just before use.

Endogenous peroxide stop solution. NaN_3 (0.1g) was dissolved in blocking solution (100 ml) just before use.

Substrate for horseradish peroxidase [0.06% (w/v) 4-chloro-1-naphthol, 0.01% (v/v) H_2O_2 / TBS]. 4-chloro-1-naphthol substrate (2 ml of a stock solution of 0.3% (v/v) solution in methanol), TBS (8 ml) and H_2O_2 (3.33 μl of 30% (v/v) solution) were mixed together just before use.

2.7.3 Procedure

After completion of electrophoretic separation of samples, the gels were transferred to blotting cassette lined with 4 sheets of filter paper on top of a Scotchbrite scouring pad. The gel was overlaid with a nitrocellulose membrane and further 4-sheets of filter paper. The cassettes were inserted into the blotting apparatus with the nitrocellulose facing the positive pole, blotting buffer added, the apparatus attached to the power pack and the current run at 200 mA at low temperature (4°C) for 16 h. After transfer was complete, the blotted membranes were retrieved from the apparatus and stained with Ponceau S. The molecular weights of standard proteins were marked before the stain was removed by addition of diluted sodium hydroxide, the nitrocellulose rinsed, dried for 1.5 h and processed for immunoblotting.

The non-specific binding sites of proteins on membranes were blocked with low fat milk powder/TBS. Levamisole or sodium azide was added where necessary to quench the endogenous alkaline phosphatase or peroxidases, respectively. After blocking, the nitrocellulose was washed 3 x 5 min with TBS and primary antibody (diluted in 0.5% (m/v) BSA/TBS) was added. After incubation (1 h) the primary antibody was discarded and membrane was washed (3 x 5 min). Secondary antibody was subsequently added and the membrane incubated for 1 h. After washing (3 x 5 min) with TBS substrate (for alkaline phosphatase or horseradish peroxidase) was added and incubated in the dark until colour development was evident. The nitrocellulose was dried and stored between filter papers for photographic capture.

2.8 Enhanced chemiluminescence

A large number of chemical reactions give rise to chemiluminescence or the emission of light photons. Unlike other western blotting secondary antibody conjugates, chemiluminescent probes, used in enhanced chemiluminescence (ECL) detection of antigen-antibody interactions, the light-producing step is a purely chemical step. This

step does not involve the precipitation of an enzyme substrate complex and light emission is captured on X-ray films (Prichard and Cormier, 1968). In the basic (non-enhanced) chemiluminescence reaction, HRP is used to oxidize a peracid salt, leading to the formation of a raised oxidation state of the haem group at the centre of HRP itself. The raised state returns to the initial (ground) state in a two-step process. At each stage a luminol radical is formed, and as this radical decays, light is emitted (Dunbar, 1994). However, in basic unenhanced reactions the light emission ceases in a relatively short period of time. Luminescence systems produced using HRP, in which blue light is generated by oxidation of luminol, often gives excellent signal to noise ratio. As compared to conventional radioactive immunoprecipitation HRP systems are extremely rapid, sensitive, relatively inexpensive, simple, quick and non-hazardous (Dunbar, 1994; Nesbitt and Horton, 1992).

Chemiluminescence reactions can be enhanced by the addition of an enhancer molecule which reacts with the haem group of HRP in place of the luminol leading to the formation of enhancer radicals. These radicals react to produce luminol radicals and light is emitted as before (Dunbar, 1994). The reaction is faster than that produced by luminol alone and is sustained for a significant period of time (Dunbar, 1994). The presence of enhancer may lead to an increase in emitted light of more than 100 fold over the unenhanced reaction (Dunbar, 1994).

ECL generally gives low background but needs to be carried out in an X-ray facility (Dunbar, 1994). The hard copy results obtained on autographic films are directly printed and are generally darker than chromogenic blots. The system also allows the same blot to be reexposed many times (Dunbar, 1994).

2.8.1 Reagents

Tris buffer saline (TBS) [20 mM Tris, 200 mM NaCl, pH 7.4]. Tris (2.42 g) and NaCl (11.7 g) was dissolved in approximately 950 ml of distilled water and adjusted to pH 7.4. The solution was made up to 1 litre with distilled water.

Blocking solution [5% (w/v) low fat milk powder/TBS]. Low fat milk powder (5 g) was added into 100 ml of TBS prepared as above. Detergents (0.3% (v/v) Brij-35, 0.3% (v/v) Tween-20 or 0.3% (v/v) Triton X-100) were added where necessary.

Endogenous peroxidase quenching solution. NaN_3 (0.1 g) was dissolved to blocking solution (100 ml) just before use.

Substrate for horseradish peroxidase [0.06% (w/v) 4-chloro-1-naphthol, 0.01% (v/v) H_2O_2 / TBS]. Stock solution (2 ml of a 0.3% (v/v) solution in methanol), TBS (8 ml) and H_2O_2 (3.33 μl of 30% (v/v) solution) was dissolved just before use.

Luminol detection solution [50% (v/v) detection solution 1, 50% (v/v) detection solution 2]. Detection solution 1 (500 μl) and detection solution 2 (500 μl) were mixed in Eppendorf tubes just before use. From commercial solutions supplied by Amersham, Pharmacia Biotech, U.K.

X-ray film development solution (Agfa Corporation, Germany). Solution 1 (10 ml) was mixed with Solution 2 (0.111 ml) and mixed. Purified water (40.4 ml) added just prior to use.

X-ray film fixing solution (Agfa Corporation, Germany). Fixer (10 ml) was mixed with distilled water (800 ml) and mixed. Hardener (1.25 ml) was added just prior to use.

2.8.2 Procedure

Blotting and immunolabelling was performed essentially as described previously in Sections 2.7. The difference was in ECL the HybondTM ECL nitrocellulose paper is used for best result. As the detection is catalysed by HRP, only HRP-linked secondary antibodies are used. After the last wash the nitrocellulose filters were taken to the autoradiograph room and all subsequent steps performed in the dark using photographic safe lights. The membrane was placed on a piece of Saran Wrap sufficiently large enough to entirely cover the membrane and was overlaid with sufficient detection solution to reach the edges of the membrane by surface tension. The membrane was incubated exactly for (1 min, RT). Excess detection solution was removed and the membrane was carefully covered with Saran Wrap, the air bubbles smoothed out and placed in the film cassette. A piece of autoradiography film was placed on top of the nitrocellulose membrane enclosed in the Saran Wrap and the ECL cassette quickly closed. The film was exposed for 15 s or sometimes more, depending on the rate of band development, quickly transferred to the photographic film developing solution (1 min) and washed by agitation. The film was subsequently transferred to the fixing solution (10 min), was washed in purified water and dried between two pieces of filter papers returned to the lightbox and photographed.

2.9 Zymography and reverse zymography

Zymography and reverse zymography are techniques used to analyse the activities of proteolytic enzymes and their inhibitors in a protein mixture. Zymography involves the electrophoretic separation of enzymes under denaturing (SDS) but non-reducing conditions (no reducing agent) in polyacrylamide gels containing a substrate. Since renaturation of proteins can be reversible or irreversible, reversibly denatured proteins separated in SDS-gels may be renatured by the addition of non-anionic detergent, such as Triton X-100. Non-ionic detergents have lower critical micelle concentration (CMC) values than SDS and quickly form micelles that surround and remove SDS molecules

renaturing most proteins (Hawkes *et al.*, 2001). The gel may subsequently be incubated in a buffer which maintains the activity of the particular proteinases under study, stained with e.g. Coomassie brilliant blue, and proteolytic activity may be detected as clear bands (digested) against a blue background of stained undegraded substrate in the gel. This method is very sensitive and can detect very low concentration of enzymes in mixed protein solutions (Hawkes *et al.*, 2001). However, if the substrate has a very high molecular weight or copolymerized in high concentration with the gel it may alter the migration of some enzymes by electrophoresis (Gabriel and Gersten, 1992).

Reverse zymography is a modification of the zymographic procedure and may be used to detect inhibitors such as TIMPs. Demonstration of the presence of an inhibitor is accomplished by electrophoretic separation of TIMP-containing samples on zymographic gel containing gelatin and the gelatin degrading enzyme e.g. MMP-9 after the removal of SDS using Triton X-100 washing step (Hawkes *et al.*, 2001), incubating the gel under conditions facilitating the gelatinolytic activity of the MMP. The proteolytic activity results in the degradation of the gelatin except in the regions protected by TIMP activity. Subsequent Coomassie brilliant blue staining allows the visualization of TIMP bands as blue (undigested gelatin bands) against a clear background created by gelatin degradation. Mixing of the enzyme and substrate together in the gel reduces the background as the enzyme gets access to the deeper parts of the gel. Overloading and running of large amount of proteins may cause detectable staining of non-inhibitory proteins and hence false “inhibition” results.

For detection of gelatinase B (MMP-9), gelatin, unlike some other substrates, does not affect the mobility of gelatinase enzymes (Makowski and Ramsby, 1996). A particular advantage of zymography of MMPs is that as proenzymes are activated by SDS and the molecular weights of both the proenzyme and active forms of MMPs are evident, information which cannot be achieved by fluorometric technique (Hawkes *et al.*, 2001) or ELISA. Moreover, a developing buffer lacking calcium, which is essential for activity or addition of metal chelators such as EDTA, which bind with the essential metal ion (Makowski and Ramsby, 1996) may be used as a negative control to confirm that the

gelatinolytic enzymes detected are MMPs. The pH can also be manipulated to prevent proteolytic activity of unwanted enzymes. For example, to prevent the activity of other granule proteins such as elastase, the pH can be adjusted to pH 8.8, a suitable pH for MMP activity.

To run control experiment for reverse zymography is not possible. However, reverse zymograms may be controlled using other techniques such as by subsequently blotting the reverse zymogram after development or blotting a duplicate gel into nitrocellulose paper and using specific antibodies to detect the inhibitor. Reverse zymography must be optimized to give good results as the gelatinase concentration in the gel is usually inversely proportional to sensitivity. While, increasing the gelatin concentration increases the sensitivity and background (Oliver *et al.*, 1997).

2.9.1 Reagents

Renaturation solution [2.5% (v/v) Triton X-100]. Triton X-100 (6.25 ml) was dissolved in distilled water in a final volume of 250 ml.

Gelatinase zymography development buffer [50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl_2 , 0.02% (w/v) NaN_3 , 0.02% (v/v) Brij-35, 2 mM PMSF, pH 8.8]. Tris (0.605 g), NaCl (1.17 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.074 g), NaN_3 (0.02 g), PMSF (1 ml of 200 mM solution), and Brij 35 [67 μl of a 30% (v/v) solution] were dissolved in ddH₂O (90 ml). The pH was titrated to pH 8.8 with HCl and the volume made up to 100 ml and was warmed to 37°C just before use.

Fixing/destaining solution: [methanol:acetic acid: water (4.5: 1:4.5 (v/v))]. Methanol (45 ml), acetic acid (10 ml) and water (45 ml) were mixed and stored at room temperature.

Staining solution: [0.1% Coomassie brilliant blue R-250 (w/v) in fixing/staining solution]. Coomassie brilliant blue R-250 (0.1 g) was dissolved in 100 ml of fixing/staining solution.

2.9.2 Procedure

For zymography protein samples were mixed with non-reducing treatment buffer and were separated on a 10% Laemmli gel in which gelatin (0.01 g/ml dissolved in running gel buffer) was co-polymerized. After separation is completed, the proteins were renatured by washing (2 x 30 min) and transferred into a container filled with development buffer and developed (16 h, 37°C). After development was complete, the buffer was discarded and the gel was subsequently stained in 0.1% Coomassie brilliant blue solution, and destained in destaining solution that contain (methanol: acetic acid: water [4.5:1:4.5, (v/v)]). The results were visualized in a light box. Enzyme activity appeared as clear bands in blue background. As a control EDTA(Na₂) (5 mM) was added to the development buffer.

For reverse zymography proteins were mixed with half of their volumes of non-reducing treatment buffer and separated on a 15% Laemmli gel prepared by co-polymerization of gelatin (0.01 g/ml) and MMP-9 (32 µg/ml) in the running gel. After separation, the proteins were renatured by washing in renaturation buffer (2 x 30 min) were transferred into a container filled with development buffer and developed (16 h, 37°C). The buffer was discarded and the gel stained in coomassie brilliant blue solution and destained in destaining solution. The results were visualized in a light box and captured. TIMP-1 inhibitory activity was seen as dark blue bands of MMP-9 activity in a clear background of MMP-9 digested gelatin.

2.10 Density gradient separation of PMNs.

Many types of cells can be separated using isopycnic or density gradient separation (Table 2.3). Stoke's law states that the velocity of movement of a cell in a centrifugal field is proportional to the difference between the specific gravity of the cell and that of the medium (Roos and De Boer, 1986). According to this law, cells with specific gravity higher than that of the medium will be sedimented and those with a specific gravity lower than that of the medium will float to the surface (Roos and De Boer, 1986). Using this method copurification of different cells at the same time is possible.

Table 2.3 Specific gravity and relative volume of human blood cells (Ross and De Boer, 1986).

| Cells | Specific gravity (g/cm ³) | Volume (Channel number) |
|--------------|---------------------------------------|-------------------------|
| Platelets | 1.054 – 1.062 | <3 |
| Monocytes | 1.055 – 1.065 | 46-54 |
| Lymphocytes | 1.060 – 1.072 | 21-27 |
| Basophils | 1.065 – 1.075 | 30-33 |
| PMNs | 1.080 – 1.084 | 40-46 |
| Eosinophils | 1.082 – 1.090 | 45-52 |
| Erythrocytes | 1.090 – 1.110 | 6-9 |

(290 mOsm and pH 7.4 at 25°C)

Isopycnic separation of blood cells can initially be performed by centrifugal separation into a light and a heavy fraction. The light fraction usually contains the mononuclear fraction (monocytes and lymphocytes, basophils and a few early precursor cells as well as containing platelets), and the heavy fraction contains erythrocytes and granulocytes (PMNs and eosinophils) (Roos and De Boer, 1986). The separation of PMNs from blood can be achieved using a medium density of 1.077 g/cm³ (Roos and De Boer, 1986; Bøyum, 1968), generated using a mixture of Ficoll (a sucrose polymer) and Isopaque (an

iodinated x-ray contrast medium). Since Ficoll/Hypaque (Isopaque) is, however, reportedly found to alter the metabolism of leukocytes (Dooley *et al.*, 1982), it is not the method of choice. Another method using Percoll (a colloidal suspension of polyvinyl pyrrolidone coated silica particles) has been developed, therefore.

Percoll is the density gradient medium choice. It has a zero tonicity and low viscosity, and can be easily made iso-osmotic and brought to a physiologic pH over a large density range using various buffers) by addition of media and solute (Borregard *et al.*, 1993). This property gives rise to a linear relation between concentration and density of Percoll. It permits cell separation at relatively low centrifugation speeds in a short time. Moreover, lack of toxicity makes this an ideal density gradient medium (Roos and De Boer, 1986; Pertoft *et al.*, 1978). PMNs isolated using Percoll gradient are also found to maintain their biological activity and microbicidal and chemotactic functions at a level comparable to those of cells isolated by centrifugal elutriation (Dooley *et al.*, 1982). Moreover, it is described that up to 1.2 g/ml of Percoll can be sterilized by autoclaving, thus can be used for experiments which needs sterile gradients (Pertoft *et al.*, 1978).

Aqueous solutions of Percoll have high density, low viscosity and low osmolality. Therefore, during centrifugation gradients are generated automatically by increasing the colloid towards the bottom of the tube containing the medium. The use of Percoll as a gradient material has an advantage because it is not taken up by cells. Other gradients, however, are permeable to cells and make the cells to shrink or swell. This changes their specific gravity and causes a problem during isopycnic separation. To overcome this problem, monocytes and granulocytes have to be pre-incubated for some time in other material before subsequent gradient centrifugation is performed. This makes them to maintain their original specific gravity (Roos and De Boer, 1986).

Other parameters which may create problems during separation are overloading and pH changes (Roos and De Boer, 1986). If the concentration of the cells loaded onto density gradients is too high, a condition called streaming results causing bulk sedimentation of cells (Roos and De Boer, 1986). The concentration of red blood cells (RBCs) which by

far are numerous fractions in blood, may sometimes give rise to this phenomenon. They are heavier than other leukocytes but their inclusion is necessary as they contribute to the creation of the desired density (1.077 g/cm^3). Since they are the heaviest cells, RBCs usually displace leukocytes into the buffy coat upon centrifugation. However, RBCs can be removed using erythrocyte lysis buffer, containing NH_4Cl and KHCO_3 . The NH_3 and CO_2 produced by the abundant carbonic anhydrase enzyme in RBC's upon penetration of these compounds into the cells results in osmotic swelling and lysis of RBC's (Roos and De Boer, 1986). The pH of the gradient can also affect the physical property of the unlysed WBC's subsequently harvested, thus a physiological pH is preferred and a lysis buffer used (Roos and De Boer, 1986).

2.10.1 Reagents

Phosphate buffered saline [PBS: 9.2 mM Na_2HPO_4 , 1.3 mM NaH_2PO_4 , 140 mM NaCl , pH 7.4]. $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (0.164 g), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.02 g) and NaCl (0.818 g) were dissolved in 80 ml of distilled water and made up to 100 ml. No pH adjustment was necessary. The solution was autoclaved (121°C , 20 min) and kept at 4°C .

Percoll dilution buffer [100 mM NaH_2PO_4 , 1.54 M NaCl]. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (1.56 g) and NaCl (9.0 g) were dissolved in distilled water in a final volume of 100 ml. This solution was autoclaved (121°C , 20 min) and stored at 4°C .

Trisodium citrate (130 mM in dd. H_2O). Trisodium citrate. $5\frac{1}{2}\text{H}_2\text{O}$ (9.55 g) was dissolved in distilled water and made up to a final volume of 250 ml. The solution was autoclaved (121°C , 20 min) and stored at 4°C .

BSA [5% (w/v) in PBS]. BSA (5 g) was allowed to dissolve in PBS (80 ml) for 1 h. The solution was then made up to 100 ml, filter sterilized through $0.22 \mu\text{m}$ filters before storing at -20°C .

Stock Percoll suspension. Percoll (18.6 ml) was mixed with Percoll dilution buffer (1.4 ml) just before use.

Percoll density gradient (1.077 g/ml) [(140 mM PBS, 9.2 mM NaCl, 9.2 mM Na₂HPO₄, 1.3 mM NaH₂PO₄, pH 7.4.) 5% (v/v) BSA, 130 mM trisodium citrate, diluted Percoll]. PBS (5.69 ml), BSA [2.5 ml of the 5% (w/v) stock solution], trisodium citrate (2.5 ml of the 130 mM stock solution) and diluted Percoll (14.31 ml) were mixed together and kept on ice. For use within ± 2 h.

Erythrocyte lysis buffer [155 mM NH₄Cl, 10 mM K₂CO₃, 0.1 mM EDTA, pH 7.4]. NH₄Cl (0.829 g), K₂CO₃ (0.138 g) and EDTA (0.003 g) were dissolved in distilled water (80 ml) and cooled on ice. The pH was adjusted to 7.4 and the volume made up to 100 ml. The solution was filter sterilized and stored at 4°C.

PMN resuspension and storage buffer [PBSG: 8 mM Na₂HPO₄, 1 mM KH₂PO₄, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 0.1% (w/v) glucose, pH 7.3]. Na₂HPO₄·2H₂O (0.142 g), KH₂PO₄ (0.0136 g), NaCl (0.818 g), KCl (0.02 g), MgCl₂·6H₂O (0.01 g), CaCl₂·2H₂O (0.0147 g) and glucose (0.1 g) were dissolved in distilled water (80 ml) and made up to 100 ml without pH adjustment. The solution was filter sterilized and stored at 4°C.

Trypan blue stock solution [0.4% (w/v) Trypan blue in 0.81% (w/v) NaCl and 0.06% (w/v) K₂HPO₄]. Trypan blue (0.04 g), NaCl (0.081 g) and K₂HPO₄ (0.006 g) were dissolved in distilled water in a final volume of 10 ml. The solution was filter sterilized and stored at 4°C.

Acid citrate phosphate dextrose anticoagulant was purchased from Sigma Diagnostics, St. Louise, MO, USA and PMN lysis solution was made up as described in Section 3.7.1.

2.10.2 Procedure

Blood (~44 ml) from a healthy individual was collected into a 50 ml tube containing ~6 ml of acid citrate phosphate anticoagulant. The cells were carefully centrifuged (200 g, 20 min, RT) to facilitate the formation of a buffy coat layer and separate the leukocytes from the platelets which remain in the plasma supernatant. Plasma above the buffy coat (5 ml) and blood below the buffy coat (5 ml) were mixed in a tube before they were carefully layered into a 15 ml tubes on top of 5 ml of cold 1.077g/cm³ Percoll gradient media was prepared according to the following formula:

$$X (0.0056) + 0.1 (0.0227) + 0.1 (0.0219) + (0.8 - X) (0.1245) = \text{desired specific gravity (g/cm}^3\text{)} - 1.$$

Where **X** = ml of PBS/ml of final suspension.

0.0056 = specific gravity of PBS – 1.

0.1 = ml of albumin and citrate/ml of final suspension.

0.0227 = specific gravity of albumin (5%) – 1.

0.0219 = specific gravity of trisodium citrate (130 mM) – 1.

0.8 – X = ml of stock Percoll suspension/ml of final suspension.

0.1245 = specific gravity of stock Percoll suspension – 1.

[i.e. = 0.2276 ml of PBS/ml of final volume and gives a solution of 290 mOsm at 25°C, pH 7.45] (Roos and De Boer, 1986).

After sample application, the gradient was centrifuged (600 x g, 30 min, 20°C) and the monocyte-containing plasma layer was removed by aspiration leaving small amount of Percoll above the PMN-erythrocyte layer. This PMN-erythrocyte layer was subsequently removed into another sterile container, suspended in a 3-fold volume of ice-cold erythrocyte lysis buffer and mixed every few min until the solution becomes clear indicating the hemolysis of the RBC's (~10 min). The tubes were centrifuged (2 min, 600 g, 4°C) and the supernatants removed. The PMN pellet was subsequently mixed

with storage buffer and immediately used or diluted in PMN lysis buffer and immediately snap frozen in liquid nitrogen before they are stored at -20°C .

To test the viability of PMNs Trypan blue solution 0.1 % was added (10 mg/ml) to cells in PBSG (20 μls) and dye exclusion was checked for with the aid of a light microscope (10 min). Concurrently the cells were also counted in an improved Neubauer haemocytometer.

CHAPTER THREE

MATRIX METALLOPROTEINASE-9: SEMI-FRACTIONATION AND CHARACTERIZATION OF ANTIBODIES

3.1 Introduction

MMPs are involved in normal tissue remodeling and are associated with many pathological conditions (Massova *et al.*, 1998). Knowledge of the structure of MMPs assists in understanding their interactions with inhibitors, the TIMPs, the domain-domain interactions that occur during activation, as well as interactions with the cell membrane and the matrix. Some background on the MMPs was explored and methods for detecting MMP-9 were developed and optimized before studies on the effect of release of MMP-9 and TIMP-1 could be undertaken. Release of TIMP-1 may also be detected by a method known as reverse zymography in which a crude preparation of MMP (such as MMP-9) is added to the running gel and the presence of inhibitor is indicated by bands of inhibition of digestion of gelatin (blue) against a clear background of digested gelatin in a gelatin gel system. For this and all optimization and method development purposes, a source of MMP was required. In this section of the dissertation such methods and sources of MMPs were explored.

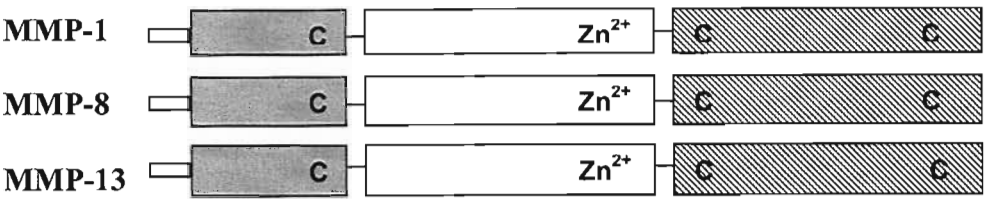
3.2 MMP structure and function

The matrix metalloproteinases (MMPs) are zinc- and calcium-dependent endopeptidases which degrade most extracellular matrix components (ECM) (Massova *et al.*, 1998; Nagase, 1997). They mainly consist of a pro-peptide, catalytic- and C-terminal region (Figure 3.1) and removal of the pro-peptide results in the activation of the zymogen. The catalytic domain is known to have two zinc ions, one catalytic and the other structural. Together with a calcium ion, the structural zinc ion is found

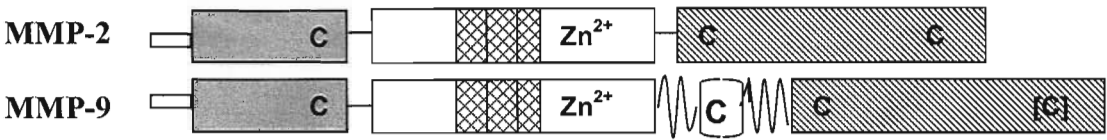
approximately 12 Å away from the catalytic zinc and is attached with three-conserved histidine residues (Massova *et al.*, 1998; Tryggrason *et al.*, 1992).

The C-terminus of the enzyme consists of a conserved hemopexin-like domain which facilitates interaction of MMPs with their tissue inhibitors of matrix metalloproteinases (TIMPs) (Massova *et al.*, 1998; Tryggrason *et al.*, 1992). Besides this domain, which is found in all except MMP-7 (PUMP-1), which lacks the C-terminal domain, MMP-2 and MMP-9 have an additional three repeat sequences of a fibronectin type II-like gelatin-binding domain which enable them to bind and cleave gelatin (Figure 3.1) (Tryggrason *et al.*, 1992; Massova *et al.*, 1998). Membrane type MMPs (MT-MMPs) have a hydrophobic domain of approximately 25 residues, representing a putative trans-membrane domain at the carboxyl terminus, and a recognition motif (RXKR) for furin-like convertases at the end of the pro-peptide domain. This is a substrate of furin which activates the MT-MMP before secretion (Massova *et al.*, 1998) (Figure 3.2). The role of the 54-amino acid long proline-rich collagen-like sequence in MMP-9 is, however, unknown and is not found in any of the secreted ECM metalloproteinase family members (Wilhelm *et al.*, 1989).

