

A STUDY OF PROTEINASES OF INVASIVE CELLS
USING
CRYOULTRAMICROTOMY AND IMMUNOGOLD LABELLING

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TEXT

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg (with aspects being done in the Department of Biochemistry, University of Georgia, Athens, Ga., and in the Department of Pharmacology, Wayne State University, Detroit, Mi., USA), from January, 1989, to December, 1993, under the supervision of Professor Clive Dennison.

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.

A handwritten signature in blue ink that reads "Edith Elliott". The signature is written in a cursive style with a small flourish at the end.

Edith Elliott

January, 1994

ABSTRACT

This study forms part of an investigation into the possible relevance of the lysosomal proteinases, cathepsins B, H, L and D, in cancer cell invasion. In this study, the main technique adopted was the Tokuyasu "cryo" method, in which the tissues were fixed, frozen and sectioned and labelled using the relevant antibodies, which were detected with protein A gold probes.

In order to implement the Tokuyasu technique, it was necessary to rebuild a knife maker, for the production of adequately sharp glass knives, and to modify a sputter-coater into a glow-discharger, for rendering carbon-coated grids hydrophilic, to promote adhesion of hydrated sections.

This study was directed towards human tissues and peptide antibodies were investigated as a means of avoiding isolation of proteins from scarce human tissue, and as a means of obtaining antibodies that will target specific regions of proteins of interest. Peptide antibodies were also considered promising for studies of proteinase trafficking and as immunoinhibiting agents, potentially useful in cancer therapy. Various prediction programmes were investigated for their effectiveness in predicting whether a given peptide sequence will elicit antibodies that will react with the native protein. Successful prediction would increase the success rate of peptide antibody production and thus lower the cost.

Leucocytes were studied as a model of an invasive cell, since they are more readily available than tumour cells and serve the purpose during the development of methods. In the course of these studies, an optimal protocol for the fixation of PMNs was developed, involving lateral fixation of cut sections, that should be useful for future studies on these cells. Elastase and cathepsins D and G were found on the surface of activated PMNs and could thus play a role in the invasive properties of these cells.

Studies on MCF-10A "normal" breast epithelial cells and their *ras*-transformed Neo-T counterparts revealed that upon transformation, lysosomes shift from a perinuclear position, to a more peripheral position. None of the cathepsins studied was found on the cell surface of either the normal or *ras*-transfected cells, suggesting that surface distribution of these enzymes may not be a requirement for invasiveness. These studies suggest that immunocytochemical investigation of cells, in the process of invading through a barrier membrane, might be profitable in elucidating the role of proteinases in invasive cancer.

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ABBREVIATIONS

APD	average particle diameter
AR	analytical reagent
ATP	adenosine triphosphate
BSA	bovine serum albumin
DAG	Diacylglycerol
DC	dendritic cell
dist.H ₂ O	distilled water
DMEM	Dulbecco's minimal essential medium
DTAF	dichlorotriazinyl amino fluorescein-
EDTA	ethylenediaminetetraacetic acid.
EGF	epidermal growth factor
EM	electron microscope
EMBL	European Molecular Biology Laboratory
ER	endoplasmic reticulum
Fc (portion of IgG)	crystallizable fragment
FCS	foetal calf serum
fMLP	N-formyl-L-methionyl-leucyl-phenylalanine
HBSS	Hanks balanced saline solution
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid
HETE	12 (S) Hydroxyeicosatetraenoic acid
HLE	human leucocyte elastase
I.E.M.	immuno-electron microscopy
I.S.O. (thread)	International Standards Organisation
IgG	immunoglobulin G
kDa	kilodaltons
KLH	keyhole limpet haemocyanin
LAMPS	lysosome associated membrane proteinase
LR White (resin)	London resin, white
M-6-P	mannose-6-phosphate
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide
MCF	Michigan Cancer Foundation
MEP	major excreted protein
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MSF	migration stimulating factor
MW	molecular weight

NHMec	7-amino-4-methyl coumarin
NHS-	N-hydroxysuccinimidyl
nm	nanometer
PBS	phosphate buffered saline
PEG	polyethylene glycol
PLT	"progressive lowering of temperature"
PMA	phorbol 12-myristate 13-acetate (see TPA)
PMNs	polymorphonuclear leucocyte (= Neutrophil)
PPP	platelet-poor plasma
PTFE	polytetrafluorethylene
PVC	polyvinyl chloride
PVP	polyvinylpyrrolidone
RER	rough endoplasmic reticulum
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCA (cycle)	tricarboxylic acid (cycle)
TGF- α	transforming growth factor- α
TPA	12-O-tetradecanoylphorbol-13-acetate (see PMA)
UV	Ultraviolet
V _o	Void volume
Z-	benzoylcarboxy-

CHAPTER 1

INTRODUCTION

1.1 Tumour malignancy and the metastatic process

The property that distinguishes malignant tumours from benign tumours is largely their ability to invade surrounding tissues and to metastasise to secondary sites (Nicolson, 1982; Maciewicz, *et al.*, 1989; Blood and Zetter, 1990). Invasion and metastasis are closely related phenomena for, in order to metastasise, the tumour cell has to invade surrounding tissue, and penetrate into a blood or lymph vessel, whereby it will be transported to another site in the body (Woolley, 1984). At the distant site, in turn, the cell must escape through the wall of the vessel to form a new metastatic focus (Woolley, 1984).

Tumour cell metastasis, as sketched out above, seems a relatively simple phenomenon, but in fact the mechanism of metastasis has many levels of complexity (Liotta, *et al.*, 1986; Gorelik *et al.*, 1988). The heterogeneity of tumours and their marked differences in characteristics make it difficult to identify a single metastatic phenotype and a single pattern of metastatic behaviour. Certain common features of the metastatic cascade which appear to correlate with metastatic potential are, however, beginning to emerge (Blood and Zetter, 1990; Condeelis *et al.*, 1992; Lester and McCarthy, 1992; Nabi *et al.*, 1992; Turley, 1992).

Most malignant tumours metastasise via the bloodstream (Blood and Zetter, 1990). Upon reaching a critical size, at which passive diffusion of nutrients and waste products can no longer occur, metastatic tumours are able to recruit new blood vessels, to sustain tumour growth, by secreting one of many so-called tumour angiogenic factors (Folkman *et al.*, 1981). Tumour cells also lose their cohesive properties (Nicolson *et al.*, 1988; Blood and Zetter, 1990) and easily escape into the fragile, highly permeable new blood vessels, which infiltrate into the tumour mass in response to angiogenic factors (Nagy *et al.*, 1989; Turley, 1992).

Once the tumour cells have entered the bloodstream, if they are to establish a secondary tumour, they have to evade the tumoricidal cells of the immune system, during transit to the secondary site. Metastatic cells accomplish this by mechanisms such as alteration in their expression of the class I MHC, a subject reviewed by Gorelik *et al.* (1988).

The adherence to and invasion through cultured endothelial monolayers has been shown to be enhanced in highly metastatic tumour cells (Kramer and Nicolson, 1979). Attachment to the target extravasation site requires adherence to specific sites on the luminal surface of the vascular endothelium, or selective attachment to the capillary endothelium of a preferred target organ (Nicolson, 1988). After induction of endothelial cell retraction (Honn *et al.*, 1989), attachment to the subendothelial basement membrane is followed by invasion through the type IV collagen, laminin and heparin sulphate of the basement membrane (Tryggvason *et al.*, 1987), a process requiring the action of hydrolytic enzymes (Liotta *et al.*, 1986). Directional movement through the barrier membrane, and growth into the new organ site follows (Liotta *et al.*, 1986).