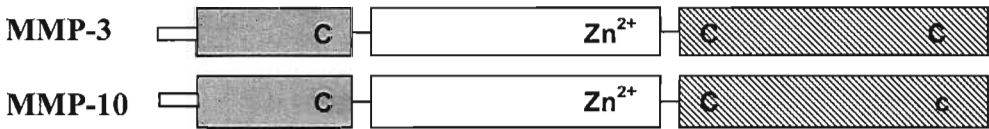
I. Collagenases



II. Gelatinases



III. Stromelysins



IV. Others

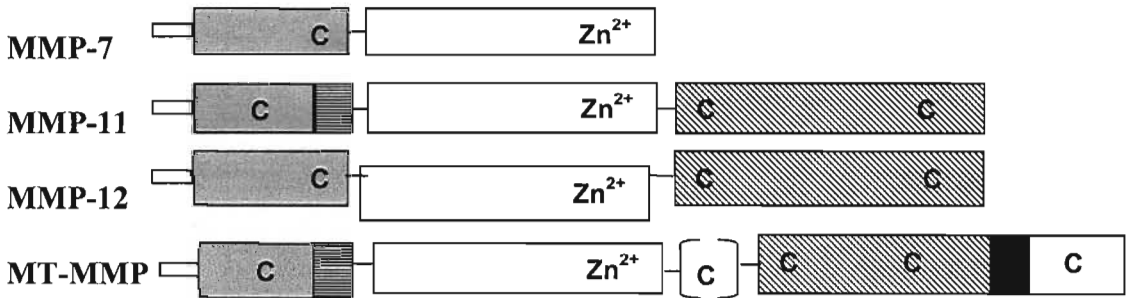


Figure 3.1 Domain structures of MMPs. Three common domains are found in MMPs, the pro-peptide domain, the catalytic domain and the C-terminal domain. Repeats similar to fibronectin type II domain in MMP-2 and 9 (▧), trans-membrane domain in MT-MMPs (■), Extra 10 to 11 residues in MMP-11 and MT-MMPs (▨), the proline-rich (collagen like sequence) in MMP-9 (〰), and the hemopexin domain in all MMP-s except for MMP-7 (▩). The cysteine in the pro-domain is for cysteine activation. C, conserved cysteine. Non conserved cysteine are in parenthesis (modified from Hooper, 1996).

The various structural domains in MMPs cannot explain all the interactions of the MMPs. However, substrate specificity is generally defined by the sequence in the substrate-binding domain and MMPs are designed to act one after the other, one MMP performing a key cleavage on the intact molecule, while others cleave the product of the primary cleavage. Collagen (Types I, II, III and X), which, with their triple helical structures are organized in such a way as to form compact highly cross-linked fibers are, for example, extremely resistant to the action of most enzymes. The two families of MMP enzymes that can degrade these collagens are the interstitial collagenase (MMP-1) and PMN collagenase (MMP-8) (Cimpean and Caloianu, 1997; Hasty *et al.*, 1990). The MMPs cleave the native collagen at a single locus near the C-terminal end (Hasty *et al.*, 1990; Murphy *et al.*, 1982). The cleavage products (gelatins) lose their triple helical structure and become substrates for various MMPs (Table 3.1). These gelatinases (MMP-2 and MMP-9) accelerate the cleavage of collagen resulting in more cleavage (hydroxyprolines) (Murphy *et al.*, 1982). These two enzymes together degrade soluble collagen and partially digested collagen two to three times more effectively than the insoluble collagen (Hibbs, 1992). Gelatinases, however, can also degrade type IV collagen which is the major component of the basement membrane (Triebel *et al.*, 1995). The enzyme specificity of gelatinases for gelatin and collagenases for collagen may usually be employed in electrophoretic and zymography techniques to demonstrate the presence or identity of such proteases and such techniques will be used in this study.

Table 3.1 Matrix metalloproteinases (modified from Hooper, 1996; Kries and Vale, 1999).

| Enzymes | Mr | | Active | Matrix substrates |
|--|---------|----------------------------|----------------------|---|
| | MMP No. | Precursor | | |
| Collagenases Interstitial/ tissue/fibroblast collagenase (3.4.24.7) | MMP-1 | 52 000 56 000* | 41 000 45 000* | Collagens I, II, III, VII, X, gelatins, entactin, link protein, aggrecan, tenascin, L-selectin, IGF-binding proteins, pro-MMPs 2 and 9. |
| PMN collagenase (3.4.24.34) | MMP-8 | 105 000* 75 000* | 65 000 | Collagens I, II, III, gelatins, aggrecan and link protein. |
| Collagenase 3 | MMP-13 | 65 000 | 55 000 | Collagen I, II, III, gelatins, aggrecan |
| Gelatinases Gelatinase A (3.4.24.24) | MMP-2 | 72 000 | 67 000 | Gelatins, collagens I, IV, V, VII, X, elastin, fibronectin, laminin, link protein, aggrecan, galectin-3, IGF-binding proteins, vitronectin, fibulin-2, FGF receptor-1, pro-MMPs 9 and 13. |
| Gelatinase B (3.4.24.35) | MMP-9 | 92 000 94 000 98 000 | 82 000 81 000 | Gelatin, collagens III, IV, V, VII, X, XI, XIV, elastin, aggrecan, link protein, proteoglycans, vitronectin, galectin-3, proMMP-2 and entactin. |
| Stromelysins Stromelysin 1 (3.4.24.17) | MMP-3 | 57 000 59 000* | 45 000 28 000 | Proteoglycans, fibronectin, aggrecan, gelatins, fibronectin, laminin, collagen III, IV, V, IX, X and XI, entactin, SPRAC, tenascin-C, vitronectin, Pro MMP 1, 8, 9 and 13, antithrombin III, PAI-2, α_1 -protease inhibitor, α_1 -antichemotrypsin, α_2 -macroglobulin, L-selectin, E- cadherin, HB-EGF, link protein, fibrin. |
| Stromelysin 2 (3.4.24.22) | MMP-10 | 57 000 | 45 000 28 000 | Aggrecan, gelatins, fibronectin, laminin, collagen I, IV, V, VII, X, entactin, fibronectin, link protein, vitronectin, elastin, tenascin, fibulin, pro-MMPS 1, 2 and 9. |
| Others Matrilysin (3.4.24.23) | MMP-7 | 28 000 | 19 000 | Proteoglycans, laminin, gelatins, collagen IV, entactin, fibronectin, link protein, vitronectin, elastin, tenascin, fibulin, proMMPs 1, 2 and 9, laminin, fibronectin, aggrecan, α_1 -proteinase inhibitor, α_2 -macroglobulin. |
| Stromelysin 3 | MMP-11 | 55 000 | 45 000 28 000 | Elastin, fibronectin, fibrinogen, laminin, entactin, collagen IV, proteoglycans, fibronectin aggrecan, link protein, vitronectin, tenascin, fibulin, α_1 -proteinase inhibitor, α_2 -macroglobulin. |
| Metalloelastase (3.4.24.65) | MMP-12 | 53 000 | 45 000 | Elastin, fibronectin, fibrinogen, laminin, entactin, collagen IV, proteoglycans, myelin basic protein, IgGs, plasminogen, α_1 -proteinase inhibitor. |
| Membrane type MMP | MMP-14 | 66 000 | 56 000 | Collagens I, II, III, gelatins, fibronectin, laminin, vitronectin, proteoglycans, proMMPs 2 and 13, α_1 -proteinase inhibitor, α_2 -macroglobulin |

*Glycosylated

3.3 Activation of MMPs

MMPs are usually secreted as proenzymes and activation may take place by proteolytic or non-proteolytic means (Nagase, 1997). *In vitro*, the non-proteolytic activation can be performed by many molecules such as SH reactive agents [iodoacetamide, 4-aminophenylmercuricacetate (APMA) (Woessner, 1991; Nagase, 1997)], HOCl, oxidized glutathione, denaturants (urea, SDS, NaSCN), heat treatment (Nagase, 1997), chaotrophs, oxidants, heavy metals, disulphide compounds and detergents (Cimpeanu and Caloianu, 1997; Hooper, 1996) (Table 3.2). Activation is, however, best achieved with organomercurials (Hibbs, 1992; Bu and Pourmotabbed, 1995), with the more hydrophobic organomercurial being the most effective (Grant *et al.*, 1992)

In vivo, most proteolytic activation of pro-MMPs is caused by tissue or plasma proteinases, or opportunistic bacterial proteinases (Table 3.2) (Nagase and Woessner, 1999). For example, during PMN diapedesis through the blood vessel walls pro-MMPs are activated by the uPA/plasmin system. The precursor of urokinase-type plasminogen activator (pro-uPA) is readily converted into active uPA after binding to a specific uPA receptor on the cell surface of PMNs (Nagase, 1997). The active uPA subsequently activates cell-associated plasminogen to plasmin which subsequently activates pro-MMPs (Figure 3.2). Active MMPs or other proteases such as trypsin, chymotrypsin, cathepsin G, and kalikrein can also activate MMPs (Schettler *et al.*, 1991; Itoh and Nagase, 1995). Nagase, (1997) suggested a two-step processes of protease-dependent activation of MMPs. The first step requires the activator protease to cleave the protease susceptible “bait” region located in the middle of the pro-peptide. In the second step this cleavage subsequently induces conformational changes in the pro-peptide and exposes the final activation site to be cleaved by a second MMP (Figure 3.2).

MMPs can also be activated before they are secreted e.g. pro-stromelysin-3 (pro-MMP-11) and pro-MT1-MMP (Nagase, 1997). Pro-stromelysin-3 has extra 10 residues in the pro-peptide domain at the junction with the catalytic domain. This sequence contains Arg-Gln-Lys-Arg at the C-terminal end of the pro-peptide. This 65 kDa proenzyme is

processed to an active 45 kDa enzyme by the Golgi-associated subtilisin-like protease, furin, and secreted as an active enzyme (Figure 3.2) (Nagase, 1997).

Pro-MMPs can also be activated via a non-proteolytic mechanisms. This is generally by “cysteine switch” mechanism, a process by which the cysteine residue in the pro-peptide region is transiently dissociated from the zinc atom in the catalytic domain and reacts with thiol modifying reagents to prevent the re-association of cysteine and zinc (Cimpean and Caloianu, 1997; Springman *et al.*, 1990; Hooper, 1996). Upon temporary activation of the enzyme, proteolytic enzymes may cleave the pro-peptide and cause permanent activation (Bu and Pourmotabbed, 1995; Springman *et al.*, 1990; Cimpean and Caloianu, 1997; Hooper, 1996).

Table 3.2 Enzyme activators of pro-MMPs (modified from Hooper, 1996).

| Activators of Pro MMPs | |
|------------------------|---|
| ProMMP-1 | Trypsin, plasmin, plasma kallikrein, chymase, MMP-3 and MMP-7 cleave Gln ⁸⁰ -Phe ⁸¹ bond only when a part of pro-peptide is removed by proteolysis. |
| ProMMP-2 | MT-MMP, MMP-1 (enhanced by heparin), MMP-7 (lesser extent) |
| ProMMP-3 | Activated by many proteases (e.g. plasmin, plasma kallikrein, tryptase, trypsin, chymase, chymotrypsin, pseudomonas elastase), but not by MMPs |
| ProMMP-7 | Trypsin, MMP-3 (slow, but accelerated in the presence of APMA) (Partially activated by plasmin, and PMN elastase) |
| ProMMP-8 | MMP-3, tissue kallikrein, PMN elastase, cathepsin G, trypsin, chymotrypsin, stromelysin and by different mercurial compounds, |
| ProMMP-9 | MMP-3, trypsin, kallikrein, stromelysin and by different mercurial compounds, (Partially activated by chymotrypsin, cathepsin G, plasmin and MMP-7) |
| ProMMP-10 | Trypsin, chymotrypsin, plasmin, but not by PMN elastase |
| ProMMP-11 | Furin |

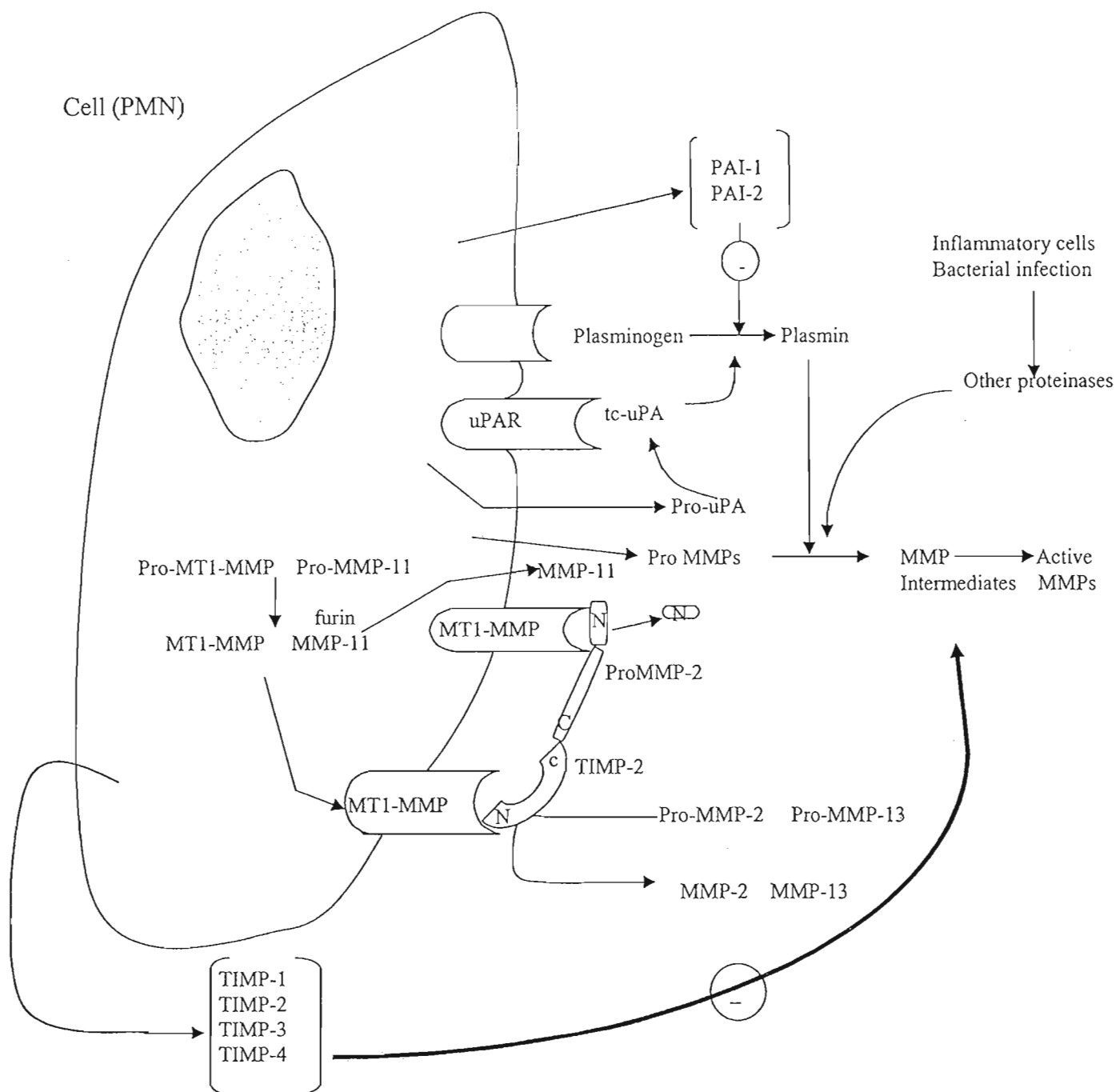


Figure 3.2 Activation pathways of pro-MMPs.

Most pro-MMPs are activated extracellularly in a stepwise manner. Pro-MMP-2 and pro-MMP-13 are activated on the cell surface by MT1-MMP. Pro-MMP-11, pro-MT1-MMP and possibly other pro-MT-MMPs are activated intra-cellularly. Pro-uPA binds to the uPA receptor and is activated on the cell surface to the two-chain uPA (tc-uPA) which in turn activates plasminogen on the cell surface. Plasminogen activator inhibitors (PAIs) interfere with this process. Plasmin then can initiate activation of the pro-MMPs. Proteinases from inflammatory cells and microorganisms can also activate pro-MMPs (modified from Nagase, 1997).

3.4 MMP-inhibitor interactions

As mentioned, complex formation may occur between some precursor MMPs and their TIMP inhibitors. Gelatinases 2 and 9 form a tight non-covalent and stable complex with their inhibitors, pro-MMP-2 binds to TIMP-2 via its C-terminus (Goldberg *et al.*, 1992), as does pro-MMP-9 to TIMP-1 (Wilhelm *et al.*, 1989). These interactions may have several functions. The pro-MMP-2/TIMP-2 complex is found to be required for the tri-molecular activation of pro-MMP-2 at the surface of the cell. The function of TIMP-1/pro-MMP-9 complex is not known (Brew *et al.*, 2000) unless it too is involved in activation (Nagase, 1997). However, TIMP-1 shields the C-terminal region of pro-MMP-9 during complex formation and protects it from inactivation by stromelysin (MMP-3) and from cleavage by PMN elastase (Ogata *et al.*, 1992; Itoh and Nagase, 1997). In such a complex, however, PMN elastase cleaves TIMP-1 and subsequently activates pro-MMP-9 (Nagase, 1997; Itoh and Nagase, 1997). In such a complex, therefore, TIMP-1 may serve to have a protective role, facilitating the activation of pro-MMP-9 in an environment in which elastase is present in high concentrations.

The activating tri-molecular complex, formed between MT1-MMP, TIMP-2, and pro-MMP-2, is bound to cell membrane. Initially the N-terminal region of TIMP-2 binds to the catalytic site of MT1-MMP and forms a bi-molecular complex. The pro-MMP-2 then binds to the C-terminal region of TIMP-2 to form a tri-molecular complex. This tri-molecular complex exposes the pro-peptide region of pro-MMP-2 which is subsequently cleaved and results in activation of the pro-MMP-2 by another nearby active MT1-MMP (Figure 3.2) (Brew *et al.*, 2000; Nagase, 1997). A tri-molecular complex is also formed between pro-MMP-9/TIMP-1 and MMP-3. In this reversible complex another molecule of MMP-3, is bound and releases active MMP-9 (Nagase, 1997). Tri-molecular complexes are also described to be more stable than bi-molecular complexes and can inhibit other MMPs. Therefore, a more stable tri-molecular complex of pro-MMP-9/TIMP-1/MMP is formed for the activation of pro-MMP-9 (Gomez *et al.*, 1997; Brew *et al.*, 2000).

MMP-8 and MMP-9 are found at high concentrations in PMNs (Oronsky *et al.*, 1973; Hibbs, 1992; Hibbs *et al.*, 1985) and are mainly secreted extracellularly (Price *et al.*, 2000; Schettler *et al.*, 1991) especially in response to PMA (Hasty *et al.*, 1986; Hibbs *et al.*, 1985) cytokines, chemokines or complement components (Schettler *et al.*, 1991) (Table 3.2, 3.3). Pro-MMP-8 may be purified from PMNs in the buffy coat of blood (Schettler *et al.*, 1991) while pro-MMP-9, due to its liability, is best purified from PMA-stimulated PMNs. PMA stimulation results in up regulation of pro-MMP-9 mRNA translation and hence an increase in the amount of MMP-9 seen (Hibbs, 1992).

The molecular weights of PMN collagenases and gelatinase, the agonists which cause their secretion, their substrates, inactivators, location and endogenous inhibitors are tabulated in Table 3.3.

Table 3.3 PMN Matrix Metalloproteinase.

| Properties | PMN Collagenase (MMP-8) | PMN Gelatinase (MMP-9) |
|----------------------------|--|--|
| Molecular weight | Pro-105/75/85, mature-58 | Pro-92, mature-82 |
| Expression induced by | PMA, cytokines, chemokines, complement components | PMA, cytokines, chemokines, complement components |
| Activation | Trypsin, organomercurials, MMP-3, tissue kallikrein, PMN elastase, cathepsin G, chymotrypsin, stromelysin and different mercurial compounds. | Low pH (2), trypsin, organomercurials, MMP-3, chymotrypsin, cathepsin G, plasmin, MMP-7, kallikrein and stromelysin and different mercurial compounds. |
| Substrate | Collagen types I, II, and III, α_2 -M, gelatins, aggrecan and link protein. | Gelatin, collagen types III, IV, V, VII, X, XI and XIV, elastin, proteoglycan, aggrecan, link protein, vitronectin, galactin-3, proMMP-2 and entactin. |
| Inactivation | EDTA, 1,10 phenanthroline | EDTA, 1,10 phenanthroline |
| Intracellular localization | Specific granules | Gelatinase granules |
| Endogenous Inhibitors | α_2 -M, TIMP-1, TIMP-2 | α_2 -M, TIMP-1, TIMP-2 |

3.5 Source of MMP-9

MMP-9 has been described as a marker for the gelatinase granules of PMNs (Kjeldsen *et al.*, 1992; Hibbs and Bainton, 1989) and is reported to be destined solely for extracellular secretion and is found in high amount in PMNs and leukemic cells (Hibbs *et al.*, 1992; Hibbs, 1985; Janowska-Wieczorek *et al.*, 1999; Wilhelm *et al.*, 1989). After activation by PMA, gelatinase is the major proteinase secreted by PMNs (Hibbs *et al.*, 1985). Stimulation of PMN in this way, therefore, is an effective method for facilitating the isolation of MMP-9 in a latent form (Hibbs, 1992), a form more stable to most denaturing conditions. The selective release of these stored proteinases also gives rise to a relatively pure preparation from which MMP-9 may be more easily isolated using a gelatin column, exploiting the high affinity of the enzyme for gelatin (Hibbs, 1992). Hibbs *et al.* (1985) also suggested that by manipulation of conditions in the PMN suspension, it is possible to harvest the gelatinase with minimal contamination from serine proteinases and other PMN granule proteins. A crude fraction of MMP-9, either from culture media or from PMN homogenates, may also be prepared using three phase partitioning (TPP) (Dennison and Lovrien, 1997).

There are several advantages in choosing TPP fractionation of sputum rather than PMNs for MMP isolation, however. The isolation of PMNs is a time-consuming, difficult and expensive procedure as compared to the collection of sputum and use of TPP fractionation. Cell culture media often used to suspend PMNs also contains additives such as serum which may complicate isolation procedures. Human saliva contains a latent 94 kDa MMP-9 which may be released to the oral cavity from the surrounding cells or from PMNs which may enter to the oral cavity through gingival sulci (Davis, 1991). Previously MMP-9 fractionation was performed using TPP (Price *et al.*, 2000) and gave good results as judged by SDS-PAGE, zymography and western blotting analysis.

Sputum was, therefore, chosen as a source of MMP-9 and TPP was chosen as a crude fractionation procedure. For the characterization of anti-MMP-9 antibody and for PMN release, however, a PMN source was used.

3.6 Three phase partitioning of sputum and analysis of fractions using western blotting and zymography

One of the problems in isolating MMP-9 by homogenization of PMNs is that release of PMN serine proteinases, especially elastase, may degrade the PMN MMPs (Murphy *et al.*, 1982). Several proteins and many enzymes are also degraded by oxygen radicals generated by stimulated PMNs. This is especially important as loss of activity may result and hence detection of e.g. TIMP-1 in reverse zymograms may fail. For immunological characterization, however, the presence of epitopes is of primary interest and loss of activity may not necessarily prevent detection. Degradation of proteins may, however, give rise to detection of MMP fragments which appear to have incorrect molecular weights if no protease inhibitors are added.

Various forms of MMP-9 have previously been described. For the pro-MMP-9 form a 92, 94 and 98 kDa have been found (Hibbs, 1992; Wilhelm *et al.*, 1989; Davis, 1991; Schettler *et al.*, 1991). An 82 and 81 kDa active form of MMP-9, varying depending upon the method of activation (Itoh and Nagase, 1995; Schettler *et al.*, 1991) have also been observed and were, therefore, anticipated. However, non-reducing treatment of the enzyme also gives, higher bands of pro-MMP-9. These are usually between 125-130 and 225 kDa and may represent an NGAL-MMP-9 hetero-dimer and an MMP-9 homodimer, respectively (Price *et al.*, 2000; Hibbs *et al.*, 1985; Hibbs, 1992; Schettler *et al.*, 1991).

3.6.1 Reagents

Reagents for reverse zymograms were prepared according to Section 2.9.2, for Western blots, Section 2.7.2, and for SDS (Laemmli) gel electrophoresis, Section 2.6.1.1.

The amount of protein to be loaded was measured by Bradford protein binding assay as described in Section 2.2.1.2.

3.6.2 Procedure

The pro-MMP-9 to be used for western ligand blot and reverse zymograms was isolated from sputum using TPP as described in Section 2.4. Sputum (20 mls) was collected from a healthy patient fasted for 12 h, proteins were subsequently precipitated with 30% (v/v) t-butanol and incremental addition of 10-40% (m/v) ammonium sulphate. Precipitated proteins collected after phase separation were re-suspended in MMP-9 buffer [50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl_2 , 0.02% (w/v) NaN_3 , 0.02% (v/v) Brij-35, 2 mM PMSF, pH 8.8] and stored at -20°C for future use. Alternatively they were mixed in equal volumes of reducing treatment buffer, separated in 10% Laemmli gel and stained in Coomassie brilliant blue solution as described in Section 2.6.1.1. A further sample for zymography was mixed with non-reducing treatment buffer and run in 10% Laemmli gel co-polymerized with 0.1% (w/v) gelatin. After separation on the SDS PAGE gel, gels were renatured in 2.5% (v/v) Triton X-100 and the zymogram developed in zymogram development buffer. A further sample was treated with reducing buffer to avoid dimer-formation, electrophoretically separated, blotted into nitrocellulose and detected with 219 $\mu\text{g/ml}$ anti-MMP-9 antibody.

3.6.3 Results

The reducing SDS-PAGE gel of the TPP-precipitated proteins showed bands at 14.3, 62.6, 66, and 73.3 kDa (Figure 3.3, A). The 66 kDa band was found to be more intense in the 10-20% and 20-30% (m/v) ammonium sulphate fractions (Figure 3.3 A, c and d, respectively), while, the 73.5 kDa band was more intense in 0-10% (m/v) and 30-40% (m/v) fractions (Figure 3.3 A, b and e, respectively). A 14 kDa band was also observed in the 30-40% (m/v) fraction of sputum (Figure 3.3 A, e).