With regard to directional movement, metastatic potential has also been linked to tumour motility, the greater the motility, the greater the metastatic potential (Nabi *et al.*, 1992). Morphological studies of AH66F and Yoshida sarcoma cells have shown that these may exhibit pseudopod extensions and leucocyte-like motility (Hosaka *et al.*, 1979). Like leucocytes, many tumour cells, including those of Walker 256 carcinosarcoma, human A2058 melanoma, HeLa cervical carcinoma, MCF-7 breast carcinoma, HT-1080 lung carcinoma, murine B16 melanoma, UV-2237 fibrosarcoma and K-1735 melanoma, as well as NIH-3T3 fibroblasts, have been reported to have the potential for chemotactic activation (Marasco *et al.*, 1985; Liotta *et al.*, 1986; Atnip *et al.*, 1987; Nabi *et al.*, 1990; Silletti *et al.*, 1991). Chemotactic agents have been found to range from host serum and extracellular breakdown products (Lam *et al.*, 1981; McCarthy *et al.*, 1985; Nabeshima *et al.*, 1986) to cytokines secreted in an autocrine fashion by the tumour itself (Liotta *et al.*, 1986; Atnip *et al.*, 1987). A tumour secreted cytokine, denoted autocrine motility factor (AMF), has been found to induce both random and directed tumour cell migration (Liotta *et al.*, 1986; Atnip *et al.*, 1987; Nabi *et al.*, 1990; Silletti *et al.*, 1991). Although the tumours mentioned above are predominantly of epitheloid origin, foetal and tumour-derived fibroblasts exhibit similar modes of migratory behaviour and have been found to secrete a migration stimulating factor (MSF) (Schor *et al.*, 1988).

Dissociation from the primary tumour and invasion into the bloodstream has been associated especially with the chemotactic action of host serum and extracellular breakdown products on various tumour cells (Lam *et al.*, 1981; McCarthy *et al.*, 1985; Nabeshima *et al.*, 1986). In this part of the metastatic process, i.e. in the chemotactic activity of some tumour cells, their attachment to target extravasation sites, and their proteolytic enzyme-assisted passage through the basal lamina, tumour cells have been

found to parallel the behaviour of normal inflammatory cells such as neutrophils (PMNs) and macrophages (Savion *et al.*, 1984; Marasco *et al.*, 1985; Blood and Zetter, 1990). Further, the receptor for urokinase type plasminogen activator has been found to polarise the expression of the protease to the leading edge of migrating monocytes/macrophages and to promote degradation of inhibitor complexes (Estreicher *et al.*, 1990). Such polarised expression of proteinases towards the "invasion front" may occur in PMNs and in invasive malignant cells.

PMNs and monocytes may, therefore, be studied as a model of invasive tumour cells, with the advantage that they are more readily accessible. A further reason for studying PMNs in the context of cancer, however, is that there is evidence that suggests that PMNs may assist in the arrest and extravasation processes of tumour cells (Orr and Warner, 1987). B16 and Lewis lung carcinoma cells, have been found in contact with PMNs throughout the arrest and extravasation step (Crissman *et al.*, 1985), and PMNs from rat peritoneum have been found to increase the attachment of rat hepatocarcinoma cells to endothelial monolayers (Orr and Warner, 1987). In a similar fashion, it has been suggested that, whereas highly metastatic tumours can independently degrade the subendothelial extracellular matrix, less aggressive tumour cells may follow the invasion path of PMNs, through vessel walls into the parenchymal tissue, or may be assisted by the proteinases secreted by these leucocytes (Blood and Zetter, 1990).

Similarly, extracellular matrix destruction at the tumour periphery, due to degradative enzymes produced by the host cells, such as neutrophils and monocytes, attracted to the tumour site (Dabbous *et al.*, 1986) and migrating vascular endothelial cells of the angiogenic process (Kalebic *et al.*, 1983), may assist the primary tumour metastatic event, the escape from the primary tumour, or site of origin.

The proteinases that are involved in leucocyte invasion and the interaction of invasive cells, host tissues and leucocytes, was, therefore, of interest in this study. Also of interest was whether, in cancer cells and PMN leucocytes, proteinases may be associated with the "invasion front" or become associated generally with the external membrane, or be released in a controlled directional fashion.