On the gelatin zymogram gels, only two fractions (0-10 and 20-30% (m/v) ammonium sulphate fractions) showed gelatinolytic activity (Figure 3.3 B, b and d, respectively). A 94 kDa gelatinolytic band not seen in the SDS-PAGE gel, was seen on the gelatin zymography gel indicating the high sensitivity of zymography for visualizing the activity of MMPs (Figure 3.3 B).

When the SDS-PAGE gel was blotted into a nitrocellulose by western blotting and probed with an MMP-9 antibody 94, 61, 45, 36, 33, 16, and 15 kDa bands appeared (Figure 3.4). Cuts of 0-10% and 10-20% (m/v) ammonium sulfate allowed resolution of a 61, 45, 36 and 16 kDa band (Figure 3.4, a and b). However, a 94 kDa possible proMMP-9 band and many apparent degradation products appeared in the 20-30% (m/v) fraction (Figure 3.4, c). In the 30-40% (m/v) fraction, only the 94 kDa proenzyme was apparent (Figure 3.4, d). It is difficult to conclude that all the bands belong to MMP-9 as there may be several MMPs such as MMP-8 and MMP-1 which may cross react with the antisera used and may be secreted in the mouth by the surrounding cells and PMNs. Cleavage products may also occur due to possible presence of oral proteases of host and bacterial origin (Okamoto *et al.*, 1996) and the observed molecular weights may differ from those anticipated (92, 82 and 81 kDa). The 94 kDa band seen (Figure 3.3 B, b and d, respectively) due to their gelatinolytic nature and molecular weight possibly represent pro-MMP-9.

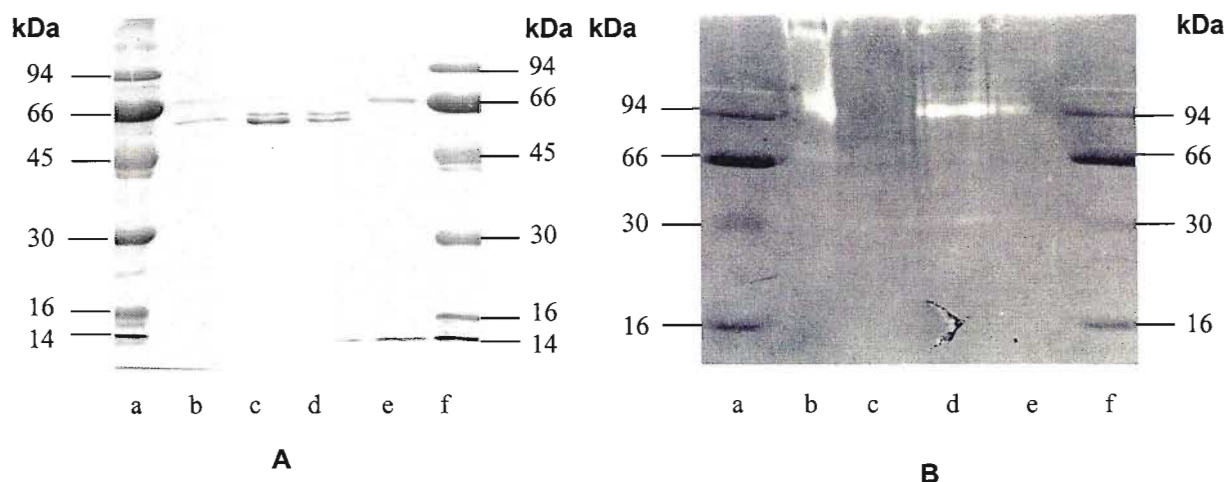


Figure 3.3 **SDS-PAGE and gelatin zymogram of sputum MMP-9 isolated by three phase partitioning (TPP).** Aliquots of TPP fractionated sputum (10 μ g/lane) were separated under reducing conditions on a 10% Laemmli SDS-PAGE gel, stained in Coomassie Brilliant Blue solution and destained (A) and under non-reducing conditions containing 1 mg/ml gelatin (B). The proteins were renatured in 2.5% (v/v) Triton X-100 and developed in zymogram development buffer. *Lane a*, M_r markers; *lane b*, 0-10% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *lane c*, 10-20% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *lane d*, 20-30% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *lane e*, 30-40% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *lane f*, M_r markers.

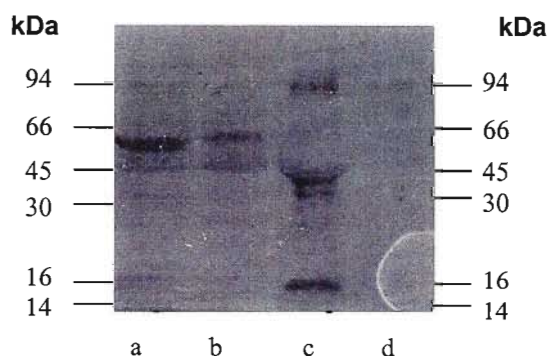


Figure 3.4 **Western blotting of sputum MMP-9 isolated by three phase partitioning (TPP) and PMN homogenates.** Aliquots of TPP fractionated sputum (10 μ g/lane) were separated in Laemmli gel under reducing conditions. The gel was blotted onto nitrocellulose and detected with 219 μ g/ml α -MMP-9 antibody. *Lane a*, 0-10% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *Lane b*, 10-20% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *Lane c*, 20-30% (m/v) $(\text{NH}_4)_2\text{SO}_4$; *Lane d*, 30-40% (m/v) $(\text{NH}_4)_2\text{SO}_4$.

3.7 Isolation, SDS-PAGE and western blotting of MMP-9 from PMNs

PMNs produce and secrete large amount of MMP-9 upon stimulation. To confirm the specificity of the antibody prepared to be used in subsequent studies and hence the suitability of the sample chosen, PMN MMP-9 was obtained by homogenization of PMNs and the anti-MMP-9 antibody was re-characterized.

3.7.1 Reagents

Reagents for western blots were prepared as per Section 2.7. Reagents for density gradient separation of PMNs were prepared as described in Section 2.10. An anti-MMP-9 antibody raised against a 220 kDa dimer of MMP-9 expressed (by Volker Zollinger, University of Bielefeld) in a *Pichia pastors* yeast expression system in chicken by Brendon Price, University of Natal, was used.

PMN lysis solution [1 M NaCl/PBS, 10% (v/v) DMSO]. NaCl (0.585 g) was dissolved in PBS (pH 7.4) (10 ml) and 1 ml of DMSO was added.

Inhibitor buffer [2 mM PMSF, 1 mM EDTA, 1% (w/v) NaN_3]. PMSF (10 μl of 200 mM solution in methanol), EDTA (5 μl of 200 mM solution), NaN_3 (0.01 g) dissolved in 1 ml storage buffer.

The amount of protein to be loaded was measured by Bradford protein binding assay as described in Section 2.2.1.2.

3.7.2 Procedure

PMNs were isolated from human blood as described in Section 2.10 and mixed into an equal amount of reducing treatment buffer or frozen at -20°C for subsequent use.

Samples (20 μ l) were separated by electrophoresis and the gel blotted into nitrocellulose for 16 h as described in Section 2.7.3. The membranes were subsequently probed with 21.9 μ g/ml anti-MMP-9 IgY antibody. Either Brij-35 or Triton X-100 was added to wash buffers to aid detection. (The importance of detergents in enhancing detection of antigens using western blotting is discussed in Chapter 4).

3.7.3 Results

Immunoblotting revealed a band of approximately 94 kDa using both detergents Brij-35 and Triton X-100 (Figure 3.5 B and C, respectively). Sharper bands were detected without the use of detergents, however (Figure 3.5, D).

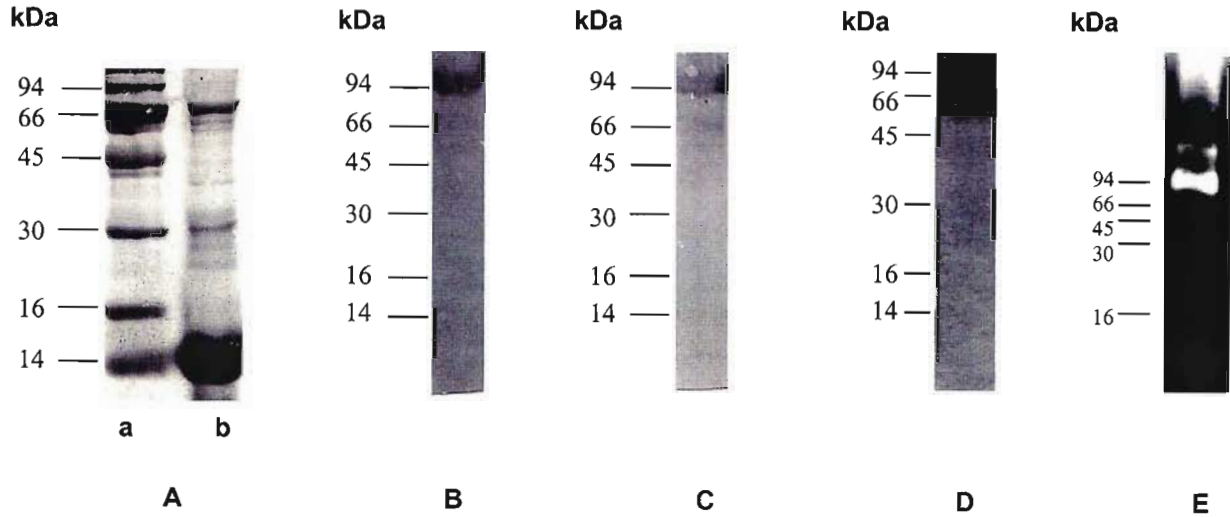


Figure 3.5 Western blotting of MMP-9 from PMN homogenates using detergents. Molecular weight markers (a) and PMNs homogenates (20 μ l) (b) were separated on (A) 12% (m/v) Laemmli SDS-PAGE gel under reducing conditions. The PMN homogenates were blotted (B, C and D) and subsequently probed with 219 μ g/ml α -MMP-9 IgY. Detergents both Brij-35 (B) and Triton X-100 (C) were added to aid detection or no detergent was added (D). PMN homogenates were also separated on a 10 % (m/v) Laemmli gel containing 0.01g/ml gelatin in the running buffer, renatured in (2.5 % v/v) Triton X-100 and developed in zymogram buffer for 16 h before staining (E).

Antibody labelling specificity for MMP-9 was more easily shown using PMN homogenates (Fig. 3.5) because, unlike sputum samples (Figure 3.4) bands were only seen at approximately 94 kDa (possibly pro-MMP-9), and at 66 kDa (Figure 3.5 B,C and D). The homogenates were also separated on a zymography gel and bands appeared at 92, 125 and approximately 230 kDa (Figure 3.5, E), confirming the gelatinolytic nature of the 92 kDa and higher molecular weight bands. The 66 kDa band, seen in blots where no detergent was used, showed no proteolytic activity and, therefore, possibly either does not represent an active form of MMP-9 or does not represent MMP-9 (Figure 3.5 D).

3.8 Discussion

MMPs are generally extracellular enzymes and are rapidly secreted upon specific stimuli such as PMA (Hibbs, 1992). Due to the ease with which secretion of gelatinase granules occurs and the substrate specificity of MMP-9, MMP-9 is believed to be the major protease involved in diapedesis of PMNs (Sengeløv *et al.*, 1995; Hibbs, 1992). Primary granule proteases, on the other hand, are secreted to the external environment mainly during frustrated phagocytosis or excessive activation of PMNs (Henson, 1971). During PMN homogenization, however, all proteins and degradative enzymes are released. The main obstacle to the isolation of pro-MMP-9 and other undegraded forms of MMP-9 in this case is the digestive activity of especially the serine proteinases (Deutscher, 1990; Murphy *et al.*, 1982). It is important to inhibit degradation of MMP-9 by serine proteases by adding PMSF to all buffers (Hibbs *et al.*, 1985). In this study MMP-9 was apparently detected in sputa by both zymography and western blotting. A gelatinolytic band of approximately 94 kDa was observed similar to that found in saliva and identified as MMP-9 by Davis (1991). In addition to the weak 94 kDa band, many other lower molecular weight, non-gelatinolytic, MMP-9 immunoreactive bands were also observed in sputa. MMPs may have similar domains which may cause the cross reactivity with polyclonal antibodies raised against other whole MMP enzymes. The anti-MMP-9 antibody which was used for this project seems to be specific, however, as

no cross-reactivity was observed with other MMPs from PMN homogenates. Since only MMP-8 and MMP-9 are found in PMNs, however, the number of possible cross-reacting MMPs which may cause immunoreactivity are reduced so it may be argued that the possible cross-reactivity of the anti-MMP-9 antibody used in this study is still unknown. The fact that the major band targeted was of the correct molecular weight for pro-MMP9 and this form was gelatinolytic seems to confirm the antibody's specificity, however. MMPs, which degrade gelatin, include MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-10, MMP-13, and MMP-14, and saliva also contains some of these enzymes. Gelatinolytic forms of these, however, do not appear to have been observed in gelatin zymograms. It would, therefore, seem that the MMP-9 antibody is specific and other reactive bands in sputum samples were perhaps MMP-degradation products. No improved immunoreactivity was observed as a result of the inclusion of detergents.

Results obtained by TPP fractionation show that the TPP method is useful for semi-fractionating enzymes. It is simpler to semi-fractionate MMP-9 from sputum than from PMNs. The purity and concentration of MMP-9 isolated from sputum fractions is lower, but the resource is more easily accessed in adequate quantities for use in reverse zymograms (for the detection of TIMP-1). The presence of contaminating proteins are also irrelevant in such a system. At this point these reagents were, therefore, judged adequate for the optimization of TIMP-1 detection by reverse zymography and for future studies on the release of TIMP-1 and MMP-9.

CHAPTER FOUR

OPTIMIZATION OF WESTERN BLOTTING, ECL, AND REVERSE ZYMOGRAPHIC DETECTION OF TIMP-1

4.1 Introduction

The tissue inhibitors of matrix metalloproteinases (TIMPs) are naturally occurring inhibitors of the MMPs, responsible for ECM degradation and ECM turnover and tissue remodelling. In the blood about 95% of all proteases are regulated by α_2 -macroglobulin (Woolley *et al.*, 1976; Ishibashi *et al.*, 1988). The large size (780 kDa) of α_2 -macroglobulin, however, does not allow the inhibitor to penetrate into the tissue (Woolley *et al.*, 1976) and the TIMPs, which are smaller (~30 kDa), are the only MMP inhibitors in tissues (Houng *et al.*, 1997). TIMPs inhibit MMPs in a one to one ratio (Willenbrock *et al.*, 1993; Langton *et al.*, 1998; Macartney and Tschesche, 1983; Stricklin and Welgus, 1983) and there are four members of the TIMP family numbered according to the order in which they were discovered i.e. TIMP-1, TIMP-2, TIMP-3 and TIMP-4. These range in molecular weight from 22 to 30 kDa (Gomish-Rüth *et al.*, 1997) and in distribution (Table 4.1). They all possess 12 cysteine residues and form six disulphide bonds which give rise to six loop structures (Williamson *et al.*, 1990) (Figure 4.1). The presence of these six disulphide bonds and six loops give the TIMP family members a compact structure and a relatively high structural similarity (40-50%) though low amino acid homology (Williamson *et al.*, 1990; Gomish-Rüth *et al.*, 1997). Their main differences are found on their C-terminal domains (loops 4 - 6 and a free tail) (Kries and Vale, 1999).

The N-terminal domain of TIMPs is the inhibitory region which binds the catalytic domain of the MMPs (Olson *et al.*, 1997). This domain is both necessary and sufficient

for MMP inhibition (Murphy *et al.*, 1991). The C-terminal domain, on the other hand, is used to bind to the C-terminal domain of MMPs during complex formation (Goldberg *et al.*, 1992; Wilhelm *et al.*, 1989). TIMPs inhibit active MMPs through a non-covalent binding (Gomez *et al.*, 1997) and a decrease in TIMP-1 and TIMP-2 levels in tissue facilitates the uncontrolled degradation of the extracellular matrix observed in pathological conditions such as rheumatoid arthritis (Osthues *et al.*, 1992). TIMP-2 and TIMP-3 are effective inhibitors for membrane type MMP (MT-MMP), while TIMP-3 also inhibits tumour necrosis factor- α converting enzyme, a metalloproteinase that is not a member of the matrixin family of MMPs (Brew *et al.*, 2000).

Table 4.1 Molecular characteristics of TIMPs

| Inhibitor | kDa. | Glycosylation | Location | Isoelectric point |
|-----------|------|------------------|---|-------------------|
| TIMP-1 | 28.5 | Glycosylated | Human skin fibroblasts, corneal fibroblasts, gingival fibroblasts, adults and foetal lung fibroblasts, human PMNs and macrophages | 5.5 - 8.0 |
| TIMP-2 | 21 | Non-glycosylated | Melanoma cells, fibroblasts, alveolar macrophages | 6.45 |
| TIMP-3 | 21 | Glycosylated | Extracellular matrix | 9.04 |
| TIMP-4 | 23 | Non-glycosylated | Heart | 7.34 |

4.1.1 Structure of TIMP-1

TIMP-1, the most ubiquitous MMP inhibitor is a glycoprotein with an approximate molecular weight of 28.5 kDa (Triebel *et al.*, 1995) (Table 4.1). It consists of 184 residues, which, like the other TIMPs, include the 12 cysteine bonds and form 6 loops (Figure 4.1) (Williamson *et al.*, 1990) which imparts a resistance to high temperature, pH, pressure, denaturing agents and oxidation (Strickling and Welgus, 1983). TIMP-1,

however, has one free sulfhydryl group which is required for its inhibitory activity and TIMP-1 activity can be abolished by alkylation of this group using iodoacetamide (Macartney and Tschesche, 1983).

Due to post-translational modification, TIMP-1 is a highly glycosylated protein. Its two N-linked glycosylation sites are composed of sialic acid, mannose, galactose and N-acetyl glucosamine residues which gives rise to approximately 30% of the molecular weight of the protein (Caterina *et al.*, 1998). Glycosylation occurs in sites (N³⁰ and N⁸²) and is heterogeneous. Due to the variability of sialic acid substitution differently glycosylated TIMP-1's may have heterogeneous pI values (Murphy and Willenbrock, 1995; Macartney and Tschesche, 1983) (Table 4.1). Glycosylation, however, does not affect inhibitory activity (Caterina *et al.*, 1998). The N-terminal domain of TIMPs, especially the region surrounding the 2nd "disulfide knot" (cys¹³ – cys¹²⁴, cys¹²⁷ – cys¹⁷⁴) (Bodden *et al.*, 1994), is the inhibitory region which binds with the catalytic domain of the MMPs (Olson *et al.*, 1997). Though it inhibits MMPs in 1:1 ratio (Taylor *et al.*, 1996), TIMP-1 does not inhibit bacterial collagenase and thermolysins (Hayakawa *et al.*, 1992).

TIMP-1 is a member of the oligosaccharide/oligonucleotide-binding proteins. Like the other oligosaccharide/oligonucleotide-binding proteins it has a binding region which consists of five-stranded β -pleated sheet rolled into a β -barrel of conical shape (Gomish-Rüth *et al.*, 1997). This β -barrel topology is homologous to that seen in proteins of the oligosaccharide/oligonucleotide-binding (OB) fold family (Murphy and Willenbrock, 1995; Murzin, 1993). The common structural features of this family include the number of β -strands and their arrangement, the β -barrel shear number, an inter-strand hydrogen bond network, the packing of the hydrophobic core, and the conserved β -bulge (Murphy and Willenbrock, 1995). Similarity with other known OB protein fold family members such as staphylococcal nuclease and *Escherichia coli* heat labile enterotoxin was confirmed by superimposition of the inhibitory region (N-TIMP) of TIMP and the active or binding domain of the other family proteins. However, these proteins do not have

similar sequences in their active sites indicating that they do not share a common ligand binding (Murphy and Willenbrock, 1995).

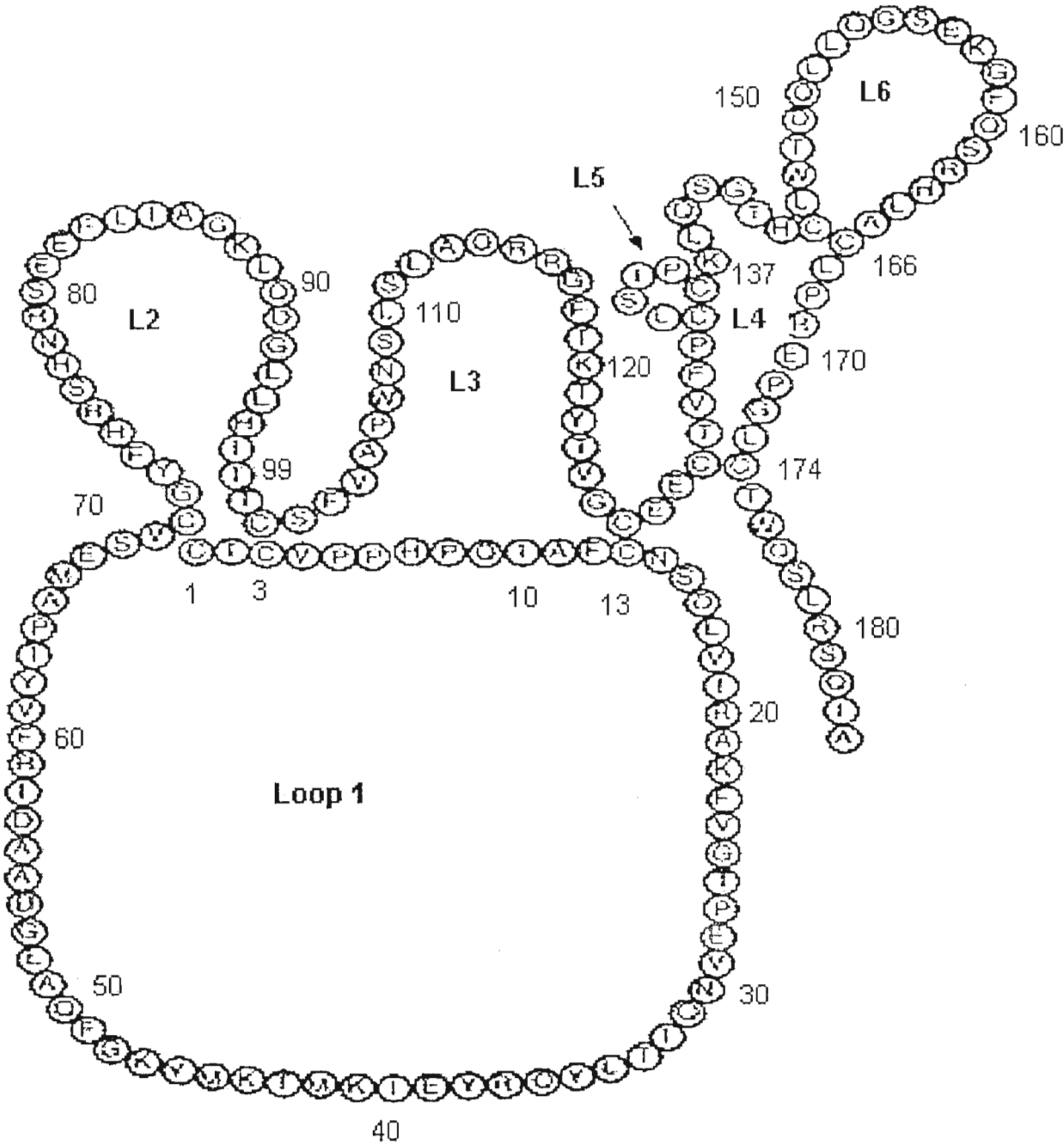


Figure 4.1 The proposed structure of TIMP-1. The diagram shows the schematic representation of the amino acid sequence of TIMP-1, including the disulfide bonds assigned by Williamson *et al.* (1990). Loops created by disulfide bridges are indicated (L1 through L6) (modified from Caterina *et al.*, 1997; 1998).

4.1.2 Multifunctional nature of TIMP-1.

Besides to its MMP inhibitory activity, TIMP-1 has shown to be involved in several body functions as listed in Table 4.2.

Table 4.2 Some reported functions of TIMP-1 (modified from Gomez *et al.*, 1996).

| Some reported functions of TIMP-1 | Reference |
|---|--|
| <ul style="list-style-type: none">• Inhibits active forms of MMPs | Gomez <i>et al.</i> , 1997. |
| <ul style="list-style-type: none">• Binds to pro MMP-9 | Goldberg <i>et al.</i> , 1992; Wilhelm <i>et al.</i> , 1989; Itoh and Nagase, 1995. |
| <ul style="list-style-type: none">• Role in embryonic bone tissue remodelling | Flenniken and Williams, 1990. |
| <ul style="list-style-type: none">• Role in gonadal steroidogenesis | Boujard <i>et al.</i> , 1995. |
| <ul style="list-style-type: none">• Role in tissue remodelling during tumour progression | Anne <i>et al.</i> , 1997; Yoshiji <i>et al.</i> , 1998; Yoshikawa <i>et al.</i> , 1999. |
| <ul style="list-style-type: none">• Growth promoting activity to wide range of cells | Hayakawa <i>et al.</i> , 1992. |
| <ul style="list-style-type: none">• Inhibits angiogenesis in bioassays | Moses, 1997. |
| <ul style="list-style-type: none">• Role in gonadal steroidogenesis, tissue remodelling of the reproductive system, and embryonic development | Gomez <i>et al.</i> , 1997. |
| <ul style="list-style-type: none">• Role in malignancy and other disease process | Gomez <i>et al.</i> , 1997. |
| <ul style="list-style-type: none">• Inhibition of angiogenesis | Gomez <i>et al.</i> , 1997. |
| <ul style="list-style-type: none">• Modulates cell morphology in vitro | Ray and Stetler-Stevenson, 1995. |
| <ul style="list-style-type: none">• Oligonucleotide/oligosaccharide binding ability | Muzin, 1993. |
| <ul style="list-style-type: none">• In vitro suppression of programmed cell death of B-cells | Gudez <i>et al.</i> , 1998. |
| <ul style="list-style-type: none">• Inhibits apoptosis of human breast epithelial cells | Li <i>et al.</i> , 1999. |
| <ul style="list-style-type: none">• Erythroid-potentiating activity | Docherty <i>et al.</i> , 1985 |

4.1.3 TIMP-1 Vesicles

The subcellular localization of TIMP-1 in PMNs was discovered by Price *et al.* (2000). This finding apparently revealed that, in PMNs, TIMP-1 is not constitutively secreted, but stored in one of the PMN vesicles. TIMP-1 was found to be located in distinct

organelles which are oval in shape, translucent, but smaller than azurophilic granules. These organelles do not contain any of the classical PMN granule marker proteins, which indicates that they were a novel organelle (Price *et al.*, 2000). Their sedimentation with the least dense specific granules in density gradient co-fractionation and their pleomorphic structure suggests that they are vesicle populations rather than granule populations (Price *et al.*, 2000). In leukaemia (HL-60) cells which are developmentally arrested in their maturation at the early promyelocytic stage, TIMP-1 is constitutively secreted (Kobayashi *et al.*, 1995; Janowska-Wieczorek *et al.*, 1999; Bar-Shavit *et al.*, 1985) like its other granule proteins (Cabec *et al.*, 1997). Price *et al.* (2000) discovered that MMP-9 and TIMP-1 colocalized in a minor vesicle population suggesting that the TIMP-1 granule is synthesized possibly after the synthesis of the gelatinase granule and MMP-9 (Price *et al.*, 2000).

The retention of TIMP-1 largely in a separate PMN granules indicates that PMNs can possibly release TIMP-1 when required and preliminary unpublished work indicated that TIMP-1 release was not regulated by calcium like other granules (Price *et al.*, unpublished data). In HL-60 cells, expression of TIMP-1 has been shown to be upregulated by several activators such as PMA, retinoic acid, vitamin D₃ and dimethyl sulfoxide (DMSO) (Bar-Shavit *et al.*, 1985) but how its release is regulated remains unknown.

TIMP-1 is not a strong inhibitor, therefore, its presence does not result in complete inhibition of MMPs thus limited proteolysis may occur in the presence of TIMP-1 or upon binding of TIMP-1 to MMPs (Murphy *et al.*, 1982).

4.1.4 TIMP-1 and its interaction with MMP-9

TIMP-1 forms a complex with the 92-kDa pro-gelatinase (pro-MMP-9) (Table 4.2) (Wilhelm *et al.*, 1989). In this complex, the C-terminal domain is bound to the C-terminal domain of pro-MMP-9 but the N-terminal domain of TIMP-1 is capable of

inhibition of other active MMPs (Itoh and Nagase, 1995) as the N-terminal domain of TIMP-1 only binds to active MMPs, but not to pro-MMPs (Murphy *et al.*, 1991). Even during complex formation with TIMP-1, pro-MMP-9 can be activated by organomercurials, trypsin and stromelysin-1, resulting in an active enzyme which can degrade types IV and V collagen (Wilhelm *et al.*, 1989; Goldberg *et al.*, 1992). Goldberg *et al.* (1992) also showed that in the absence of TIMP-1, pro-MMP-9 could form a covalent homodimer or a complex with MMP-1. In the presence of TIMP-1, however, the formation of a pro-MMP-9 homodimer complex with MMP-1, and activation of pro-MMP-9 by MMP-3 is prevented. TIMP-1 also cannot bind to the pro-MMP-9 homodimer. The TIMP-1/MMP-9 complex and all other complexes can be dissociated by 0.1 % SDS (Goldberg *et al.*, 1992; Triebel *et al.*, 1995). Dissociation of the enzyme and inhibitor by this procedure does not inhibit their activities (Itoh and Nagase, 1995; Triebel *et al.*, 1995).

4.2 Optimization of TIMP-1 detection

The aim of the project is to investigate the release of TIMP-1 and MMP-9 during complement-mediated phagocytosis of polystyrene beads. Previously Price *et al.* (2000) observed that TIMP-1 and pro-MMP-9 were not secreted into the PMN phagosome during phagocytosis of IgG-opsonized latex beads. Clearly this showed that MMP-9 and TIMP-1 have no intracellular role in such a case. However, in this study the indirect role of TIMP-1 and how it might be involved in the process of C1q-mediated internalization of particles is of interest.

For such a study the release of TIMP-1 may be monitored or measured in supernatants of PMNs incubated in the presence of polystyrene beads and complement. Under such test conditions the release of TIMP-1 and MMP-9 may be very low and, therefore, the sensitivity of detection methods had to be investigated and optimized and the best sensitive method chosen.

4.2.1 Western blot detection of TIMP-1 from PMN homogenates

Western blotting, western ligand blotting and enhanced chemiluminescence were all techniques which were considered to be among the best, most sensitive and straightforward methods of detecting TIMP-1. Before all these methods could be compared and the most sensitive method chosen for the final experiments on TIMP-1 release, the anti-TIMP-1 antibodies to be used had to be optimized.

In order to characterise antibodies to be used for TIMP-1 detection, a suitable source of TIMP-1 was required and PMN homogenates were chosen. As discussed in Chapter 3, the primary problem of homogenization is that proteins and enzymes are released from cells into an environment that is not ideal for their survival in an undegraded state. Itoh and Nagase, (1995) showed that TIMP-1 and MMP-9 can be degraded by PMN serine proteases in a time-dependent manner and can produce lower molecular weight forms of both proteins. When TIMP-1 was incubated with elastase, for example, it was degraded and lost its inhibitory function (Huang *et al.*, 1997). Reactive oxygen radicals can also degrade TIMP-1 (Stricklin and Hoidal, 1992). Thus, the addition of appropriate proteinase inhibitors and anti-oxidants in the homogenization buffer was required.

Even with the proteolytic activity of TIMP-1 degrading enzymes blocked many different molecular weight forms of TIMP-1 have been reported. The deglycosylated core protein molecule of TIMP-1 has a molecular weight of 20 kDa (Carmichael *et al.*, 1986) but, several glycosylated forms have been reported. TIMP-1 is generally reported to have an approximate molecular weight of 30 kDa (Price *et al.*, 2000; Cawston *et al.*, 1986; Hayakawa *et al.*, 1992). This most highly glycosylated form of TIMP-1 was reported by Triebel *et al.* (1995), Strickling and Welgus (1983), and Ritter *et al.* (1999) to have a molecular weight of 28.5 kDa. Other forms such as 24.5 kDa (Maccartney and Tsechesche, 1983), 26.4 kDa (Strickling and Welgus, 1983), 27.5 kDa (Cawston *et al.*, 1986), 28 kDa (Cawston *et al.*, 1986; Murphy *et al.*, 1981; Nagayama *et al.*, 1984), and 29 kDa (Drouin *et al.*, 1988) have also been identified. TIMP-1 also forms a 66 kDa homodimer (Price *et al.*, 2000). The 3 N-terminal inhibitory loops of TIMP-1 have also

been described to occur in 3 forms, a 24 kDa and 19.5 kDa glycosylated form and a 13 kDa unglycosylated form (Murphy *et al.*, 1991). The N-terminal form is responsible for binding and inhibition of active MMPs and N-terminal truncation of TIMP-1 severely decreases binding to proMMP-9 (Murphy *et al.*, 1991). The N-terminal form is responsible for binding and inhibition of active MMPs and an N-terminal truncation of TIMP-1 severely decreases binding of TIMP-1 to pro-MMP-9 (Murphy *et al.*, 1991).

For this study anti-MMP-9 antibodies (characterized in Section 3.7.1) against a 220 kDa dimer of MMP-9 expressed in *Pichia pastoris* yeast and anti-TIMP-1 antibodies against human TIMP-1 (isolated from synovial fluid by Marc-Oliver Luther, Department of Biochemistry, University of Bielefeld, Germany) raised in chicken, were used as well as two commercial antibodies, one against the C-terminus of TIMP-1 and a mouse monoclonal antibody, to ensure that the bands identified were TIMP-1. All antibodies were characterized and optimized in this study using methods such as western blot, enhanced chemiluminescence and various detergents.

4.2.1.1 Reagents

Anti-MMP-9 antibodies (characterized in Section 3.7.1) against a 220 kDa dimer of MMP-9 expressed in *Pichia pastoris* yeast (a gift from Volker Zollinger, University of Bielefeld) and anti-TIMP-1 antibodies against human TIMP-1 (isolated from synovial fluid by Marc-Oliver Luther, Department of Biochemistry, University of Bielefeld, Germany), raised in chicken, were a kind gift from Dr. Brendon Price, University of Natal, South Africa. A rabbit antibody against the C-terminus of TIMP-1 and mouse anti-TIMP-1 monoclonal antibody was purchased from Sigma Chemicals (St. Louis, Mo).

PMN lysis buffer [1 M NaCl/PBS, 10% (v/v) DMSO]. NaCl (0.585 g) was dissolved in PBS (pH 7.4) (10 ml) as described in Section 2.10.1 and 1 ml of DMSO was added.

Inhibitor buffer [2 mM PMSF, 1 mM EDTA, 1% (w/v) NaN₃]. PMSF (10 µl of 200 mM solution in methanol), EDTA (5 µl of 200 mM solution) and NaN₃ (0.01 g) dissolved in 1 ml storage buffer.

The reagents for the Bradford protein binding assay were prepared as described in Section 2.2.1.1.

4.2.1.2 Procedure

For western blotting PMNs were isolated from human blood using a Percoll density gradient 1.077 g/cm³ as described in Section 2.10.2. Some of the PMNs were immediately suspended in PMN lysis buffer, snap frozen in liquid nitrogen and stored at -20°C. When these PMNs were thawed, they were thawed in Inhibitor buffer. Another batch of PMNs isolates were mixed with treatment buffer and analysed within 24 h. All treated and untreated samples (1.59 µg), however, were separated by a Tris-tricine gel electrophoresis using reducing conditions and blotted (16 h). The membrane was subsequently probed with (7.2 µg/ml) chicken anti-TIMP-1 antibody with detergents (Brij-35, Tween-20 and Triton X-100) added into blocking- and antibody diluent solutions to a concentration of 0.3 % (v/v). Binding of the chicken anti-TIMP-1 antibody or rabbit anti-C-terminal antibody was detected using alkaline phosphatase conjugated rabbit anti-chicken antibody (1/100 000) or a mouse anti-rabbit antibody (1/30 000) and an NBT/BCIP substrate. All blots developed at the same time to allow comparison of reactivity with the use of different detergents.

4.2.1.3 Results and discussion

Initially western blot experiments were optimized by dot blots (results not shown) which showed that endogenous PMN alkaline phosphatase and peroxidases needed to be

quenched using levamisole or NaN_3 . The optimal concentration of chicken anti-TIMP-1 and rabbit anti-TIMP-1 antibody was easily determined by this method but the mouse monoclonal antibody could not be optimized using such a technique. This may be due to conformational change in TIMP-1 induced after SDS-PAGE which may either expose or destroy the epitope targeted by the specific antibody used (Nesbitt and Horton, 1992).

During western blot detection of TIMP-1, both the chicken and the rabbit anti-TIMP-1 antibodies appeared specific as they revealed bands of reactivity previously found for TIMP-1. However, even after optimization, using normal western blotting procedures, bands detected in western blots were relatively faint (Figure 4.2 E).

To overcome possible conformational changes in epitopes due to the treatment of proteins during the electrophoresis and blotting processes, detergents were employed in an attempt to enhance immunoreactivity. Detergents may alter or refold the proteins to their original conformation and are commonly used in western blotting and western ligand blotting for removal of non-specifically binding antibodies. Among the detergents used, the non-ionic detergents such as Tween-20, Nonidet P-40 and Triton X-100 (Davies *et al.*, 1994), Brij-35 (Zeng *et al.*, 1996) are used most commonly. These non-ionic polyoxyethylene type detergents, in incubation buffers, may induce renaturation of blotted proteins while other types of detergents do not (Klinz, 1994). Proper re-folding and stabilization of proteins only occurs when such detergents are present at a concentration above their critical micelle concentration (CMC) (Tandon and Harawitz, 1987) which would be [(0.09 mM) Brij-35, (0.049-0.059 mM) Tween-20 and (0.2-0.9 mM) Triton X-100] (Neugebauer, 1990; Bhairi, 1997). Besides inducing increased antigen detection by renaturation, protein movement and flexibility is also increased and may induce the appropriate conformation for binding by antibodies and other proteins (Zeng *et al.*, 1996).

The western blotting experiments performed with such detergents showed the most intense bands at 31 kDa when Brij-35 was used (Figure 4.2 B). The molecular weight of this band is comparable to the 31 kDa TIMP-1 inhibitor band purified from skin

fibroblasts by Welgus *et al.* (1979). Brij-35 has been shown to be important in the extraction of low solubility proteins. However, the intensity of the immunoreactivity of TIMP-1 in the current study was decreased or abolished when Tween-20 or Triton X-100 was used (Figure 4.2 C and 4.2 D). When no detergents were used the bands were less evident possibly due in part to a high background and possibly also to the absence of a detergent's surfactant action in removing weakly bound antibodies (Figure 4.2 E). Using the chicken antibody, even though the most intense bands appeared in blots treated with detergents were those with a 31 kDa band, other minor bands were also observed (Figure 4.2 arrows, B, C, D and E). The immunoreactivity of these bands were enhanced by some detergents and suppressed by others (Figure 4.2 compare B, C, D and E). It was evident that the inclusion of inhibitors and anti-oxidants (DMSO) favoured the preservation of higher molecular forms of proteins [Figure 4.2 A, compare "a" (no inhibitors) and "b" (inhibitors)] and (Figure 4.2 B, C, D and E, compare "a" and "b"). DMSO is known to prevent oxidative degradation of TIMP-1 (Stricklin and Hoidal, 1992). This is of particular interest as it seems that all detergents seem to favour the reactivity of higher (~30 kDa) over lower (~15 kDa) molecular weight forms of TIMP-1.

PMN serine proteases degrade TIMP-1 from the N-terminal domain in a time-dependent manner, and Itoh and Nagase (1995), have reported 16 and 17 kDa breakdown products of this protein. Therefore, it was not surprising to observe a 14.5 and 16.5 kDa form or possible total digestion of TIMP-1 in samples where no inhibitors (1 mM EDTA, 2 mM PMSF, 1% (w/v) NaN_3) were added (Figure 4.2 B, C, D, E, sample "a"). The time-dependent degradation of TIMP-1 is also illustrated by the fact that, when fresh PMN homogenates (containing no anti-oxidants and inhibitors) were blotted and Tween 20 used in washes and diluents, only a 31 kDa band of undegraded TIMP-1 was detected (Figure 4.3 B). In other blots where no inhibitors were included the molecular weight of bands decreased with time (Figure 4.2, B, C, D and E sample "a")

The lower molecular weight fragments of TIMP-1 from untreated samples blotted after longer period of storage were strongly detected even in the absence of detergents, showing that no refolding was required for chicken antibody binding and detection of

these forms of TIMP-1 (Figure 4.2 E, sample “a”). Generally for the PMN homogenates which were treated with anti-oxidants and inhibitors, however, only one band i.e 31 kDa was obtained (Fig.4.2, B, C, D and E, sample “b” compare with “a”, arrows).

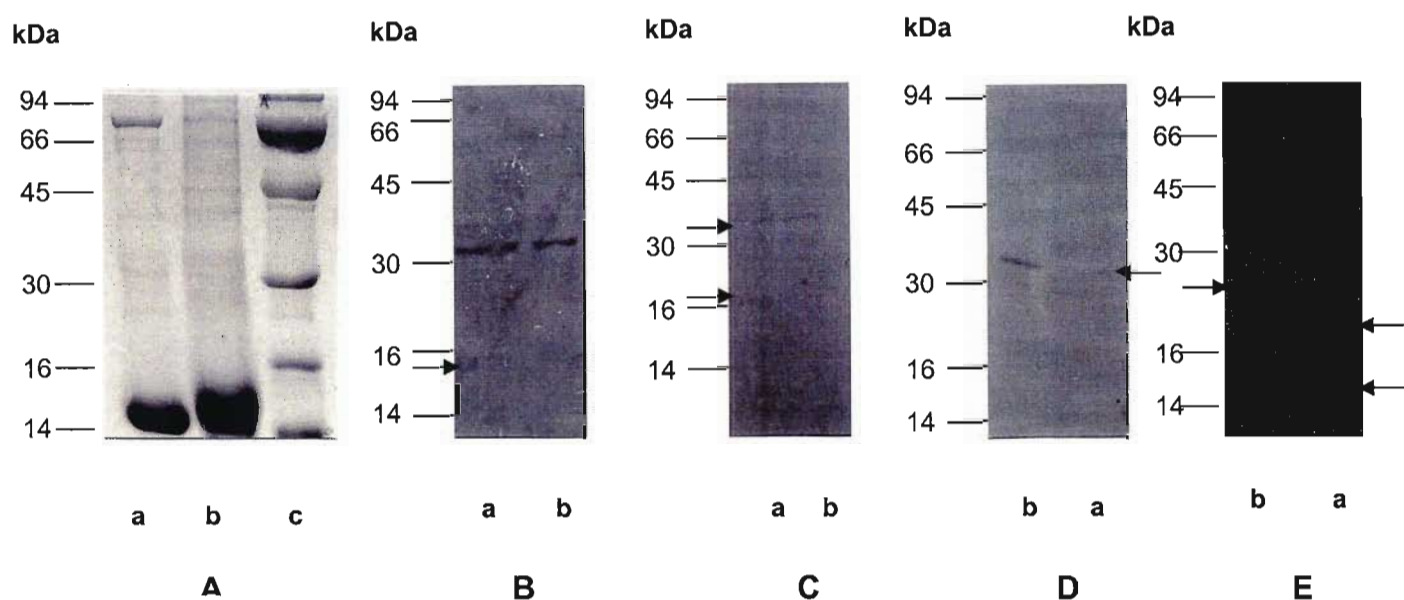


Figure 4.2 Western blotting of TIMP-1 from human PMN homogenates using detergents.

PMNs were isolated from human blood over Percoll density gradient 1.077 g/cm³ as described by Roos and De Boer (1986) and either diluted with equal amount of reducing treatment buffer (a) or were first suspended in 1 M NaCl/PBS, and 10% (v/v) DMSO, snap frozen in liquid nitrogen and thawed into a buffer containing 1 mM EDTA, 2 mM PMSF, 1% (w/v) NaN₃ before mixing into equal amount of reducing treatment buffer (b), molecular weight markers (c). PMN homogenates (1.59 µg) (a) or (b) were separated on a Tris-tricine gel (A) and blotted (16 h) (B, C, D, E). The membrane was probed with (7.2 µg/ml) anti-TIMP-1 antibody with detergents added into blocking, and antibody diluent solutions in a concentration of 0.3 % (v/v) in all except one specimen (no detergent) (E), Brij-35 (B) Triton X-100 (C) Tween 20 (D). Antibody binding was detected by alkaline phosphatase conjugated rabbit anti-chicken antibody (1/100 000) and NBT/BCIP substrate.

The chicken antibody generally bound to the lower molecular weight, possible degradation fragments of TIMP-1 and to the mature molecule and no high molecular weight forms > 31 kDa (Figure 4.2) where the rabbit antibody bound 30 kDa and greater (Figure 4.4). Since TIMP-1 is usually degraded from the N-terminal region, such a labelling pattern may indicate that either there is no epitopes in the N-terminal region that is recognized by the chicken antibodies or the N-terminal region needed to be removed in order to expose the epitopes which form the target of the chicken antibodies.

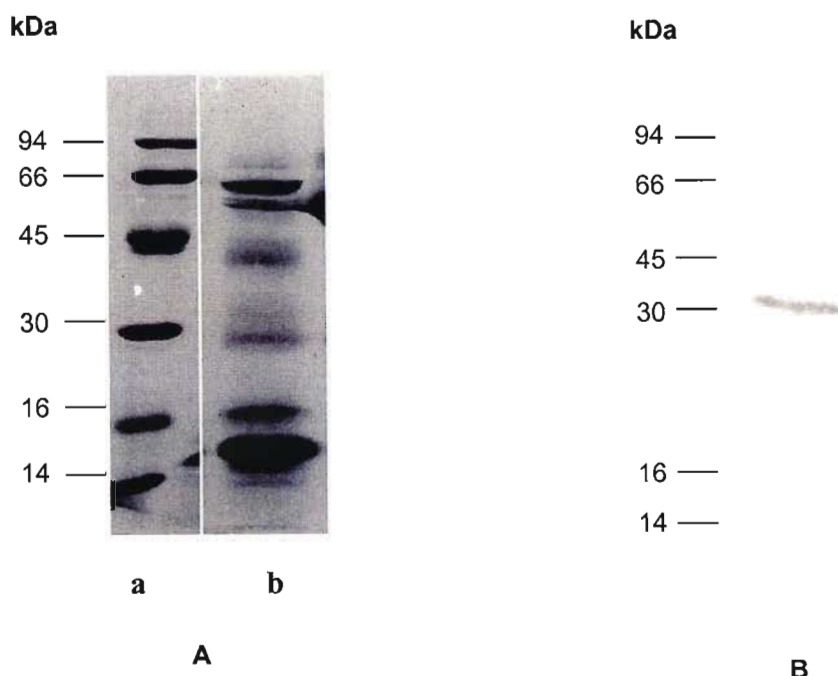


Figure 4.3 **Western blotting of TIMP-1 from untreated human PMN homogenates.** PMNs were isolated from human blood over Percoll density gradient 1.077 g/cm^3 as described by Roos and De Boer, (1986) and immediately diluted with equal amount of reducing treatment buffer (no protease inhibitors or anti-oxidants). Homogenates (20 μl) (A,b) molecular weight markers (A,a) were separated on a reducing Tris-Tricine gel (A), and blotted (16 h) (B). The membrane was probed with (7.2 $\mu\text{g/ml}$) chicken-anti-TIMP-1 antibody with Tween-20 added into blocking, and antibody diluent solutions in a concentration of 0.3 % (v/v). Antibody binding was detected by alkaline phosphatase conjugated rabbit anti-chicken antibody (1/100 000) and NBT/BCIP substrate.

As mentioned, the rabbit anti-TIMP-1 antibody against the C-terminal region of the molecule, labelled the higher molecular weight bands, 30 kDa and greater (Figure 4.4 B and C weakly, and D most successfully) except in blots treated with Triton X-100 where a lower~15 kDa band was also detected (Figure 4.4 D, arrow).

In the presence of Brij-35, a 31 kDa probable TIMP-1 band, seen with the chicken antibodies, was not detected by the rabbit antibodies (Figure 4.4 B, arrows). Both Brij-35 and Triton X-100 assisted the detection of the presumptive 66 kDa TIMP-1-homodimer previously described by Price *et al.* (2000), using the chicken antibodies (Figure 4.4, B and D). Unlike the chicken antibody, the rabbit antibody recognized

higher molecular weight forms almost exclusively indicating that higher bands may contain the C-terminal epitope, where low molecular weight forms may not, or where the higher molecular weight forms contain the C-terminal epitope domain which may become accessible to the rabbit antibody with certain detergent treatments.

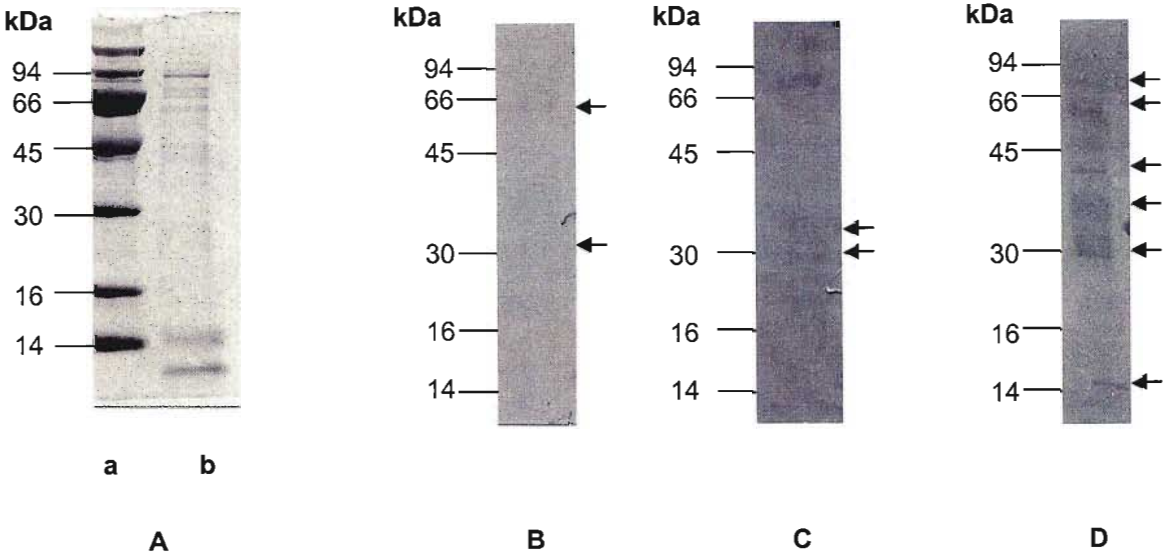


Figure 4.4 Western blotting of TIMP-1 from human PMN homogenates using rabbit antibody. Molecular weight markers and PMN homogenates (1.59 μ g) separated on a reducing Tris-Tricine gel (A) before blotting into nitrocellulose for 16 h (B, C, D). The membranes were subsequently probed with 0.2 μ g/ml rabbit anti-TIMP-1 antibody (B, C, D) with detergents added into blocking, and antibody diluent solutions to a concentration of 0.3 % (v/v) blotted homogenates were probed with Brij-35 (B) Tween-20 (C) Triton X-100 (D).

In experiments where Tween-20 and the rabbit antibody was used, bands at about 73, 33 and 30 kDa, but no lower molecular weight bands, were detected (Figure 4.4 C). Triton X-100 treatment similarly revealed a greater number of higher molecular weight bands at 73, 66, 43 and 30 kDa (Figure 4.4 D) with the rabbit antibody. The detection of a 15 kDa form of TIMP-1 using Triton-X 100 and the rabbit antibody was unexpected as it was previously reasoned that the C-terminal domain may be absent in low molecular weight forms of TIMP-1 (approx. 15 kDa) as no reactivity was generally seen with the rabbit anti-C-terminal peptide antibody. It was reasoned that the C-terminal fragment

targeted by the rabbit antibody must be present and especially accessible in a Triton X-100-treated blot (Figure 4.4, D, arrows). If this is so, this band should also have been detected in blots treated with Brij-35 or Tween-20 but may not have reacted with the rabbit antibody due to the unfavourable conformation of the epitopes in Brij-35- or Tween-20-treated blots (Figure 4.4 B and 4.4 C).