1.2 Proteinases and invasion

Strong evidence indicating that proteinases contribute to the invasive potential of metastatic tumours now exists (Sloane *et al.*, 1981; 1982; 1986; 1987; Ryan *et al.*, 1985; Rozhin *et al.*, 1987; Tryggvason *et al.*, 1987; Krepela *et al.*, 1987; Keren and Le Grue, 1988; Qian *et al.*, 1989; Moin *et al.*, 1989; Zucker *et al.*, 1985; Cardozo *et al.*, 1992; Nazeer *et al.*, 1992; Wibe *et al.*, 1992). The studies reported in this thesis are one facet of a broader study of the role of proteolytic enzymes in invasive and metastatic cancer, and of possible immuno-therapeutic approaches that may be used to prevent metastasis and eliminate tumourous tissues. In the broader studies, attention is being directed largely, although not exclusively, to the so-called cathepsins and, in the studies reported here, attention is focused especially on cathepsin B in tumour cells, and cathepsin G and elastase in PMNs. PMNs were used mainly as a model of an invasive cell and a discussion of these cells and their proteinases will be deferred until Chapter 4.

In normal, non-leucocytic cells cathepsins are lysosomal proteases, having a wide range of proteolytic specificities (Barrett and Kirschke, 1981), and play a role in the digestion of endocytosed proteins and in the turnover of intracellular protein. Lysosomal enzymes are typically glycoproteins that are synthesised, in high molecular weight zymogen form, in the rough endoplasmic reticulum and are trafficked, via the Golgi apparatus, to the lysosome. In the Golgi apparatus they acquire a M-6-P "address label" which is recognised by the M-6-P receptor, which directs them to the lysosome, via the late endosome. *En route*, they are proteolytically processed, from high-molecular weight pro-forms, to the smaller, mature form. The cathepsin B precursor pro-form is processed to the single-chain mature form in the lysosome (Nishimura *et al.*, 1988). In keeping with their natural intracellular lysosomal location, the mature, lysosomal cathepsins mostly have pH and stability optima of *ca.* pH 5.6 (Bond and Butler, 1987).

Metastasis of tumours has been linked with the increased activity of cathepsins in malignant tumours, identified using classical biochemical techniques (Maciewicz *et al.*, 1989; Krepela *et al.*, 1990; Lah *et al.*, 1992a). It is now apparent, however, that the measurement of activity alone, is an inadequate approach for assessing metastatic potential (Krepela *et al.*, 1989). Correlation between cathepsin B activity and metastatic potential, for example, is often of a qualitative rather than a quantitative nature (Sloane *et al.*, 1990) as differences in cathepsin B activity in malignant tumours appears to be due to differences in regulation at many levels.

Alterations in cathepsin B activity may reflect changes in synthesis (Moin *et al.*, 1989; Qian *et al.*, 1989), activation and processing (Mort and Recklies, 1986; Qian *et al.*, 1989) and intracellular trafficking and delivery of the cathepsin (Recklies *et al.*, 1982a; 1982b; Mort and Recklies, 1986; Sloane *et al.*, 1987), resulting in its secretion (Baici and Knopf, 1986) or association with the surface of the invasive cells (Krepela *et al.*, 1987; Sloane *et al.*, 1990). The apparent increased activity of cathepsins in tumour tissues, however, may also be the result of impaired natural cathepsin inhibitors, the cystatins or "stefins" (Lah *et al.*, 1989), or a decreased level of these inhibitors in malignant tumours (Corticchiato *et al.*, 1992; Lah *et al.*, 1992a).

An increased expression of the cathepsin B gene in malignant tumours has been linked with both increased cathepsin B activity, and with an apparent alteration of the intracellular trafficking and subcellular distribution of cathepsin B (Sloane *et al.*, 1990), leading to the enzyme being secreted or becoming membrane-bound. It might be imagined that, in order to facilitate invasion, the secreted or membrane bound forms of cathepsin B must, necessarily, also be active and stable at physiological pH. Mature cathepsins are generally reported to be unstable and inactive at physiological pH (Koga *et al.*, 1991; Cardozo *et al.*, 1992) whereas immature, precursor, forms are generally regarded as being more stable, though usually inactive (Recklies *et al.*, 1982b; 1985; Burnette *et al.*, 1983; Buttle and Abrahamson, 1990).