Trimming of TIMP-1 is usually known to occur from the N-terminal end, however, especially when in a complex active with MMPs (Itoh and Nagase, 1995). Since the rabbit antibody to a C-terminal peptide recognises these forms especially well when blots are treated with Triton X-100 it seems that Triton X-100 exposes the C-terminal epitopes in the blotted protein. The chicken antibody must recognise a different epitope to that recognized by the rabbit antibody, however, as Triton X-100 produced a lower signal with the chicken antibody (Figure 4.2 C and Figure 4.4 D). It would, therefore, appear that the immunoreactivity of various forms of TIMP-1 or other antigens may be enhanced or depressed by the use of specific detergents or specimen pretreatments.

4.2.2 Western ligand blotting of TIMP-1 in PMN homogenates.

TIMP-1 has the special property of being able to bind its target MMPs after being blotted (Osthues *et al.*, 1992) and even in its reduced form (Price *et al.*, 2000). This type of complex was confirmed by Osthues *et al.* (1992) who detected TIMP-1 using western ligand blotting of TIMP-1 i.e. a pretreatment with pro-MMP-9/MMP-9 followed by detection of any band of MMP-9. This method is important especially if cross reactivity with TIMP-2 is suspected (Oliver *et al.*, 1997). As detergents were effective in altering the conformation of epitopes in blotted proteins, detergents were also introduced to assist binding of ligands in western ligand blotting.

4.2.2.1 Reagents

Reagents for the isolation of PMNs were prepared as described in Section 2.10.1. PMN lysis buffer and Inhibitor buffer was prepared as described in Section 3.7.1. Reagents for western ligand blotting were prepared as described in Section 2.6.2.1 and 2.7.2. The amount of protein to be loaded was measured by Bradford protein binding assay as described in Section 2.2.1.2.

4.2.2.2 Procedure

For western ligand blotting, PMNs were isolated from human blood as described in Section 2.10.2. The isolated PMNs were immediately suspended in PMN lysis buffer and snap frozen in liquid nitrogen. When required PMNs were thawed into an inhibitor buffer (Section 2.10.2) and mixed into equal amount of reducing treatment buffer (Section 2.6.1.1). Homogenates (0.8 µg/lane) were separated on a Tris-tricine gels stained and destained (Section 2.6.1.1). A replicate gel was blotted into nitrocellulose for 16 h. The nitrocellulose was blocked with TBS/milk and treated with TBS/milk containing 4.26 µg/ml of a crude 20-30% (m/v) NH₄SO₄ TPP fraction containing pro-MMP-9 (prepared in Section 3.6.3). Various detergents [0.3% (v/v) Brij-35, Tween-20 or Triton X-100] were added to blocking, and antibody diluents. Membranes were probed for MMP-9 using a chicken anti-MMP-9 antibody (219 µg/ml) (Section 3.6.2) and detected with rabbit anti-chicken (1/100 000) alkaline phosphatase conjugated secondary antibody and NBT/BCIP substrate.

4.2.2.3 Results and discussion

Pro-MMP-9 (92 kDa) and mature MMP-9 (~81 kDa) were evident in blots of PMN homogenates not overlaid with crude pro-MMP-9 (Figure 4.5 F). Bands other than

those seen in Figure 4.5 F, after treatment with pro-MMP-9 may, however, be assumed to be due to binding of MMP-9 by TIMP-1. When no MMP-9 was overlaid and blotting was performed no “TIMP-1 bands” were detected proving the specificity of reactivity of the MMP-9 antibody and the detection system.

As mentioned, the C- and N-terminal regions of TIMP-1 can form a complex with active or pro-MMP-9 (proMMP-9), respectively. TIMP-1 binds non-covalently via its C-terminal domain to the C-terminal domain of active MMPs (Itoh and Nagase, 1995), whereas it binds via the N-terminal inhibitory domain to pro-MMP-9 (Murphy *et al.*, 1991). Different bands may be unpredictably revealed by western ligand blotting, due to the differential binding of either the pro and/or mature MMP-9 enzymes in the crude MMP preparation and the effects of the detergents used. Hence the pattern of labelling may be difficult to interpret, irrespective of how the TIMP-1 is processed.

Extra MMP-binding TIMP-1 bands appeared in the ligand blot when Brij-35, and to a lesser extent Tween-20, were used in the overlay and blotting process (Figure 4.5 B and C, respectively). In western ligand blotting detection of TIMP-1, Brij-35 showed the best detection of an approximately 25 kDa form of TIMP-1, revealing bands of reactivity at approximately 25, 30, 40 and 59 kDa (Figure 4.5 B). These bands may arise due to binding of mature MMP-9 to the C-terminal residues of TIMP-1 which seem to be exposed in the higher molecular weight forms of blotted TIMP-1, according to the labelling results of the rabbits against the C terminal of TIMP-1. Alternatively they may arise due to binding of pro-MMP to the N-terminal domain of TIMP-1 present in uncleaved high molecular weight forms of blotted TIMP-1.

The addition of Tween-20 to buffers (Figure 4.5 C) revealed additional bands at 14, 15 and 22 kDa, possibly representing degraded forms of TIMP-1. As mentioned, the only blots which showed lower molecular weight TIMP-1 fragments were those that were blotted in the presence of Tween-20 (Figure 4.5 C). Trimming of TIMP-1 in the presence of enzymes such as elastase (which is also present in the PMN homogenates) also usually occurs from the N-terminus. It may be hypothesized, therefore, that Tween-

20 favours the binding of the C-terminal region of active MMP-9 to the C-terminus of truncated TIMP-1 (Figure 4.5, C) [whereas Brij-35 possibly favours the interaction of the N-terminal inhibitory region of blotted TIMP-1 which is largely missing in most lower molecular weight forms of TIMP-1 below 20 kDa (the deglycosylated uncleaved form) with proMMP-9, hence few of these bands are revealed in the Brij blots (Figure 4.5 B)].

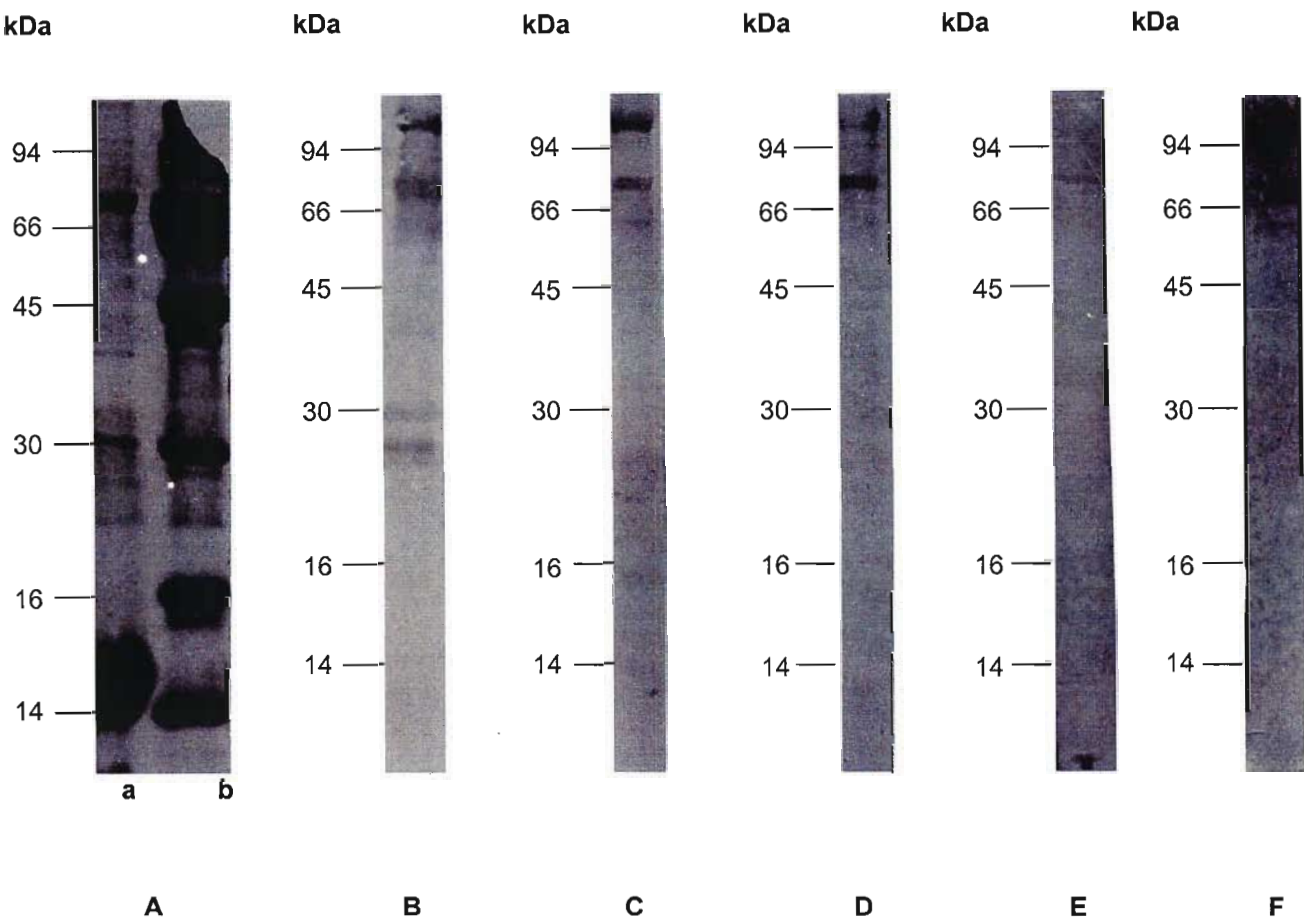


Figure 4.5 Western ligand blotting and western blot detection of TIMP-1 and MMP-9, respectively. PMN homogenates (0.8 µg) were separated on a reducing Tris-tricine gel (A) before blotting into nitrocellulose (B, C, D, E, F). The nitrocellulose was treated with TBS/milk (F) or TBS/milk containing 4.26 µg/ml pro-MMP/MMP-9 (B, C, D, E). Various detergents [0.3% (v/v)] added to all blocking, and antibody diluents: Brij-35 (B), Tween-20 (C) Triton X-100 (D) with one exception (no detergent) (E). Membranes were probed for MMP-9 (219 µg/ml) and detected with alkaline phosphatase conjugated secondary antibody and NBT/BCIP substrate.

The addition of Tween-20 to buffers (Figure 4.5 C) revealed additional bands at 14, 15 and 22 kDa, possibly representing degraded forms of TIMP-1. As mentioned, the only blots which showed lower molecular weight TIMP-1 fragments were those that were blotted in the presence of Tween-20 (Figure 4.5 C). Trimming of TIMP-1 in the presence of enzymes such as elastase (which is also present in the PMN homogenates) also usually occurs from the N-terminus. It may be hypothesized, therefore, that Tween-20 favours the binding of the C-terminal region of active MMP-9 to the C-terminus of truncated TIMP-1 (Figure 4.5, C) [whereas Brij-35 possibly favours the interaction of the N-terminal inhibitory region of blotted TIMP-1 which is largely missing in most lower molecular weight forms of TIMP-1 below 20 kDa (the deglycosylated uncleaved form) with proMMP-9, hence few of these bands are revealed in the Brij blots (Figure 4.5 B)].

Triton X-100 showed only 76.5 and 104 kDa bands of MMP-9 showing inability to assist binding of TIMP-1 to overlaid MMP-9 (Figure 4.5 D) and gave very similar results to those where no detergent was added (Figure 4.5, E). Results showed that detergents such as Brij-35 and Tween-20 seem to facilitate binding of MMP-9 to various forms of TIMP-1 (Figure 4.5 B and C). Therefore, western ligand blotting and western blotting indicates that particularly Brij-35 possibly facilitates more protein-protein interaction and greater mobility of blotted TIMP-1 allowing binding between MMP-9 and blotted TIMP-1 or the binding of antibodies to particular TIMP-1 epitopes present in the uncleaved form of TIMP-1 (30 kDa, 20 kDa and the inhibitory N-terminal domain, Murphy *et al.*, 1991).

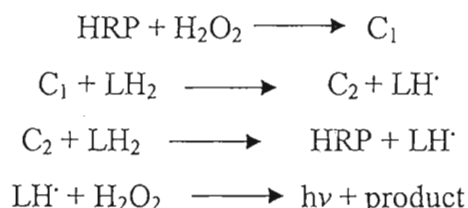
For the non-overlaid blot (Figure 4.5 F) the (NGAL/ MMP-9) complex 125-130 kDa bands obtained was western blotting by others (Hibbs *et al.*, 1985, 1992; Goldberg *et al.*, 1992) was absent. This was anticipated as such complex binding are absent when a sample is treated with reducing buffer (Hibbs *et al.*, 1982; 1985).

Similar studies by Price *et al.* (2000), using western ligand blots for the detection of TIMP-1 from PMN homogenates, showed only a 30 and a 66 kDa band. In the current studies, however, binding of MMP-9 was shown to be ligand-concentration-dependent, as 0.17 µg/ml of the crude TPP MMP-9 fraction did not show binding of TIMP-1 (results not shown), whereas addition of 4.26 µg/ml of this fraction revealed the additional bands which possibly correspond to TIMP-1.

4.2.3 Enhanced chemiluminescence (ECL) detection of TIMP-1 in PMN homogenates

Enhanced chemiluminescence (ECL) methods are generally preferred to western blotting methods, because chemiluminescence combines high sensitivity, and speed of image development. Blots can be probed and easily imaged using X-ray type paper (Olesen *et al.*, 2000). ECL is 10 times more sensitive than the most effective chromogenic substrate, alkaline phosphatase (Graf and Friedl, 1999). Techniques comparable sensitivity involve radioactivity. These methods, however, are often not used due to the hazardous nature of radioisotopes (Nesbitt and Horton, 1992). The main components of the ECL system are the HRP conjugated secondary antibody and luminol. In the presence of the peroxidase enzyme, luminol and H₂O₂, luminol donates two electrons to the HRP-H₂O₂ complex to free the enzyme complex in a two step system, followed by several reactions to give light (Prichard and Cormier, 1968).

The reaction can be represented:



Where C₁ represents the classical ES complex between HRP and H₂O₂,

C₂ represents a 1-equivalent reduction of C₁ by luminol (LH₂) to produce a luminol radical (LH).

Thus the light producing step is a purely chemical one that does not directly involve participation of an enzyme complex (Prichard and Cormier, 1968).

Usually chemical enhancers are added to create vigorous reactions. The light emitted is then captured by hyperfilm ECL or an X-ray type paper with a gelatin emulsion containing silver bromide which forms a latent image which is subsequently developed using conventional photographic developer solution.

Optimization of ECL system requires less primary and secondary antibodies than conventional western blotting due to its high sensitivity compared to chromogenic systems. This method was, therefore, compared with conventional western blot and western ligand blotting methods of the detection of TIMP-1.

4.2.3.1 Reagents

Reagents for SDS-PAGE was prepared as described in Section 2.6.2.1. Reagents for western blotting were prepared as described in Section 2.7.2. PMN lysis buffer was prepared as described in Section 2.10.1. Endogenous peroxide quenching solution, substrate for HRP, luminol detection solution and other ECL reagents were prepared as described in Section 2.7.2. Bradford protein binding assay reagents were prepared as described in Section 2.2.1.2.

4.2.3.2 Procedure

For ECL detection of TIMP-1, PMN homogenates were isolated from human blood as described in Section 2.10. After isolation PMNs were immediately suspended in PMN lysis buffer (Section 2.10.1) and snap frozen in liquid nitrogen. When required PMNs were thawed into a buffer containing 1 mM EDTA, 2 mM PMSF, 1% (w/v) NaN₃ and mixed into equal amount of reducing treatment buffer (Price *et al.*, 2000). PMN homogenates (0.8 µg/lane) and molecular weight markers (5 µl/lane) were separated on

a Tris-tricine gel and blotted into nitrocellulose as described in Section 2.7.3. The membranes were probed with 7.2 µg/ml chicken anti-TIMP-1 IgY or 10 µg/ml anti-molecular weight IgY antibody with detergents [0.3% (v/v), Brij-35, Tween-20 or Triton X-100] added to blocking and antibody diluents (TBS/BSA). Binding of antibody was subsequently detected with rabbit anti-chicken HRPO conjugated secondary antibody (1/100 000). Enhanced chemiluminescence was performed using luminol as substrate exposed into X-ray film (15 s) and the film was developed in X-ray film development solution and fixed in fixer solution (Section 2.8.1).

4.2.3.3 Results and discussion

Blots treated with Triton X-100 and Tween 20 showed a 30, 24, 20, 16, 15, 14, and ~12 kDa bands (Figure 4.6 A and B). The blot treated with Brij-35 also showed very faint 30 and 14 kDa bands (Figure 4.6 C), all of which possibly represent TIMP-1. Unlike the western blotting and western ligand blotting, therefore, the best detection of TIMP-1 was observed when Triton X-100 and Tween-20 were used in all steps (Figure 4.6 A and B).

This enhanced detection of TIMP-1 using detergents still shows that Tween-20 seems to favour reactivity of the lower molecular weight, possible C-terminal domain of TIMP-1 degradation products as shown with ECL (Figure 4.6 B) and previously shown in western ligand blotting (Figure 4.5 C) where MMP-9 is overlayed. This was not as evident in conventional western blots with this detergent (Figure 4.2 B). Whereas the inclusion of Brij-35 seemed to promote good binding of MMP-9 to blotted TIMP-1 in western ligand blots (Figure 4.5 B) and immunoreactivity with the chicken antibody (Figure 4.2 B) it did not seem to enhance ECL results to the same extent (Figure 4.6 C), an inexplicable result. ECL additionally showed chicken antibody reactivity with these low molecular weight bands of TIMP-1 using Triton X-100 (Figure 4.5 A). This detergent did not seem to favour western blotting of MMP-9 (Figure 4.5 D) or reactivity of these forms in conventional binding (Figure 4.2 C). These results are hard to explain

in the light of almost approximate results in conventional western blotting unless antibodies are exhibiting prozone effects.

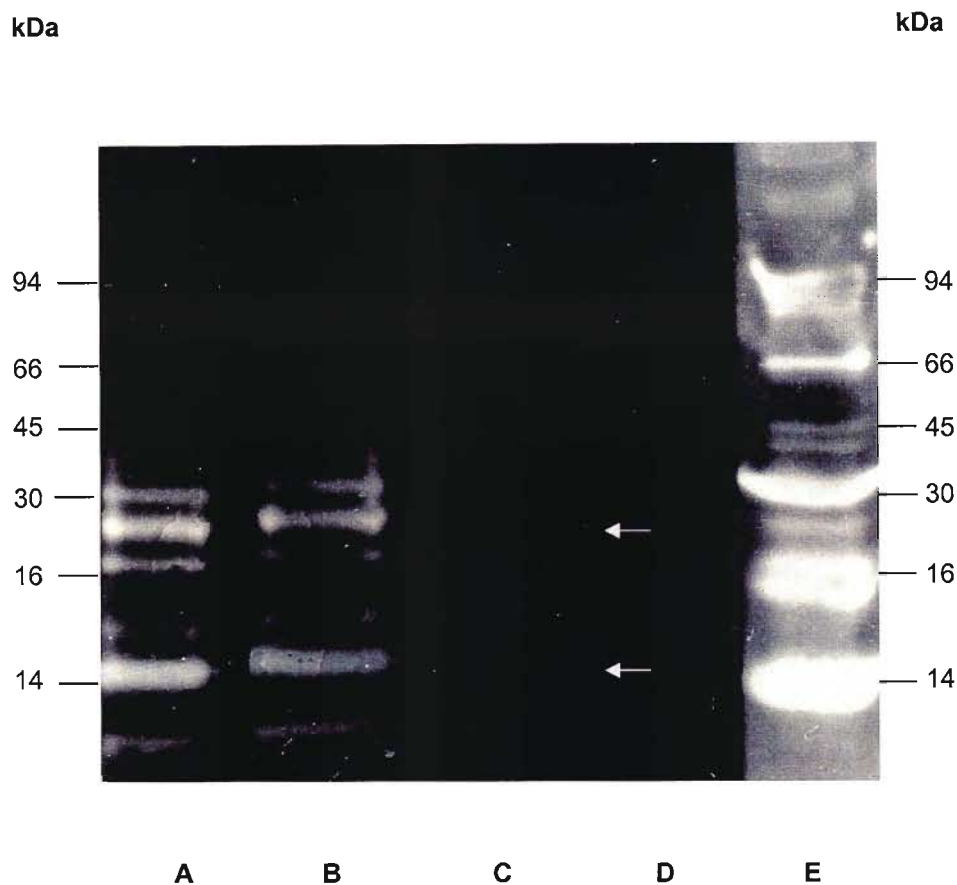


Figure 4.6 Enhanced chemiluminescence detection of TIMP-1 from PMN homogenates. PMN homogenates (0.8 μ g) were separated on a Tris-tricine gel and blotted into nitrocellulose membranes. The membranes were probed with 7.2 μ g/ml anti-TIMP-1 IgY (A, B, C, D) and 10 μ g/ml anti-molecular weight IgY (E). Detergents [0.3% (v/v)] added to blocking, and antibody diluents Brij-35 (C), Tween-20 (B) Triton X-100 (A) and no detergent (D). The membrane was then detected with HRPO conjugated secondary antibody (1/100 000). Enhanced chemiluminescence was performed using luminol (Amersham Pharmacia Biotech.) as substrate and exposed onto x-ray films (15 sec). The film was then developed and fixed.

4.2.4 Reverse zymographic detection of TIMP-1 from PMN homogenates

Optimization of reverse zymography to a picogram sensitivity level for TIMPs has previously been performed by Oliver *et al.* (1997). The method, however, was optimized using recombinant MMP-9. Using such a system 60 and 40 pg of TIMP-2 and TIMP-1,

respectively was detected. To optimise detection of TIMP-1 for the current study reverse zymograms were performed using PMN homogenates and MMP isolated as described in Section 3.6.

4.2.4.1 Reagents

Electrophoresis reagents for Tris-tricine gel electrophoresis were prepared as in Section 2.6.2.1. Renaturation solution, gelatinase zymography development buffer, fixing/destaining solution, and staining solution were prepared as described as in Section 2.9.2. PMN lysis buffer was prepared as described in Section 2.10.1. The amount of protein to be loaded was measured by Bradford protein binding assay as described in Section 2.2.1.2.

4.2.4.2 Procedure

For reverse zymographic analysis, PMNs were isolated from human blood as described Section 2.10, suspended in PMN lysis buffer and snap frozen in liquid nitrogen, thawed into a PMN lysis buffer (Section 3.7.1) and mixed into equal amount of reducing treatment buffer (Section 2.6.1.1). Various amount of PMN homogenates (79.5 ng - 636 ng) were separated on a Tris-tricine gel (Section 2.6.2.2). The same fractions were separated by reverse zymography with 15% (w/v) Laemmli gel co-polymerized with 0.01 g/ml gelatin and 32 µg/ml crude pro-MMP-9 (Section 3.6.2). The gels were washed in 2.5% (v/v) Triton X-100 and incubated in development buffer at 37°C (Section 2.9.2) and the gels were stained and destained before being visualized and photographed in a light box and captured (Section 2.9.2).

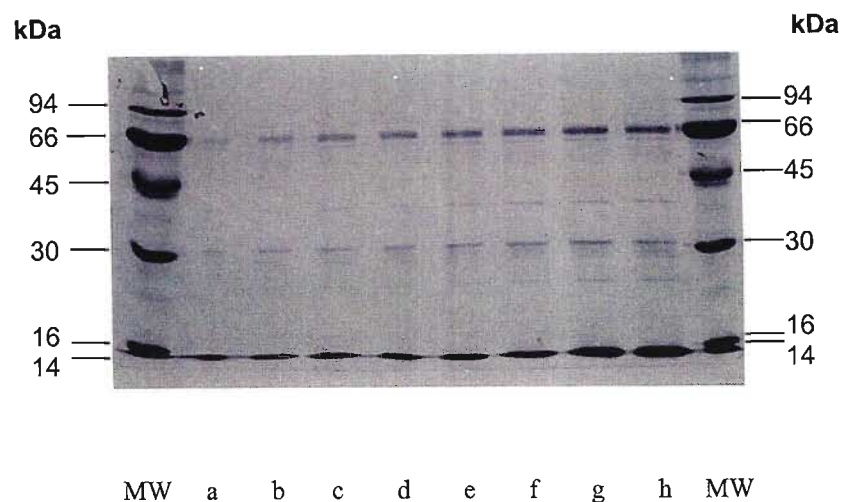
The identity of the presumptive TIMP-1 protein bands was also confirmed by western blotting of PMN homogenates. i.e. separation on a 15% Tris-tricine gel, blotting into nitrocellulose for 16 h and probing with 7.2 µg/ml chicken anti-TIMP-1 IgY in TBS/BSA buffer followed by (1/100 000) dilution of alkaline phosphatase-conjugated

rabbit anti-chicken secondary antibody containing 0.3 % (v/v) Brij-35. Binding was visualized with NBT/BCIP substrates (Section 2.7.3).

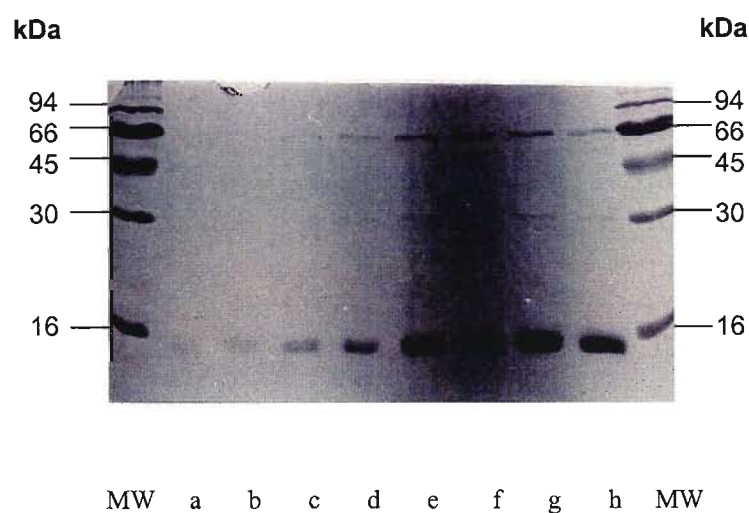
4.2.4.3 Results and discussion

PMN proteins were seen to separate into 3 major bands at 66, 30 and 15 kDa (Figure 4.7 A). Reverse zymogram analysis was more sensitive than Tris-tricine separation and Coomassie blue staining and seemed to resolve an intense degraded inhibitory TIMP-1 band at 14 kDa and minor bands at 30 (Figure 4.7, B). The 14 kDa band may represent the degraded inhibitory form of TIMP-1 produced by PMN serine proteinases (Itoh and Nagase, 1995), the serine protease inhibitors and other inhibitors of TIMP-1 being comparatively ineffectual in preventing degradation of TIMP-1 (Figure 4.7 B and Figure 4.8).