It is not strictly true that mature and precursor forms of cathepsin B are inactive at physiological pH. In the presence of large protein substrates such as laminin, mature cathepsin B is stabilised at physiological pH, so that significant enzyme activity is retained for up to 12 h at pH 7.4 (Sloane, *et al.*, 1990; Moin *et al.*, 1992). Large protein substrates might thus stabilise secreted mature cathepsin B, or mature enzyme generated *in situ* from intrinsically more stable precursors. Precursors of cathepsin B are known to have a greater stability at physiological pH, but were reported to be inactive (Mort *et al.*, 1980). More recently, however, high molecular weight forms of cathepsin B from mouse and human mammary tumours (Recklies *et al.*, 1982a; 1982b), have been isolated and found to be both stable and active at pH 7.2 (Mort and Recklies, 1986). Studies on human cathepsin B from mammary tumours (Mort and Recklies, 1986) and from sputa (Buttle *et al.*, 1988) suggest that one way in which a stable, active enzyme may be generated is for the pro-piece to become "truncated". The pro-enzyme has been found to be activated by such truncation, but remains stable, under conditions where the mature enzyme would be rapidly

inactivated (Mort and Recklies, 1986; Buttle *et al.*, 1988). The truncated forms have molecular weights intermediate between the pro-form and the mature enzyme.

Osteoclasts involved in bone re-modelling (Eeckhout, 1990) provide a useful model of a mechanism whereby mature lysosomal enzymes may function in invasion. In this process, intimate contact between the osteoclast and the target tissue is required and proteolytic enzymes may be released into a low pH micro-environment, produced in a pocket formed by the membrane of a closely adherent osteoclast and the bone being re-modelled. Similarly, in tumours, mature enzymes may be secreted into a compartment between the adherent tumour cell and the membrane through which the invasion is to take place. Such pockets have been termed "extracellular lysosomes". Breast cancer cell lines have been shown to degrade endothelial basement membrane in this way, invasion requiring intimate contact between the cancer cell and the target membrane (Yee and Shiu, 1986). A similar system may be operative in the normal functioning of macrophages (Silver *et al.*, 1988; Delaissé *et al.*, 1984) and in neutrophils (PMNs), moving out of the vasculature to a site of infection (Furie *et al.*, 1987).

Erroneous trafficking, with concomitant aberrant processing of cathepsin B seems to be a consistent finding in malignant cells (Mort *et al.*, 1980; 1983; Krepela *et al.*, 1989; Pagano *et al.*, 1989; Buttle and Abrahamson, 1990). Lysosomal enzymes, secretory proteins and plasma membrane proteins, are synthesised on the rough endoplasmic reticulum (RER). After losing their signal peptide, they undergo co-translational glycosylation of selected Asn residues and glycolytic trimming of these oligosaccharides, in the lumen of the endoplasmic reticulum. They are then transported to the Golgi stacks where they undergo a variety of post-translational modifications, most of which occur at a decreased pH. Oligosaccharides on secretory and membrane glycoproteins are processed to sialic acid-containing complex-type units, while most lysosomal enzymes acquire the critical addition of the M-6-P recognition signal, required for trafficking to the lysosome via the M_r 215 000 receptor. Lysosomal enzymes, synthesised as pre-pro-zymogen precursors, have the pre- (signal) sequence cleaved in the RER, while pro-sequence cleavage is initiated in the pre-lysosomal compartment and completed in the lysosome.

The M-6-P receptor recycles between the trans-Golgi stack and the pre-lysosomal compartment, where an acidic pH causes the release of the bound newly-synthesised enzyme. The released enzyme is then trafficked in vesicles to the lysosome (Brown *et al.*, 1986). A smaller, M_r 46 000, M-6-P receptor recycles between

the external membrane of the cell and the peripheral endosomal compartments, not via the Golgi apparatus, and is hence not involved in the major lysosomal enzyme trafficking pathway (Kornfeld, 1986).