This was also shown by western blot analysis by the presence of a 14 kDa form of TIMP-1 (Figure 4.8). The minor 30 kDa band observed in the reverse zymogram was also confirmed as TIMP-1, being immuno-reactive with anti-TIMP-1 antisera (Figure 4.8). The 66 kDa band evident in the reverse zymogram (Figure 4.7 B) was, however, not evident in the western blot (Figure 4.8). Brij-35 was used in western blotting and usually favours detection of lower not higher bands (Figure 4.2, B). This is not surprising. The blot should perhaps be repeated without detergents. The reverse zymogram revealed TIMP-1 bands as little as 80 ng of PMN homogenate. Thus, this method appears very sensitive when compared to western blotting.



A



B

Figure 4.7 Tris-tricine separating and reverse zymographic detection of TIMP-1 from PMN homogenates.

Homogenates loaded (a, 79.5 ng; b, 159 ng; c, 238 ng; c, 318 ng; d, 397 ng; e, 477 ng; f, 556 ng; and g, 636 ng) were separated on a Tris-tricine gel and followed by staining and destaining (A). For reverse zymography the same fractions were separated in 15% Laemmli gel co-polymerised with 0.01 g/ml gelatin and 32 µg/ml crude TPP fractionated MMP-9 (B). The proteins were renatured in Triton X-100 and incubated in developing buffer for 16 h before staining.

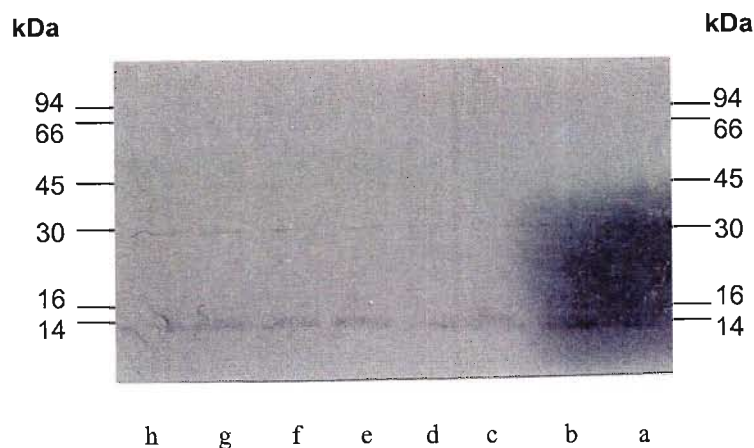


Figure 4.8 **Western blot analysis of TIMP-1 from PMN homogenates.**
Homogenates loaded from right to left (0.0795 μ g, 0.159 μ g, 0.238 μ g, 0.318 μ g, 0.397 μ g, 0.477 μ g, 0.556 μ g, and 0.636 μ g) were separated on a Tris-tricine gel (used for reverse zymography) and blotted into nitrocellulose. Nitrocellulose membranes were probed with 7.2 μ g/ml chicken anti-TIMP-1 IgY and rabbit anti-chicken alkaline phosphatase linked IgG antibody followed by NBT/BCIP substrate.

4.2.5 Discussion

The TIMP-1 protein seems to be unique in its apparent ability to be mobile after blotting, a characteristic shown by its ability to bind overlaid MMP-9 in western ligand blots. It is known that the N-terminal region of TIMP-1 contains the inhibitory domain, while the C-terminal region contains the domain which binds the MMP (usually active MMP, except in the case of proMMP-9) (Itoh and Nagase., 1995). Since the rabbit peptide antibody seems to bind only the higher molecular weight forms of TIMP-1 (30 kDa and greater) it would seem that the higher molecular weight forms contain the C-terminal domain but the lower molecular weight forms (below 30 kDa) may have lost this due to the proteolytic activity of e.g. human leukocyte elastase (Nagase, 1997). If this is so then it would appear that the chicken polyclonal antibody may recognize an epitope at or near the inhibitory domain, a domain that is not exposed in the blotted higher molecular weight forms, irrespective of which detergent was used.

Brij-35 added to the western blotting wash and diluents favoured the binding of MMP-9 to the higher molecular weight forms of blotted TIMP-1 (25 kDa and greater) while Tween-20 favoured the binding of MMP-9 to the lower molecular weight forms (25 kDa and below). It would seem that Brij-35 made the C-terminal domain accessible for MMP-9 binding but not the inhibitory N-terminal domain. In ordinary blots using chicken antibodies only the 30 kDa band was bound (and not higher M_r forms) as the N-terminal domain does not seem to be exposed for binding by the antibody upon Brij-35 exposure. Tween-20, however, seems to favour binding of MMP-9 to the lower molecular weight forms of TIMP-1 (25 kDa and less) in western ligand blots i.e. must theoretically expose the N-terminal or C-terminal domain of the blotted TIMP-1.

Tween-20 does not, however, enhance the immunoreactivity of the lower molecular weight forms of TIMP-1 using the chicken antibody as would be anticipated. The action of the detergents are, therefore, quite subtle and difficult to interpret and predict but may be useful for demonstration of different forms of TIMP-1.

CHAPTER FIVE

C1q-MEDIATED PMN STIMULATION IN PHAGOCYTOSIS

5.1 Introduction

The binding and conversion of factor C1, of the classical complement cascade, to C1q on the surface of foreign particles, is possibly the most important step in the activation of the innate immune complement-mediated system for the control of microorganisms via phagocytosis and digestion. The C1q component alone is required to trigger phagocytosis and digestive processes whereas other complement components require other additional stimuli. The release of certain PMN proteases may, however, interrupt this process by inactivation of e.g. C1-inhibitor and prevent the binding and exposure of C1q binding sites for further binding of complement factors on to the surface of the microorganisms. It is our hypothesis that certain organisms may deliberately exploit such a mechanism in order to evade phagocytosis and killing by phagocytes such as PMNs. In order to investigate this possibility and the possible role of MMP-9 and TIMP-1 in such strategy, a C1q-coated latex bead model system was used to first investigate the effect of C1q opsonization and binding of PMN receptors on the release of TIMP-1 and MMP-9. Such an approach was necessary as it was not known (a) whether TIMP-1 and MMP-9 are usually released prior to and/or during C1q-mediated phagocytosis and, (b) whether TIMP-1 release, is able to protect C1-inhibitor from MMP-9 inactivation, allowing disassembly of C1 on the surface of bacteria, exposure of C1q, binding to PMN C1q receptors and initiation of phagocytosis and phagosome maturation into a killing body. This needed to be established before our hypothesis could further be explored.

Inactivation of C1-inhibitor, by PMN enzymes was first shown by Pemberton *et al.* (1989) and later by Knäuper *et al.* (1991). As previously mentioned in Chapter 1, three

of the PMN enzymes have now been confirmed to degrade and inactivate C1-inhibitor. These are elastase (Pemberton *et al.*, 1989; Gigli and Tausk, 1988, Brower and Harpel, 1981), MMP-8 and MMP-9 (Knäuper *et al.*, 1991). However, under normal circumstances, inactivation of C1-inhibitor is unlikely to be due to elastase or collagenase (MMP-8) as activation of PMNs by receptors binding to C1q-coated surfaces does not seem to cause the release of primary and secondary granules containing these enzymes (Goodman and Tenner, 1992; Kishore and Reid, 2000) and few or no primary granule enzymes are reported to be secreted during phagocytosis of opsonized particles (Schettler *et al.*, 1991). Secretion of MMP-9-containing granules may, however, occur (Schettler *et al.*, 1991) and hence MMP-9 may inactivate C1-inhibitor and prevent the dissociation of C1r and C1s from C1 complex and binding of the C1q receptors. As the intact C1 molecule is known to be incapable of binding to PMN receptors, C1 is thus rendered unable to initiate phagocytosis and superoxide production or maturation of the phagosome (Tenner and Cooper, 1980), some information about C1-inhibitor and its role is deemed necessary at this point.

5.2 C1-inhibitor

C1-inhibitor is a plasma protein of the serine proteinase inhibitor superfamily (serpins) and is synthesized in the liver (Bock *et al.*, 1986). The molecule is highly glycosylated with N- and O- linked carbohydrates constitute a total of 49% of its molecular weight (Bock *et al.*, 1986). Thus of the 104 kDa molecule only 52 kDa constitutes the core protein (Figure 5.1)(Bock *et al.*, 1986). Deglycosylation with N-glycanase and O-glycanase, or both, does not have any major effect on its functional activity (Reboul *et al.*, 1987). It also has two disulphide bonds, one connecting residues 101-406 and the other connects residues 108-183 of the molecule (Bock *et al.*, 1986).

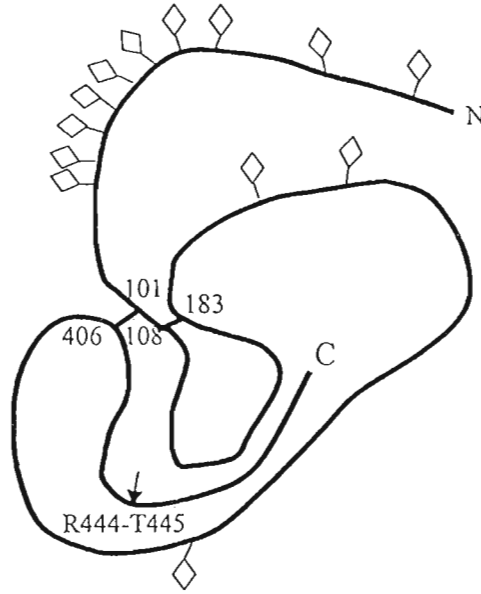


Figure 5.1 Schematic diagram of mature, C1-inhibitor. N and C indicate the N-and C-termini of the 478-residue polypeptide chain. The disulphide bridge pattern is shown with bars; residue numbers refer to disulfide-bounded cysteine residues. Diamonds marks known sites of oligosaccharides (Asn-3, -47, -59, -216, -231, and -330, Ser-42, Thr-26, -49, -61, -66, -70 and -74). The peptide bond cleaved by C1s during complex formation is indicated with an arrow (Bock *et al.*, 1986).

In the complement cascade C1-inhibitor requires complex formation of C1-inhibitor with C1s for activation (Brower and Harpel, 1981). Like other serpins, the active site of C1-inhibitor is located in an exposed loop near the C-terminus of the molecule (Knäuper *et al.*, 1991). C1s binds Arg 444 in its P1 site and Thr 445 in the P'1 site (Figure 5.1), during complex formation, releasing a small C-terminal fragment and forming a covalent inactive C1s: C1-inhibitor complex (Bock *et al.*, 1986; Salvesen *et al.*, 1985). PMN elastase may cleave C1-inhibitor at residues 37 (I-L), 40 (V-S) and 440 (I-S) of the N- and C-terminal region and result in the destruction of inhibitory activity (Bock *et al.*, 1986) (Figure 5.2). MMP-8 and MMP-9 cleave residues 439 (A-I) and 441 (S-V) closer to the reactive site (Figure 5.2)(Knäuper *et al.*, 1991; Pemberton *et al.*, 1989). Inactivation of C1-inhibitor by MMPs can be prevented by the addition of EDTA and 1, 10-phenanthroline (Knäuper *et al.*, 1991) or peptide inhibitors (Grey *et al.*, 1992).

et al., 1994) (Figure 5.3). In initiation of respiratory burst, however, it is the C chain that is required (Ruiz *et al.*, 1999) (Figure 5.3). Previously activation of complement was thought to be initiated only by C1q bound to the Fc region of antibodies. However, recently C1q has been shown to bind directly to molecules involved in many inflammatory diseases and molecules such as DNA, Alzheimer's proteins (Eggletton *et al.*, 1998), viral components, lipid A, other components of bacteria and parasites (Bobak *et al.*, 1987), β -amyloid protein (Jiang *et al.*, 1994) and C-reactive protein (CRP)(Eggletton *et al.*, 1998). The binding site on such molecules is distinct from the mannose-binding lectin (MBL) pathway which binds mannose residues, and is associated with two serine proteinases (MASP-1 and MASP-2). This mimics the activity seen in C1q-C1r₂C1s₂ complex (Eggletton *et al.*, 1998). Here the MBL complex binds to a pathogen surface and MASP-1 and MASP-2 are activated to cleave C4 and C2 (Eggletton *et al.*, 1998) forming C3 convertase from C2b bound to C4b, as in the C1q-C1r and C1s classical pathway (Figure 1.3).

During C1q-mediated opsonization, the collagen-like region of C1q (C1qCLR), with a molecular weight of 180 kDa, is necessary to trigger C1q-mediated phagocytosis and superoxide production by PMNs (Eggletton *et al.*, 1998; Ruiz *et al.*, 1995; 1999) and even MMP-cleaved C1q fragments may trigger respiratory burst (Ruiz *et al.*, 1999). Whether binding initiates phagocytosis or superoxide production, however, depends on which C1q receptor is bound. Binding of C1qCLR to the C1qRp receptor (126 kDa) results in initiation of phagocytosis while binding of C1qCLR to the C1qRO₂⁻ receptors (60 kDa) generates superoxide production (Ruiz *et al.*, 1999; Kishore and Reid, 2000).

Like C1q, other collectins such as MBL and pulmonary surfactant protein A (SP-A) have been shown to enhance Fc receptor- or complement receptor-mediated phagocytosis. However, neither SP-A nor MBP stimulate superoxide production in PMNs (Ruiz *et al.*, 1995; Goodman and Tenner, 1992). They were, therefore, considered less important in the current study and steps were taken to ensure only the C1q-mediated system was studied.

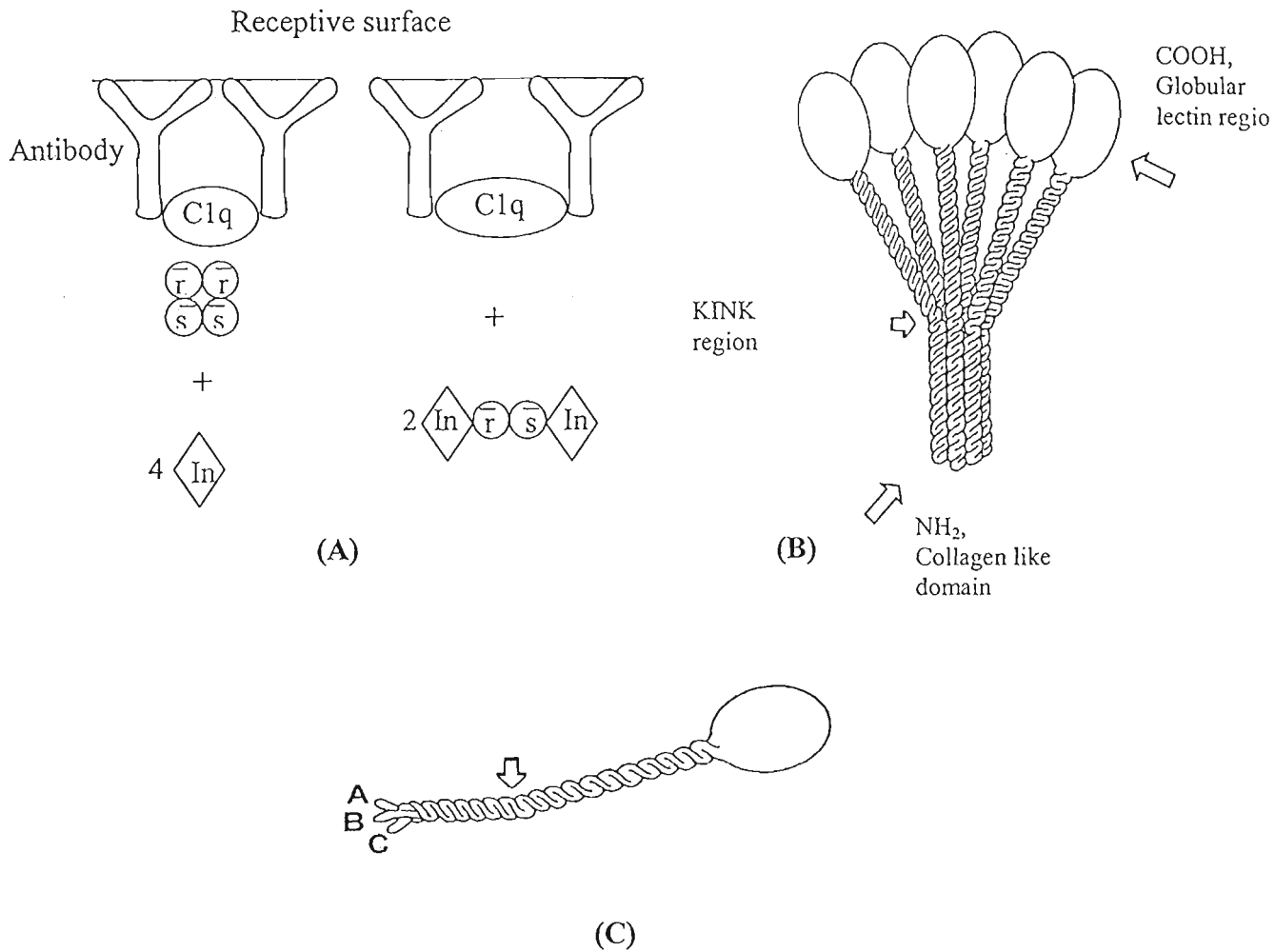


Figure 5.3 Early assembly of the complement system. A) After binding of C1 to the surface of the particle or antibody molecule, C1-inhibitor (In) dissociates C1r (r) and C1s (s) to initiate the classical complement pathway (Adapted from Ziccardi and Cooper, 1979). B) The C1q molecule. C) A single triple helical structure of C1q (adapted from Ruiz *et al.*, 1995).

It is known that during stimulation of PMNs by C1q-opsonized particles, phagocytosis and respiratory burst is initiated (Eggleton *et al.*, 1998; Tenner and Cooper, 1982; Eggleton *et al.*, 1998; Goodman and Tenner, 1992). Primary and secondary granule release does not occur (Goodman and Tenner, 1992) but MMP-9 granules may be released. Whether TIMP-1 is released after such stimulation is, however, unknown.

Not much is known about the signalling mechanism of C1q-mediated phagocytosis and respiratory burst triggered by C1qRp- and C1qRO₂⁻ receptors. Unfortunately in previous experiments performed by Tenner and Cooper (1982), to study such effects, C1q-coated latex beads that were too large to be internalized by PMNs were used. Therefore, C1q-mediated phagocytosis was not observed and results were left incomplete. Such experiments were, therefore, repeated during the current study using smaller beads.

5.4 Binding characteristics of complement components

Several proteins, as well as complement ligands involved in opsonization and removal of foreign particles and immune complexes, are found in serum. The binding characteristics of these components, however, differs. The first component of the classical pathway for complement activation, C1q, for example, is the only complement opsonin that forms electrostatic interactions with charged surfaces. This property is due to the charged globular lecithin region of this molecule and its overall highly basic character (positively charged), the reason for binding to weakly acidic (negatively charged) surfaces (Tenner *et al.*, 1981; Butko *et al.*, 1999; Guan *et al.*, 1991). C1q can also bind to the Fab portion of antibodies by electrostatic interactions, an ionic strength-dependent property (Duncan and Winter, 1998).

Opsonization via the alternative pathway is initiated by the active cleavage products of complement factors C3, C4 and C5 (C3b, C4b and C5b) (Gigli and Tausk, 1988; Janatova, 1988). All three components have similar binding properties (Law *et al.*, 1984; Sahu *et al.*, 1994; Janatova, 1988). They have thioester binding sites which are protected by hydrophobic regions, which, if exposed to water, become inactivated (Janatova, 1988; Law *et al.*, 1981) (Figure 5.4). Cleavage of the subunits by their convertase enzymes exposes the thiol ester-binding site which quickly binds with hydroxyl or amide residues. This causes the formation of a stable covalent ester bond with surface sugars or amino acids (Law *et al.*, 1979; 1981; 1984; Sahu *et al.*, 1999) and prevents the deposition of complement far from the site of activation, thus preventing tissue damage

(Janeway *et al.*, 2001). The cysteine and glutamine residues which form a thioester bond in C3b (Figure 5.4) and C4b, in the classical pathway, are replaced by serine and alanine residues in C5b (Janatova, 1988). C4b is also activated in the same manner by exposure to a thioester reactive site. The difference between C3b and C4b is that C4b preferably binds to amine groups rather than hydroxyl groups (Sahu *et al.*, 1994).

C1q forms primary electrostatic interactions with charged surfaces whereas C3b, C4b and C5b initially interact with hydrophobic surfaces via their protected hydrophobic thioester binding sites. This is fortuitous as it enabled either C1q or C3b, C4b and C5b to be semi-selectively adsorbed onto latex beads, depending upon the surface of the bead selected. In the current study a bead with a charged, carboxyl-modified surface was, therefore, selected to favour the binding of C1q.

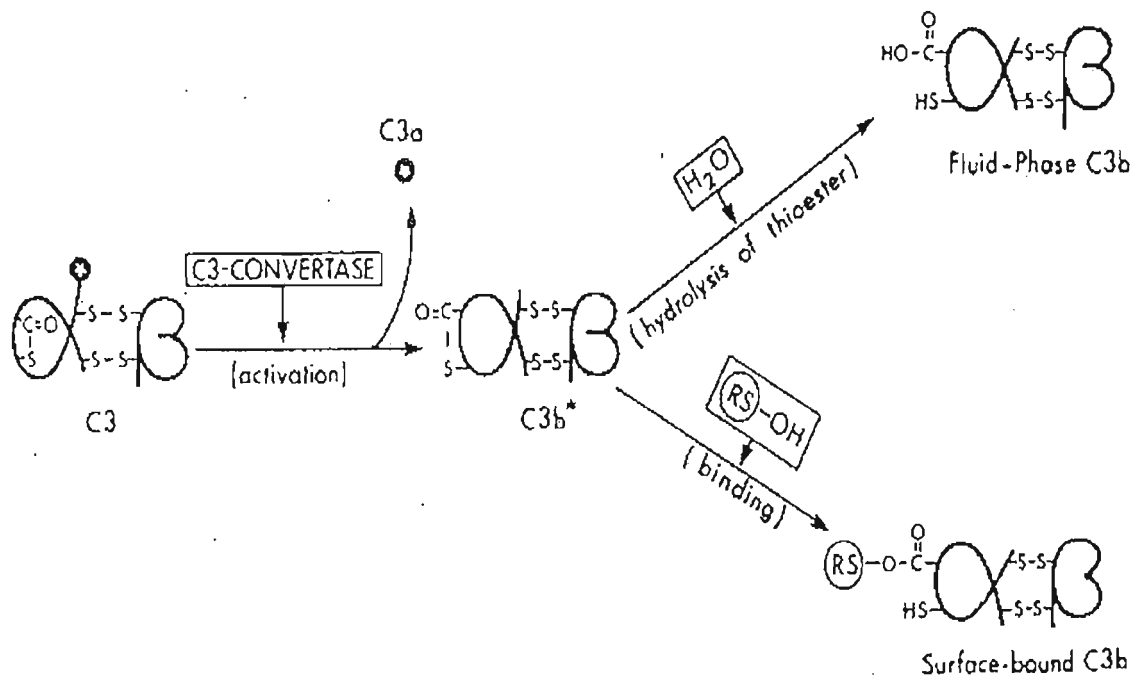


Figure 5.4 Hypothetical binding mechanism of C3 (from Law *et al.*, 1981).

5.5 Binding of proteins to polystyrene beads.

Due to the lability of complement and difficulties experienced in transporting commercial complement fractions from the supplier for use, it was decided to use fresh whole serum, appropriately diluted to favour the appropriately C1q interactions with charged beads.

Proteins normally readily adsorb to polystyrene beads. Unmodified polystyrene beads have polymeric surfaces which favour the binding of more hydrophobic residues whereas carboxylated beads may favour charged interactions. There are many proteins beside complement in serum (such as albumin, α , β and γ globulins). It was, however, reasoned that antibody binding should not be a problem since at low concentration antibodies bind to polystyrene beads in a random way but when applied at higher concentrations, such as found in serum (Table 5.1), crowding of antibody favours the binding of the Fc regions to the bead (Instruction manual, Bangs Laboratories Ltd.) and due to the hydrophobicity of the Fc region, this would be unlikely to occur. If antibodies did bind via the Fab region, they would assist in fixing C1q via the classical pathway (Table 5.1). Therefore, any of IgG in serum would either not bind to carboxylated beads or would facilitate the binding of C1q.

Since C3, C4 and C5 initially bind to hydrophobic residues and subsequently C3 and C5 interact with hydroxyl residues and C4 reacts with amide and hydroxyl residues, carboxylated polystyrene beads were considered a good choice as these would not favour the binding of C3, C4 or C5. The binding of the charged globular region of C1q, the opsonin required for this study would, however, take place and hence only the C1q-binding receptors on the surface of the PMN would be bound. For this reason any phagocytosis and respiratory burst would be due to the binding and exposure of C1q to the bead surface and hence due to the signalling via C1q-binding receptors. It was also reasoned that, if other serum proteins did bind or competed for binding on the beads this would not be a problem as long as some C1q molecules were bound. The binding of complement was, therefore, assessed using a complement-fixation indicator system.

Table 5.1 The properties and serum levels of the human immunoglobulin isotypes (modified from Janeway *et al.*, 2001).

| Property | Immunoglobulin | | | | | | | | |
|---|------------------|------------------|------------------|------------------|-----|------------------|------------------|------|--------------------|
| | IgG ₁ | IgG ₂ | IgG ₃ | IgG ₄ | IgM | IgA ₁ | IgA ₂ | IgD | IgE |
| Molecular weight | 146 | 146 | 165 | 146 | 970 | 160 | 160 | 184 | 188 |
| Serum level (mean adult mg/ml) | 9 | 3 | 1 | 0.5 | 1.5 | 3.0 | 0.5 | 0.03 | 5x10 ⁻⁵ |
| Classical pathway of Complement activation | ++ | + | +++ | - | +++ | - | - | - | - |
| Reactivity with Streptococcal protein A | + | + | ± | + | - | - | - | - | - |

5.6 Optimization of complement (C1q) coating of polystyrene beads.

Most phagocytosis studies using C1q opsonization used pure C1q from serum which contains around 34-246 µg/ml (Eggleton *et al.*, 1994; Eggleton *et al.*, 1998; Tenner *et al.*, 1981). Serum, therefore, is a good source of C1q. Since complement is labile and isolation of pure C1q would have been time-consuming and would have had to be carried out rapidly and under aseptic conditions or isolates would have had to be sterilized in some way, due to the time constraint, it was decided to use fresh serum and establish the minimum volume of serum required to coat beads without affecting C1q binding and activity, using a complement fixation test.

5.6.1 Reagents

Carboxyl-modified polystyrene beads (0.93 μm) were purchased from Bangs Laboratories Ltd. and supplied at a concentration of 100 mg/ml.

Physiological saline (150 mM NaCl). NaCl (0.878 g) was dissolved in 80 ml of purified water and made up to 100 ml and sterilized by autoclaving at (121°C, 20 min).

5.6.2 Procedure

Serum was obtained from whole blood and allowed to clot at 4°C overnight, the clot was removed by centrifugation (2 000 x g, 5 min) and the supernatant serum was immediately frozen at -70°C.

C1q-coated polystyrene beads have previously been used for PMN phagocytosis and respiratory burst experiments (Tenner and Cooper, 1982). Thus the concentration of C1q (and hence volume of serum) used for the experiments was based on this data where the maximum amount C1q used was $\pm 4.4 \mu\text{g}$ (Eggleton *et al.*, 1994; Tenner *et al.*, 1981). Since serum contains 200 $\mu\text{g/ml}$ of C1q, it was calculated that 5 μg would be available in 25 μl . Therefore, a minimum of 25 μl of serum was added to each tube.

Previously Robert and Quastel (1963) also showed that binding of IgG to polystyrene beads (at a concentration lower than 1 mg/ml of beads) is proportional to the concentration of beads. The concentration of polystyrene beads to be used in the experiment was, therefore, chosen considering the concentration found to be optimal by Robert and Quastel (1963). Eggleton *et al.* (1994) also suggested that C1q at a concentration of 2.2 μg or less is sufficient to opsonize 10^5 latex beads for PMN stimulation. Therefore, a concentration of approximately 5 μg C1q or 25 μl of serum was used to opsonize approximately 2×10^7 beads.

A measure of the amount of serum to be used and hence complement bound to polystyrene beads was required. In order to establish the amount of serum (and hence complement) bound a complement fixation indicator system was used. This would provide an indication of the amount of complement in serum and subsequently of free complement, unfixed by exogenously added polystyrene beads. The level of serum complement free or bound to beads was determined using an IgG-sensitized erythrocyte (RBC) indicator system (i.e. sheep RBCs sensitized with anti-sheep IgG, where lysis would indicate complement fixation or the presence of free complement). The absorbance of RBC to be used in the complement fixation system was adjusted to $A_{541} = 0.42$ (10^9 cells/ml). RBCs were washed three times in physiological saline, sedimented by centrifugation and diluted to 10^8 cells/ml by diluting 10 times (Gee, 1983). Serum volumes ranging from 25 μ l [(0.13 % v/v) of the final volume] to 87.5 μ l [(0.46% v/v)] with 12.5 μ l increments, were added into microtiter plates. Sensitized RBCs (25 μ l) and physiological saline was added to make a final volume of the 87.5 μ l and plate was incubated at 37°C for 30 min (Table 5.2). Wells containing the minimum amount of serum showing lysis were chosen as the minimum volume used for the test system to determine the extent of C1q-binding to the carboxylated polystyrene beads in 30 min. This volume of serum and increments of 10 μ l and an equal volume of physiological saline was added to 1 μ l (10 μ g) of polystyrene beads in 75 μ l of PMN storage buffer (PBSG) in various wells of a microtiter tray and incubated (30 min). Controls which included, the omission of beads (Table 5.3). All suspensions were incubated (30 min, 37°C) before RBCs (25 μ l) were added and incubated further (1 h, 37°C). Haemolysis patterns between tests, in which beads were added and controls, in which beads were omitted, were compared to determine the minimum amount of serum (C1q) to be added for total absorption onto the surface of polystyrene bead i.e. the point where no hemolysis occurs in the test wells (bead-containing) while hemolysis occurs in the equivalent control.

5.6.3 Results

A minimum volume of 50 μl of a 1/100 dilution of freshly isolated serum diluted to a final volume of 162.5 ml [(0.31 % v/v)] in physiological saline was shown to be the minimum amount of serum required to ensure the sensitivity of the complement fixation-sensitive RBC indicator system for use in the assessment of binding of C1q to latex beads (Table 5.2).

Table 5.2 Haemolysis assay on isolated serum

| Assay tubes | *Serum | Erythrocytes | PS | H ₂ O | Reaction | |
|---------------------|-------------------|----------------------|-------------------|-------------------|----------|---------|
| | (μl) | ($10^8/\text{ml}$) | (μl) | (μl) | Test | Control |
| A | 25 | 25 | 137.5 | — | — | — |
| B | 37.5 | 25 | 125 | — | — | — |
| C | 50 | 25 | 112.5 | — | + | — |
| D | 62.5 | 25 | 100 | — | + | — |
| E | 75 | 25 | 87.5 | — | + | — |
| F | 87.5 | 25 | 75 | — | + | — |
| Control | | | | | | |
| (100% lysis) | — | 25 | — | 162.5 | + | + |
| (Spontaneous lysis) | — | 25 | 162.5 | — | — | — |

* Serum diluted 1:100 in physiological saline (PS) before assay.

+ Indicates complete lysis. Partial lysis was not considered.

In the coating of latex beads for C1q-mediated uptake, a minimum of 170 μl of a 1/100 dilution of serum to 170 μl of physiological saline to 10^7 beads was required for C1q-coating. However, the volume of serum used must contain 4.4 μg of C1q which is contained in 25 μl of serum (Eggleton *et al.*, 1994). In the complement fixation test (Table 5.3) the equivalent of 1.7 μl of serum i.e. 0.34 μg of C1q was found to be the

minimum amount which could be used to coat 10^7 latex beads and give a detectable end point with the RBC indicator system.

Table 5.3 Complement fixation test to demonstrate binding of C1q.

| Test (μl) | | | | | | | | | | | |
|--|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| *S | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
| PS | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
| Beads | 1 μl | | | | | | | | | | |
| *S | 160 | 170 | 180 | 190 | 200 | | | | | | |
| PS | 160 | 170 | 180 | 190 | 200 | | | | | | |
| Beads | 1 μl | | | | | | | | | | |
| Control (μl) | | | | | | | | | | | |
| *S | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
| PS | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
| *S | 160 | 170 | 180 | 190 | 200 | | | | | | |
| PS | 160 | 170 | 180 | 190 | 200 | | | | | | |
| Incubation (30 min, 37°C) in microtiter plates | | | | | | | | | | | |
| ↓ | | | | | | | | | | | |
| Addition of 25 μl (10 ⁸ cells/ml) RBC and incubation (1 h, 37°C). | | | | | | | | | | | |
| ↓ | | | | | | | | | | | |
| Result | | | | | | | | | | | |
| Test | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
| (*S) | - | - | - | - | - | - | - | - | - | - | - |
| (μl) | 160 | 170 | 180 | 190 | 200 | | | | | | |
| | - | - | - | - | - | | | | | | |
| Cont. | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
| (*S) | - | - | - | - | - | - | - | - | - | - | - |
| (μl) | 160 | 170 | 180 | 190 | 200 | | | | | | |
| | - | + | + | + | + | | | | | | |

*S diluted 1:100 in physiological saline (PS) before assay.

5.7 C1q-mediated PMN phagocytosis experiments

For studies on phagocytosis in PMNs using IgG-coated or -uncoated polystyrene beads a maximum uptake of beads was previously shown to take place during the first 20 minutes of incubation (Robert and Quastel, 1963). As extended incubation periods may cause the release of secondary granule enzymes (Goodman and Tenner, 1992) and 20

minutes was previously shown not to cause extensive release of primary granule enzymes (Schettler *et al.*, 1991) a 20 minute incubation time was used in this study.

Our ultimate goal was to correlate superoxide production with C1q-mediated uptake and MMP-9 and TIMP-1 release. Since Eggleton *et al.* (1994) and Tenner and Cooper (1982) had shown that change in temperature does not affect the expression of C1q receptors or superoxide generation, experiments were, therefore, performed at room temperature. Phorbol ester (PMA) was also used to assess the effect of activation of the PKC pathway while other controls included the uptake of uncoated beads.

5.7.1 Reagents

Reagents for Laemmli gel electrophoresis were prepared as described in Sections 2.6.1.1 and for Tris-tricine electrophoresis as described in Section 2.6.2.1, for zymography as described in Section 2.9.1, for western blotting as described in Section 2.7.2 and for PMN isolation as described in Section 2.10.1.

Carboxyl-modified polystyrene beads (0.93- μ m) were purchased from Bangs Laboratories Ltd and supplied at a concentration of 100 mg/ml.

0.2 M Stock phosphate buffer. K_2HPO_4 (3.48 g) and KH_2PO_4 (2.72 g) were each dissolved in 100 ml of purified water and equal volumes of both solutions were mixed to make 0.2 M stock phosphate buffer of pH 6.8.

0.05 M Phosphate buffer. One part of stock phosphate buffer was diluted in three parts of purified water to give a buffer of pH 6.8.

Phosphate buffered saline (PBS: 10.4 mM K_2HPO_4 , 1.83 mM $NaH_2PO_4 \cdot 2H_2O$, 150 mM NaCl). K_2HPO_4 (0.364 g), $NaH_2PO_4 \cdot 2H_2O$ (0.057 g) and NaCl (1.75 g) was dissolved in

180 ml of purified water, the pH was adjusted to pH 7.4 and made up to a final volume of 200 ml in a sterile, pyrogen free container. The solution was autoclaved (121°C, 20 min) and kept at 4°C until used.

5.7.2 Procedure

Since endotoxins activate PMNs, reagent glassware was first depyrogenated by heating (250°C, 2 h) followed by autoclaving (121°C, 20 min) before reagent preparation. Utensils and reagent containers which could not be exposed to high temperature of dry heating were extensively washed with sterile ultra pure water. All manipulations and reagents were sterile and handled aseptically in a laminar flow hood.

PMNs were isolated and re-suspended in PMN resuspension and storage buffer as described in Section 2.10.2. Cell viability was assessed using the trypan blue (10 mg/ml) exclusion assay to ensure viability exceeded 98%.

Polystyrene beads were washed three times with a sterile, pyrogen-free PBS pH 7.4, diluted 10 times to 10 µg/µl i.e. 2×10^7 beads/µl and were used for all experiments. To each of three tests in Eppendorf tubes, 75 µl of PBS, 25 µl of fresh serum and 1 µl (10 µg) of beads were added, mixed and incubated (37°C, 30 min). The beads were washed three times in PBS pH 7.4 (5 400 x g, 5 min), and storage buffer (50 µl), serum (25 µl) and PMNs (1×10^6) were added and allowed to interact (20 min, room temperature). The beads and PMNs were precipitated by centrifuged (1 300 x g, 1 min) and the supernatants and PMN/bead pellets were separated and stored at -70 °C until used for assay for TIMP-1 and MMP-9. All tests were performed in triplicate. One test was fixed with 100 µl of fixative solution for processing for electron microscopy (Section 5.8.2).

Control experiments were performed simultaneously and consisted of PMNs incubated in storage buffer (75 µl) without serum, or with uncoated beads either in the presence of serum (25 µl) and storage buffer (50 µl) or with only storage buffer (75 µl) and

otherwise processed as described for the tests. Further controls did not included beads but contained serum (25 μ l) and storage buffer (50 μ l), or storage buffer (75 μ l) only. The last control contained storage buffer (75 μ l) and PMA (90 ng) and no beads.

The protein concentration of the supernatants were measured by Bradford dye binding assay as described in Section 2.2.1.2 and samples (2.5 μ l) separated by electrophoresis using Laemmli gel electrophoresis as described in Section 2.6.1.2 or Tris-tricine gel electrophoresis as described in Section 2.6.2.2. For the detection of MMP-9 and TIMP-1 gels separated by electrophoresis were blotted onto nitrocellulose and detected by western blotting as described in Section 2.7.3. MMP-9 detection was also performed by running the samples in Laemmli gel co-polymerized with gelatin (zymography) as described in Section 2.9.1. To test the release of primary granule enzymes supernatants were dotted into nitrocellulose and detected with anti-elastase antibodies.

5.7.3 Results

Little TIMP-1, release was seen. The TIMP-1 that was released seem to be degraded to 14.5 kDa (Figure 5.5 B). Degradation may be due to the release of primary granule enzymes, especially elastase (Itoh and Nagase, 1995), and/or oxidative processes (Stricklin and Hoidal, 1992). Degradation was possibly due to oxidative process as PMN samples incubated in buffer only showed no elastase release (results not shown), but a TIMP-1 band at 14.5 kDa (Figure 5.5 B). Other samples also showed low level elastase release (results not shown). Though levels of proteins loaded varied slightly most TIMP-1 was released when PMNs were incubated in storage buffer (Figure 5.5 B, f) and to a lesser extent upon uptake of coated beads (Figure 5.5 B, c) or uncoated beads in the absence of serum (Figure 5.5 B, d).

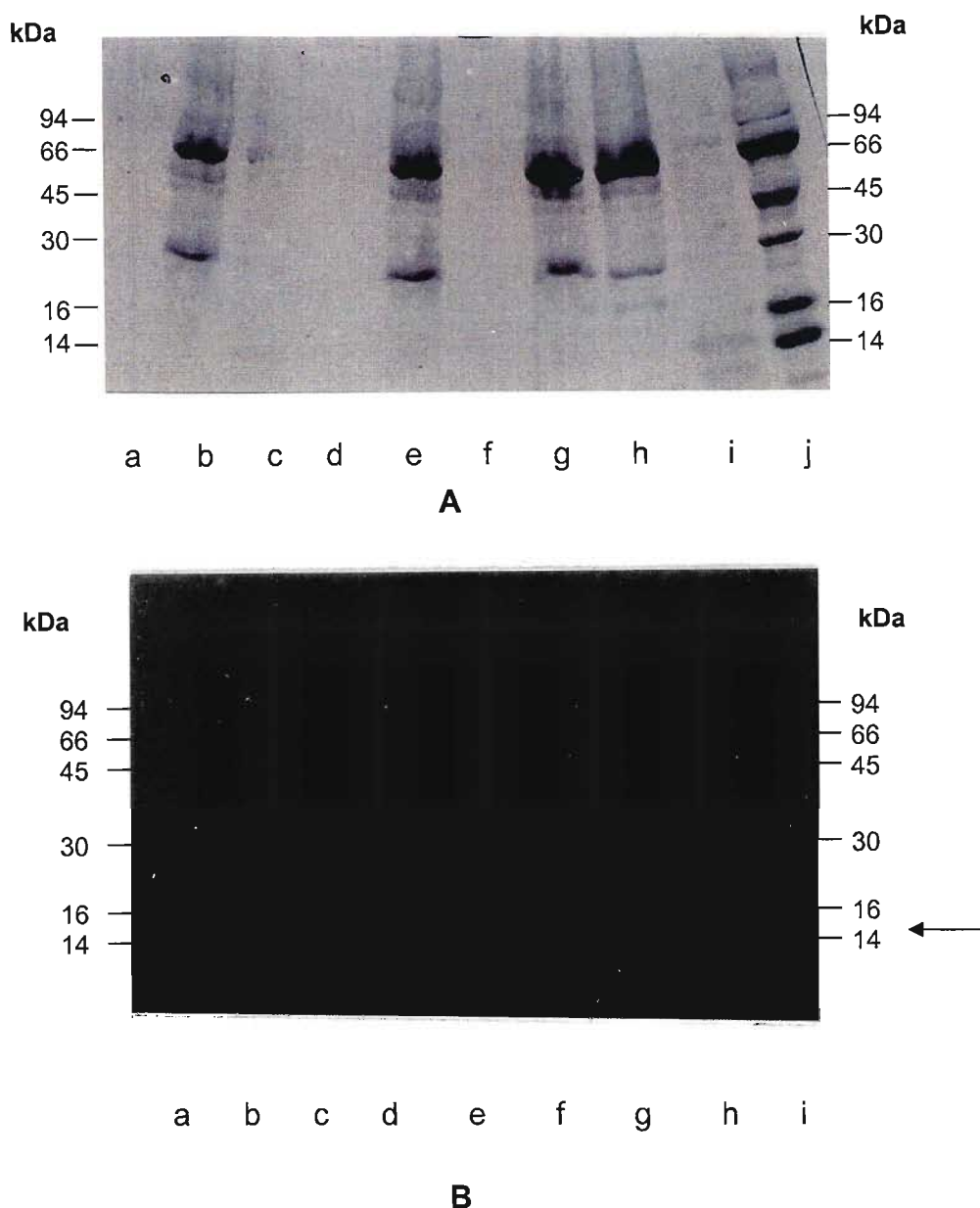


Figure 5.5 **TIMP-1 release during C1q-coated latex bead uptake studies.** Freshly isolated PMNs were re-suspended in PBSG buffer and 1×10^6 cells were added to a) PBSG (75 μ l) + PMA (90 ng), b) serum (25 μ l) + coated beads (2×10^7) + PBSG (50 μ l), c) PBSG (75 μ l) + coated beads (2×10^7), d) PBSG (75 μ l) + uncoated beads (2×10^7), e) serum (25 μ l) + PBSG (50 μ l) + uncoated beads (2×10^7), f) PBSG (75 μ l), g) serum (25 μ l) + PBSG (50 μ l). Controls included h) diluted serum i) PMN homogenates. Suspensions were incubated for 20 min RT, centrifuged (1 300 g, 1 min) and supernatant (2.5 μ l each) were reduced with equal amount of treatment buffer and run in (A) Tris-tricine gel as follows a) 0.07 μ g, b) 1.54 μ g, c) 0.09 μ g, d) 0.067 μ g, e) 1.48 μ g, f) 0.067 μ g, g) 1.02 μ g, h) 2.12 μ g, i) 0.096 μ g, j) molecular weight markers. (B) Gel was blotted into nitrocellulose for 16 h and detected with 7.2 μ g/ml chicken anti-TIMP-1 antibody followed by rabbit anti-chicken antibody (1/100 000). Colour was developed by NBT/BCIP system.

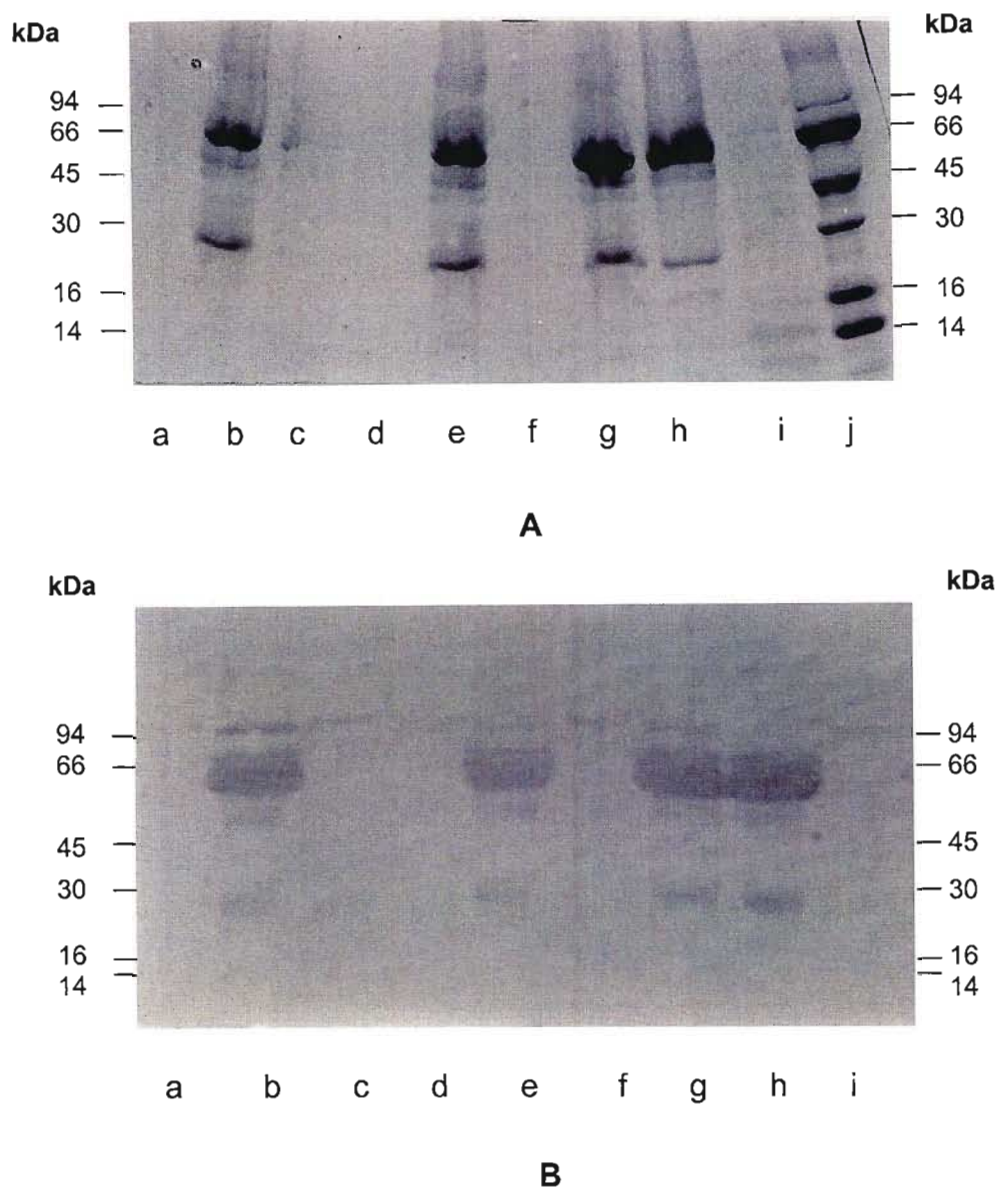


Figure 5.6 **MMP-9 release during C1q-coated latex bead uptake studies.** Freshly isolated PMNs were resuspended in PBSG buffer and 1×10^6 cells were added to a) PBSG (75 μ l) + PMA (90 ng), b) serum (25 μ l) + coated beads (2×10^7) + PBSG (50 μ l), c) PBSG (75 μ l) + coated beads (2×10^7), d) PBSG (75 μ l) + uncoated beads (2×10^7), e) serum (25 μ l) + PBSG (50 μ l) + uncoated beads (2×10^7), f) PBSG (75 μ l), g) serum (25 μ l) + PBSG (50 μ l). Controls included h) diluted serum i) PMN homogenate. Suspensions were incubated for 20 min RT, centrifuged (1 300g, 1 min) and supernatants (2.5 μ l each) reduced with equal amount of treatment buffer and run in (A) Tris-tricine gel as follows a) 0.07 μ g, b) 1.54 μ g, c) 0.09 μ g, d) 0.067 μ g, e) 1.48 μ g, f) 0.067 μ g, g) 1.02 μ g, h) 2.12 μ g, i) 0.096 μ g, j) molecular weight markers. B) blotted into nitrocellulose for 16 h and detected with 219 μ g/ml chicken anti-MMP-9 antibody followed by rabbit anti-chicken antibody (1/100 000) and NBT/BCIP system.

The 94 kDa pro-MMP-9 form of MMP-9 seemed to be released due to the binding of C1q-coated beads by PMNs (Figure 5.6 B, a-g, compare with serum control lane h). Increased release of MMP-9 was, however, triggered in the experiments where PMNs were incubated with coated beads in the presence of serum (Figure 5.6 B, b).

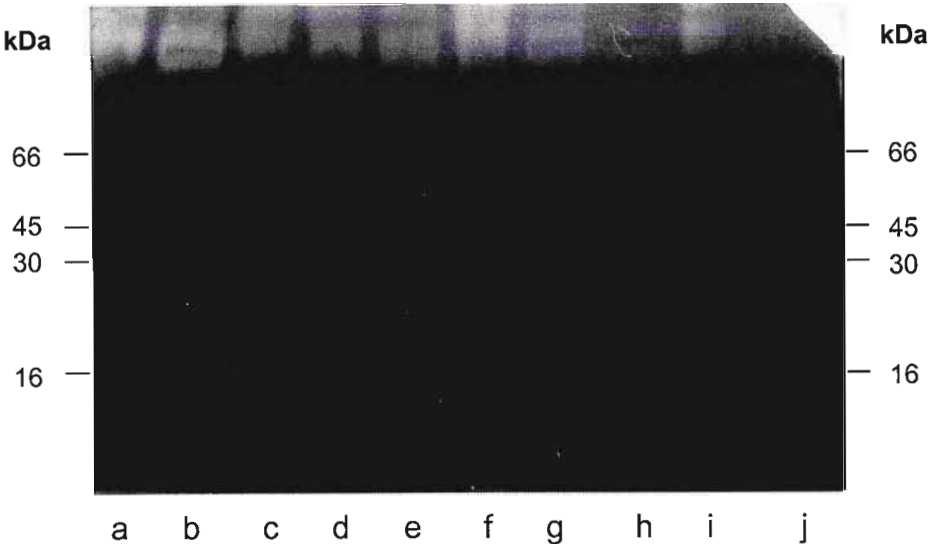


Figure 5.7 Zymographic results of MMP-9 release during C1q-coated latex bead uptake studies. Freshly isolated PMNs were re-suspended in PBSG buffer and 1×10^6 cells were added to a) PBSG (75 μ l) + PMA (90 ng), b) serum (25 μ l) + coated beads (2×10^7) + PBSG (50 μ l), c) PBSG (75 μ l) + coated beads (2×10^7), d) PBSG (75 μ l) + uncoated beads (2×10^7), e) serum (25 μ l) + PBSG (50 μ l) + uncoated beads (2×10^7), f) PBSG (75 μ l), g) serum (25 μ l) + PBSG (50 μ l). Two controls were included h) diluted serum i) PMN homogenates. The suspensions (2.5 μ g each) were incubated for 20 min at room temperature and centrifuged (1 300 g, 1 min). Supernatants were treated with equal amount of non-reducing treatment buffer and run in (A) Laemmli gel containing 1% (w/v) gelatin as follows a) 0.07 μ g, b) 1.54 μ g, c) 0.09 μ g, d) 0.067 μ g, e) 1.48 μ g, f) 0.067 μ g, g) 1.02 μ g, h) 2.12 μ g, i) 0.096 μ g, j) molecular weight markers. The gel was subsequently renatured in Triton X-100 (2.5 % v/v) for 1 h and developed in development buffer (0.05 M Tris-HCl, 5 mM CaCl_2 , 2 mM PMSF, and 0.02% (v/v) Brij-35, pH 8.8) for 16 h. The gel was subsequently stained with 0.1 % Coomassie blue and destained.