Bearing the above trafficking scheme in mind, five possible mechanisms that could account for the secretion of proteinases of various levels of processing and glycosylation may be envisaged:

- i) a mutation in the cathepsin B gene/mRNA itself, affecting trafficking/processing,
- ii) an increase in synthesis of cathepsin B that overwhelms the M-6-P receptors, leading to by-passing of the low pH compartments responsible for processing, and to default secretion of an unprocessed enzyme,
- iii) an alteration in glycosylation or in phosphorylation that affects the enzyme's binding to the M-6-P receptor causing default secretion,
- iv) an alteration in number or regulation of recycling of the M-6-P receptors,
- v) a change in the pH of the lysosomal/endosomal compartments, leading to failure of the M-6-P receptor shuttle system.

Though the mechanisms responsible for the aberrant trafficking and secretion of the cathepsins in tumours are still largely unresolved, a pattern seems to be emerging. Transformation and dedifferentiation seems to result in the selective increase in synthesis of usually one of the lysosomal proteinases. In most cases, there also appears to be some alterations in the glycosylation and processing patterns.

High levels of cathepsin B have been isolated from homogenates of metastatic tumours (Maciewicz *et al.*, 1989; Lah *et al.*, 1992b), and increases in mRNA transcripts have been observed in most murine and human tumours, relative to normal tissues (Achkar *et al.*, 1990; Murnane *et al.*, 1991). No abnormalities in gene sequences coding for cathepsin B have, however, been observed (Qian *et al.*, 1991). Gene promoters seem normal in B16a tumours (Qian *et al.*, 1991), so the increased cathepsin B levels may be ascribed to the increased stability of the larger mRNA transcripts seen in cancer tissues (Jones and Cole, 1987). Differences in transcript size, due to differences in the 3' untranslated regions (UTRs), have been found to have a significant effect on the translation efficiency (Kruys *et al.*, 1987) and, coupled with the increased stability of the mRNA, may result in increased cathepsin B expression in some tumours.

Two main sizes of transcript, of *ca* 2 kb and 4 kb, have been found to predominate in most tumours (Capony *et al.*, 1989; Qian *et al.*, 1991), and a 5 kb

transcript has also been reported (Qian *et al.*, 1991). The 2 kb and 4 kb transcripts are apparently not tumour specific but are found in greater amounts at certain stages of tumour development: for example, they are reported to be increased 4-fold during tumour invasion (Murnane *et al.*, 1991). Some authors associate the 2 kb transcript with transformation, rather than with the proliferation of tumour cells (Capony *et al.*, 1989).

It might be anticipated that overproduction of a lysosomal enzyme, such as cathepsin B, might lead to saturation of the M-6-P receptors, and consequent secretion of most lysosomal enzymes by a default secretory mechanism. Studies on transformed or tumour cells, however, indicate that the lysosome has a normal complement of lysosomal enzymes, even if the synthesis and secretion of one lysosomal proteinase is greatly increased. The normal population of lysosomal enzymes may be due to an as-yet-undiscovered mode of trafficking of lysosomal enzymes to the lysosome (Kornfeld, 1990). With the secretion of a preponderance of one enzyme, it is difficult to imagine how a normal population of enzymes in the lysosome could result from the recapture and uptake of secreted lysosomal enzymes by a cell-surface M-6-P receptor. Mere saturation of receptors is, therefore, unlikely to be responsible for the secretion of proteinases. By-passing of the low pH environment of the lysosome, due to default secretion could, nevertheless, explain why it is precursor enzymes that are secreted. If secretion is by default, then all of the lysosomal enzymes should be secreted, though the over-produced enzyme would be secreted in the greatest proportion.