Zymogram results for MMP-9 showed that only the ≥ 94 kDa forms of pro-MMP-9 secreted or in the PMN homogenate were active (Figure 5.7 a-g and i, respectively). No activity was detected in serum (Figure 5.7 h) and activity comparable to that seen in non-PMA stimulated cells was observed in the sample in which PMNs were incubated with PMA (Figure 5.7, a).

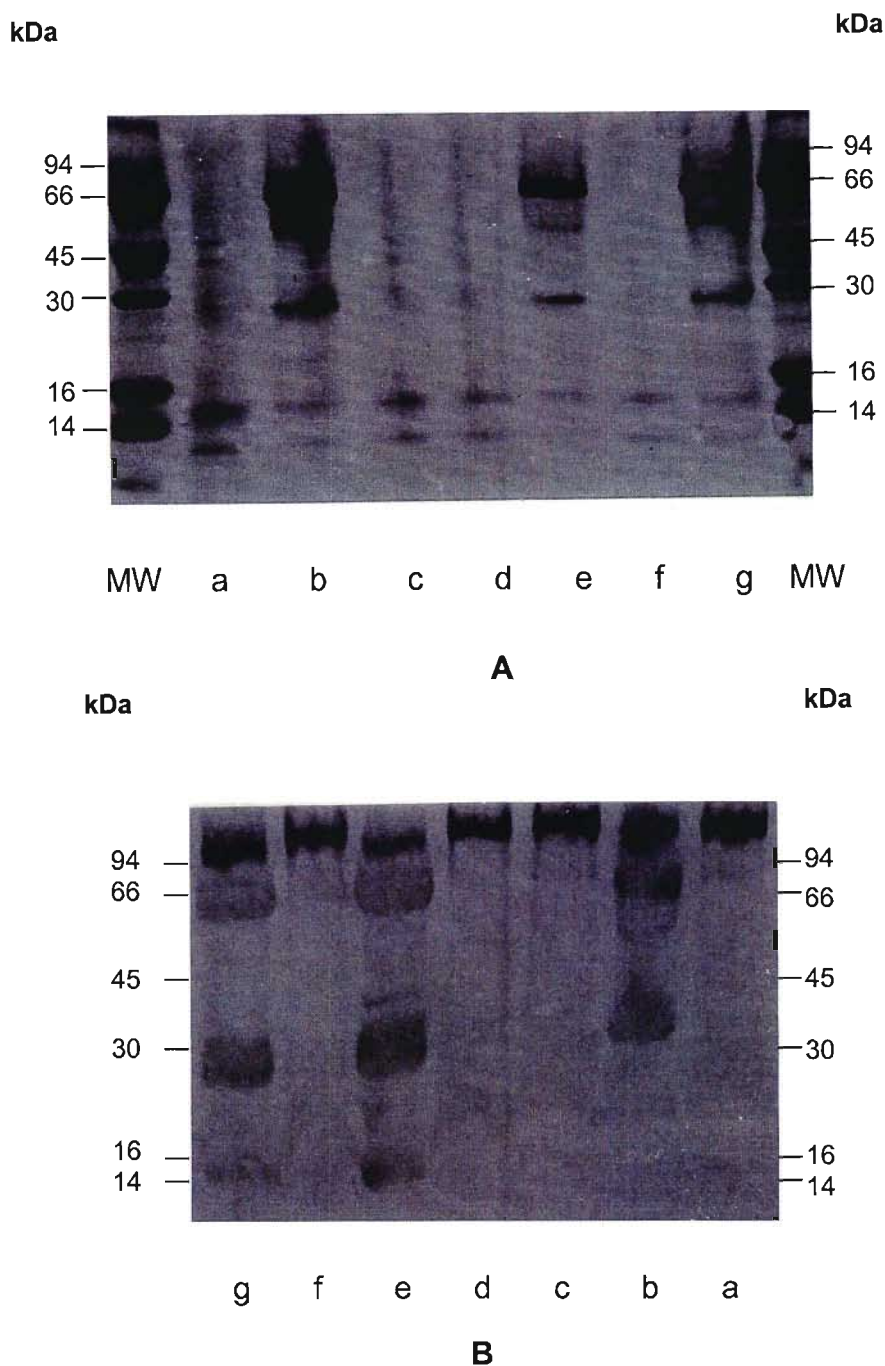


Figure 5.8 **MMP-9 in the pellet from during C1q-coated latex bead uptake studies.** Freshly isolated PMNs were re-suspended in PBSG buffer and 1×10^6 cells were added to a) PBSG (75 μ l) + PMA (90 ng), b) serum (25 μ l) + coated beads (2×10^7) + PBSG (50 μ l), c) PBSG (75 μ l) + coated beads (2×10^7), d) PBSG (75 μ l) + uncoated beads (2×10^7), e) serum (25 μ l) + PBSG (50 μ l) + uncoated beads (2×10^7), f) PBSG (75 μ l), g) serum (25 μ l) + PBSG (50 μ l), (MW) molecular weight markers. The suspensions were incubated for 20 min at room temperature and centrifuged (1 300 g, 1 min). The pellets were reduced with equal amount of treatment buffer and run in (A) Tris-tricine gel (B) The gel was blotted into nitrocellulose for 16 hours and detected with 219 μ g/ml chicken anti-TIMP-1 antibody followed by rabbit anti-chicken antibody (1/100 000). Colour was developed by NBT/BCIP system.

MMP-8 is also able to degrade gelatin. However, MMP-8 activity was absent in zymograms showing that there was no significant release of specific granules (Figure 5.7).

No TIMP-1 was detected in pellet samples indicating that, under the test conditions, all TIMP-1 was released (results not shown). Detection for MMP-9, however, revealed a > 94 kDa, a 65 kDa, 25 kDa and a 15 kDa band in most samples (Figure 5.8 B). As maximum release of MMP-9 was observed in PMNs incubated with coated beads and serum, low level of MMP-9 was detected in the pellet confirming that MMP-9 was mostly released (Figure 5.8 B, b). Like in supernatant samples, lower bands were also observed in pellets which were incubated in serum and may represent degraded products of MMP-9.

5.8 Bead processing for uptake studies

In order to have a clear picture of the effect of MMP-9 and TIMP-1 release on C1q-mediated uptake of latex beads in the test system, the third test in the triplicate set of tests set up as required in 5.7.2 was processed for electron microscopy. Robert and Quastel (1963) had previously shown the uptake of IgG coated or uncoated polystyrene beads by PMNs. However, uptake of C1q coated polystyrene beads had not been studied before.

5.8.1 Reagents

Manipulations involving OsO₄ were all carried out in a fume hood.

Sodium cacodylate buffer (0.2 M (CH₃)₂(AsO₂Na)). Sodium cacodylate (2.14 g) was dissolved in 80 ml of purified water, the pH adjusted to pH 7.2-7.4 by the addition of HCl and the solution was made up to 100 ml.

3% (w/v) Glutaraldehyde in phosphate buffer. Glutaraldehyde (3 g) was dissolved in 12 ml of purified water, stock phosphate buffer (0.2 M), 25 ml, was added and made up to 80 ml with purified water. The pH was adjusted to pH 6.8 – 7.4 and made up to 100 ml with purified water.

Fixative solution, 3% (m/v) glutaraldehyde in 0.05 M sodium cacodylate buffer. 0.2 M cacodylate buffer (25 ml) and 25% (w/v) glutaraldehyde (12 ml) were made up to 80 ml with purified water, the pH was adjusted to pH 6.8-7.4 with HCl and the volume made up to 100 ml.

4% (w/v) Osmium tetroxide. OsO₄ (1 g) was dissolved in 25 ml of purified water.

2% (w/v) Osmium tetroxide in 0.05 M sodium cacodylate buffer. 1 ml of (0.2 M stock sodium cacodylate buffer, 2 ml of 4% OsO₄ and 1 ml of purified water) was mixed in a fume hood.

EPON-ARALDITE. EPON 812 (1 part) and ARALDITE CY212 (1 part) (Inbed, Electron Microscopy, Washington, U.S.A.) and dodecenyl succinic anhydride (DDSA) (3 parts) (Agar Scientific Limited, Cambridge, U.K.) were mixed by dissolving.

5.8.2 Procedure

Pellets prepared for electron microscopy (Section 5.7.2) were fixed with 100 µl of fixation solution and processed as described in Table 5.4 for electron microscopy analysis.

Table 5.4 Standard procedures for embedding with EPON/ARALDITE.

| Action | Treatment | Exposure |
|---------------------------|--|----------------------|
| Primary Fixation | 3% Glutaraldehyde in 0.05 M sodium cacodylate buffer | overnight |
| Rinsing | 0.05 M sodium cacodylate buffer | 2 x 30 min |
| Secondary fixation | 2% (w/v) OsO ₄ in 0.05 M sodium cacodylate buffer | 2-4 h |
| Rinseing | 0.05 M sodium cacodylate buffer | 2 x 30 min |
| Dehydration | 30% (v/v) ethanol | minimum of 10 min |
| | 50% (v/v) ethanol | minimum of 10 min |
| | 70% (v/v) ethanol | (overnight) |
| | 80% (v/v) ethanol | minimum of 10 min |
| | 90% (v/v) ethanol | minimum of 10 min |
| | 100% (v/v) ethanol | minimum of 10 min |
| | 100% (v/v) ethanol | minimum of 10 min |
| | 100% (v/v) ethanol | minimum of 10 min |
| | Propylene oxide | 2 x 30 min |
| Embedding (Epon/Araldite) | *25 % EPON: 75% Propylene oxide plus DMP | 2 h caps on |
| | *50 % EPON: 50% Propylene oxide plus DMP | 2 h caps on |
| | *75 % EPON: 25% Propylene oxide plus DMP | overnight caps off |
| Embedding | 100 % EPON plus DMP | 24 h caps on |
| Embedding | 100 % EPON plus DMP | in oven (48 h, 70°C) |

*5 drops of DMP - (2,4,6-[(tri (dimethylaminoethyl) phenol)] to be added per 5 ml EPON each time.

Sections were cut in a LKBIII ultramicrotome and picked up on a 200 mesh copper grid, stained with lead citrate and uranyl acetate by Mr. Vijay Bandu, Center of Electron Microscopy, University of Natal. Grids were examined in a Philips CM-120 Biotwin transmission electron microscope operating at an accelerating voltage of 80 kV and photographed.

5.8.3 Results

For the C1q-coated beads which were incubated in the presence of serum (containing C1-inhibitor) (Figure 5.9, arrows). A greater number of beads were phagocytosed in the presence of serum (C1q-coated) than without serum (Figure 5.10 arrows).

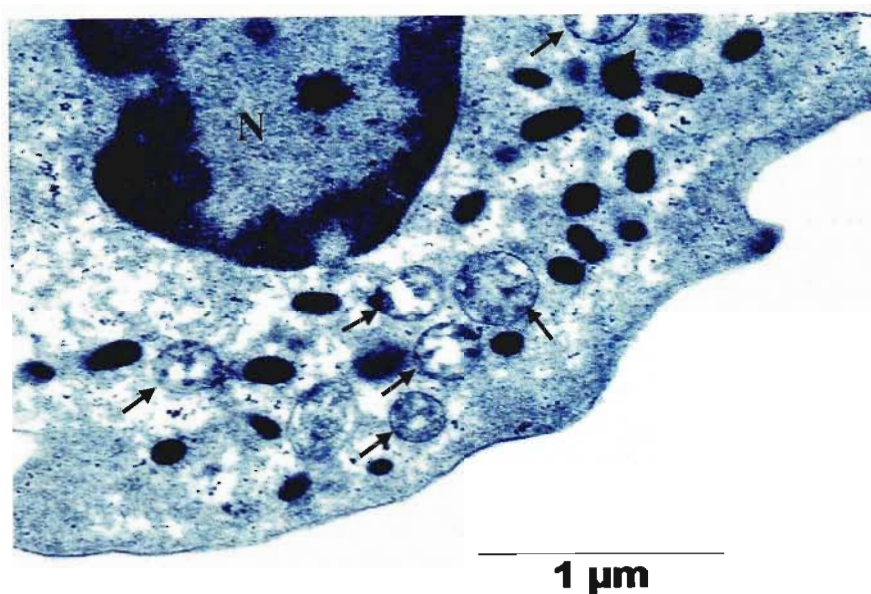


Figure 5.9 Uptake of C1q-coated polystyrene beads in the presence of serum. PMN phagocytic vacuoles containing polystyrene beads after phagocytosis was allowed to proceed for 20 min. Pellets were fixed with 100 μ l of fixation solution (3% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.95), embedded and sections cut, transferred to grids and stained for transmission electron microscopy. Polystyrene beads (arrows) observed are slightly dissolved by propylene oxide processing.

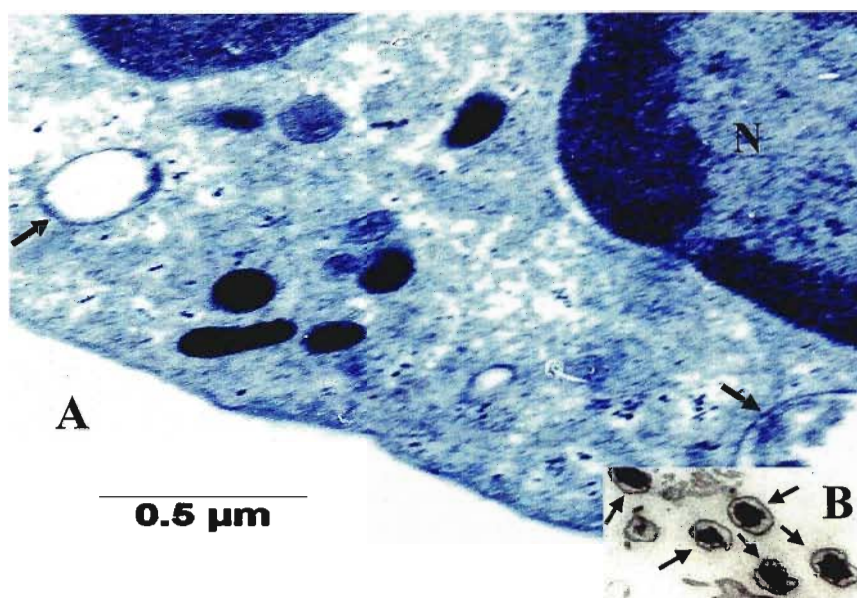


Figure 5.10 Uptake of C1q-coated polystyrene beads in the absence of serum. (A) PMN phagocytic vacuoles containing polystyrene beads after phagocytosis was allowed to proceed for 20 min. The pellet was fixed with 100 μ l of fixation solution (3% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.95). The pellet was embedded, sections cut, picked up on grids and stained for transmission electron microscopy. Polystyrene beads (arrows) are observed as slightly dissolved by propylene oxide processing. (B) Uninternalized beads (X 1 500).

5.9 Discussion

As previously mentioned, active MMPs have been shown to be required for killing of pseudomonas microorganisms (Osiewicz *et al.*, 1999). This killing process has also been reported to be complement-dependent (Wang and Soloway, 1999; Osiewicz *et al.*, 1999). The current study shows that C1q (complement) coating of polystyrene beads, in the presence of serum and other complement factors such as C1-inhibitor, appears to trigger an increased secretion of pro-MMP-9 by PMNs relative to TIMP-1. According to Osiewicz *et al.* (1999) and Wang and Soloway (1999), this situation would be associated with increased killing of microorganisms. This appears to indicate that a mechanism by which increased killing by PMNs may occur i.e. by which PMNs may secrete more pro-MMP-9 than would be normally the case has been discovered in the current study. However, active MMP-9 has been shown to be required for killing of microorganisms and MMP-9 is secreted in its inactive form. The results seem to show that secreted TIMP-1, mainly degraded to its 14.5 kDa but inhibitory form, is present at a lower level than MMP-9. Degradation by low levels, of elastase or by reactive oxygen species (Stricklin and Hoidal, 1992) may be responsible for the form of TIMP-1 seen but may also activate pro-MMP-9. Therefore, excess secreted MMP-9 (the form proposed to be required for killing of microorganisms) may become active via a similar mechanism, and hence may facilitate the killing of microorganisms by some unknown mechanisms.

In our system, however, we do not see any activated pro-MMP-9 unless it is introduced in serum. Perhaps MMP-9 normally becomes activated by proteases secreted by other cells present *in vivo*, therefore. According to our experimental results, TIMP-1 seems to be regulated more by calcium (in storage buffer) than by binding of C1q to the surface of microorganisms and PMNs. The fact that PMNs have TIMP-1 vesicles (granules) suggests that TIMP-1 secretion is not just constitutive (as seems to be implied by the low levels of TIMP-1 seen in all test samples and supernatants). During phagocytosis of C1q opsonized particles, increased numbers of gelatinase granules and some TIMP-1-containing granules were secreted. It is possible that this higher level of MMP-9 activity is required to shut down the activity of C1-inhibitor allowing increased generation of C4

and C2 cleavage products resulting in uncontrolled activation of kallikreins and hence increased swelling or fluid entry to the tissue (Janeway *et al.*, 2001; Harrison, 1983; Reboul *et al.*, 1987). This would most likely be accompanied by influx of antibodies, effector- and antibody-producing white blood cells. Such effects should assist in overcoming bacterial infections and may be the non-phagocytic route by which MMP-9 may be involved in the killing of microorganisms. C1-inhibitor is also the only molecule that dissociates C1r and C1s from C1q. After the disassembly of the C1-complex, exposure of C1q and activation of a few PMNs via C1q receptor-binding has occurred, the release of MMP-9 from PMNs, may be necessary to inactivate C1q inhibitor so that further C1 complexes remain un-dissociated and thus incapable of binding and activating more PMNs, preventing activation of too many PMNs.

The ratios of TIMP-1 and MMP-9 present in various supernatant needs to be assessed, before any definite conclusions or hypotheses may be further verified and the ratios after various incubation periods also need to be correlated with phagocytic uptake and the generation of respiratory burst.

CHAPTER SIX

GENERAL DISCUSSION AND FUTURE DIRECTIONS

From the results in this study it was evident that TIMP-1 and MMP-9 are (almost constitutively) secreted at low levels when PMNs were incubated in certain buffers. It is now clear, however, that the binding of putative C1q receptors (though they do trigger some release of TIMP-1) results in increased secretion of MMP-9, creating a condition previously shown to be necessary for the killing of microorganisms (Osiewicz *et al.*, 1999). It is also clear that TIMP-1 release is not as affected by the triggering of the putative C1q complement receptor, as is MMP-9, but is affected by the levels of extracellular calcium. This is extremely interesting as it implies that TIMP-1 release may be regulated via a different mechanism to the MMPs. As mentioned in Chapter 1, C1q-mediated signal transduction is thought to be via intracellular calcium fluxes and actin polymerization, but not via a PKC-mediated mechanism (Goodman and Tenner, 1992). The fact that increased MMP-9 secretion, which seems to mirror what happens with MMP-8 (Schettler *et al.*, 1991), is increased upon triggering this receptor seems to indicate that secretion of the MMPs, and hence gelatinase and specific granules, are regulated via such a PLC/DAG/IP₃- dependent pathway (Figure 1.4) and that TIMP-1 release, and the TIMP-1 granules is not. This suggests that if the mechanism involved in the regulation of TIMP-1 can be identified, such a pathway may be a possible target for therapeutic intervention in MMP-mediated inflammatory conditions. Putative pathways activated, therefore, should be verified using inhibitors which specifically block the signal transduction routes.

The studies of Price *et al.* (2000), using calcium and a calcium ionophore, indicated that TIMP-1 secretion is not regulated by Ca⁺⁺, however, the present study seems to partially verify and partially contradict such a conclusion. Even though some TIMP-1 secretion was triggered by extracellular calcium, secretion seemed to be at a basal (low)

level. The amount of calcium in the serum (2.5 mM), however, is even greater than what is found in storage buffer (1 mM). These experiments should, therefore, be repeated in the presence of physiological and various higher levels of calcium and other ions found in plasma (Ca^{++} , 2.5 mM; Mg^{++} , 0.75 mM; K^{+} , 4.8 mM) and should be repeated using physiological saline, to exclude the effects of these ions. This would finally establish whether extracellular calcium or other ion levels are responsible for the TIMP-1 secretion seen in this study. It would be unlikely that secretion of a granule would be regulated by the extracellular levels of an ion, but it is always possible and should be investigated.

Further studies should also be performed using different PMN agonists, such as fMLP, and the triggering of other complement receptors to try to identify the intracellular signal transduction pathway by which TIMP-1 is released. This knowledge is, in any case, very important and is required for prediction of how PMN responses may be manipulated for different purposes. i.e. to block the release of some granules while triggering others as this could be of great importance in handling inflammatory disease and invasive cancers.

The mechanism by which increased secretion of MMP-9 was induced also needs to be checked. PMA was included in studies to check whether MMP or TIMP-1 secretion was upregulated via a PKC-dependent mechanism. It is now realized that the inclusion of PMA in the latex bead experiments may be very useful as PMA, at different levels, may enhance or depress the expression of certain PMN receptors. C1q binding and stimulation of MMP-9 release may be confirmed by stimulation of PMNs with PMA as incubation of PMNs with PMA at concentrations of < 10 ng/ml may cause PMNs to shed their C1q receptors but increase the surface expression of CR1 and CR3. Incubation with PMA at a concentration of > 10 ng/ml, causes a decrease in C1q and CR1 receptors but not CR3 receptors (Eggleton *et al.*, 1994). If greater than 90 ng/ml of PMA was used in the current study CR3-mediated granule release would have been triggered. If MMP-9 and TIMP-1 secretion was not induced by the opsonin (which would be C3b) in the case of the CR3 receptor, this would imply that C3b was either not assembled, as assembly stopped at C1q, or that the CR3 receptor does not trigger the

release of MMP-9 or TIMP-1. If no MMP-9 release was seen when C1q receptors were shed (due to high levels of PMA). This would support a C1q receptor-mediated release of MMP-9. Another control which may have been included to check that MMP-9 release occurred via a C1q-mediated receptor pathway and not due to the assembly of other complement factors, would have been to include rosmarinic acid. Rosmarinic acid is an organic substance which contains four hydroxyl groups which compete for binding for the receptive surfaces on the microorganisms (with C4 and C3) (Sahu *et al.*, 1999). If, after addition of rosmarinic acid, MMP-9 is still secreted this would further indicated the involvement of C1q. However, C4 may also bind through amine residues (Sahu *et al.*, 1994) so other alternatives, such as the use of specific inhibitors for signalling pathways, should perhaps also be used to verify the binding of C1q and signal transduction pathway triggered.

Why active MMPs are required for killing of microorganisms, however, is still at this stage unclear. Active MMP-9, is most likely to be involved in limited cleavage of the extracellular matrix allowing movement towards a site of infection, through the barrier ECM (Price *et al.*, 2000). This activity would have to be tightly regulated by the almost simultaneous release of TIMP-1 as MMP-9 may cleave many other proteins which have collagen-like domains. Cleavage of C1-inhibitor by PMN MMP-8 and MMP-9 has previously been shown (Knäuper *et al.*, 1991). The release of low levels of TIMP-1 may be required to inactivate the low levels of MMPs present under most conditions, allowing the C1 complex to be dissociated by C1-inhibitor, C1q binding to PMN receptors, phagocytosis and respiratory burst, all important processes for the removal and killing of microorganisms. C1q can also be degraded by elastase (Ruiz *et al.*, 1995). Since the fragments of C1q can trigger an increased generation of superoxide production (Ruiz *et al.*, 1995), stimulation of elastase and MMP release can also potentially increase the effectiveness of respiratory burst and hence killing of microorganisms.

This study seems to indicate the importance of serum factors (possibly complement) in the release of MMP-9. The role of MMP-9, and TIMP-1 in the killing of microorganisms seems still very unclear, however, and needs further investigation. A

study of the effects of MMP-9 and TIMP-1 release on opsonisation and subsequently respiratory burst should be pursued, both using polystyrene beads, and in the presence of bacteria. Where beads are replaced by microorganisms a different mechanism of release of MMP-9 and TIMP-1 could be brought about by bacterial interference with PMN signal transduction. In the absence of such experiments no conclusions on this subject can be drawn, however. It is known that certain microorganisms such as the *pseudomonas* species secrete elastases which may activate MMP-9 (Okamoto *et al.*, 1997) and may also inactivate C1q-inhibitor, preventing phagocytosis which may occur via a C1q-mediated mechanism. TIMP-1 also does not inhibit bacterial collagenases (Hayakawa *et al.*, 1992). Bacterial components may, therefore, induce the differential release of MMP-9 and TIMP-1 and result in different outcomes.

The effect of MMP-9 and TIMP-1 release on phagocytosis and respiratory burst using purified proteins and checking the activity of MMPs after PMN stimulation and inhibitor secretion, is still untested and should be further investigated. The inactivation of C1-inhibitor under such conditions may also be assessed. Knowledge of whether TIMP-1 is totally inhibitory to MMP activity under various conditions would be useful in understanding the role of TIMP-1 release in facilitating infection and preserving anti-inflammatory conditions. Even though the complement system has such an important task in the clearance of microorganisms, as has been discussed, its components may be inactivated by some PMN enzymes and the release of these enzymes may also have both adverse and beneficial effects. It is recommended that further tests be carried out in a reductionist fashion using proteins in pure forms, to carefully further explore these effects.

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