Simple default secretion may only occur in cases where the M-6-P address label has not been attached to the lysosomal enzyme during post-translational modification, such as occurs in I-cell disease (Kornfeld, 1990). In most cases, uptake studies on the secreted enzymes show that they are correctly phosphorylated (Dong *et al.*, 1989). Default secretion, therefore, seems most often to be related to a defect in the M-6-P receptors. Accumulating evidence points to the involvement of disturbances in the cellular pH, due to alterations in the metabolic patterns of transformed cells (Pouyssegur *et al.*, 1980). Such pH disturbances may give rise to disturbances in M-6-P receptor trafficking and in lysosomal enzyme processing (Brown *et al.*, 1986).

The pH may be shifted in either direction. Studies with cell culture systems indicate that changes in extracellular pH are accompanied by parallel shifts in intracellular pH (Jähde *et al.*, 1989; Tannock and Rotin, 1989). A decrease in pH leads

to a shift in the distribution of lysosomes and endosomes to the cell periphery (Heuser, 1989), and to the secretion of **mature** lysosomal enzymes (Krepela *et al.*, 1989). A lower than usual pH in the Golgi and secretory vesicles could result in premature dissociation of lysosomal enzymes from the M-6-PRs and in premature (autocatalytic) cleavage, resulting in the secretion of mature enzymes. Secretion of mature lysosomal enzymes has mainly been demonstrated in cell cultures, however, and may be a phenomenon of cell cultures only.

Conversely, an increase in cellular pH may give rise to the secretion of **precursor** lysosomal enzymes, which is more common (Sloane *et al.*, 1990). An increased pH is known to stop the trafficking of M-6-PRs, as M-6-PRs remain in the late endosome, unable to dissociate from their bound, newly-synthesised, lysosomal proteinases (Brown *et al.*, 1986). Dissociation does not occur, because this requires a low pH environment, and, consequently, recycling of the receptor also does not occur (Brown *et al.*, 1986). This may lead to a receptor shortage and the general default secretion of unprocessed precursor proteinases (Achkar *et al.*, 1990).

Failure of the acidification of lysosomes and late endosomes, resulting in the secretion of precursor lysosomal enzymes, occurs in normal cells, treated with lysosomotropic agents, but has also been observed in hepatocarcinomas, and is induced during transformation (Achkar *et al.*, 1990). Transfection with the *ras* gene has also been shown to increase the pH of the endosome-lysosome system in human fibroblasts (Jiang *et al.*, 1990). These observations suggest a connection between pH, oncogene transfection and secretion of proteinase precursors.

Defects in the acidification of processing organelles may be due to depletion of the ATP required for ATPase-dependent acidification of the lysosome-endosome-Golgi system, a result of the altered carbohydrate metabolism characteristic of malignant tumours. A switch from respiration to aerobic glycolysis is a characteristic, with few exceptions, of tumour cells (Pouyssegur *et al.*, 1980), and is closely associated with the malignant phenotype (Weinhouse, 1972; Birnbaum *et al.*, 1987). Aerobic glycolysis would yield substantially less ATP, than would respiration through the TCA cycle, and this may cause an ATP deficiency. The reason for the change in metabolism is unknown, although it has been speculated that it may be due to altered expression of certain enzymes (Weinhouse, 1972).

Paradoxically, although the switch to aerobic glycolysis might occasion a failure in acidification of the lysosome-endosome-Golgi system, it appears to lead to a

decrease in pH in the immediate extracellular environment, to pH 6.5 to 6.8 (Gullino *et al.*, 1965; Jähde *et al.*, 1992). This may be a consequence of the production and secretion of lactic acid as the end-product of glycolysis, even in the presence of a sufficiency of oxygen (Jähde *et al.*, 1992). It is interesting to observe that neutrophils, which are naturally invasive cells, also seem to rely mainly on glycolysis for energy, though the significance of this in relation to their invasive capability, is unknown.

In cancer cells, the ATP depletion could also result in the malfunctioning of glycosylation steps in the Golgi, and in consequent mis-direction of cellular traffic. In transformed murine NIH-3T3 fibroblasts, a high molecular weight precursor form of cathepsin L (called MEP - the major excreted protein) has been found to be secreted, as a secondary product of the *ras* oncogene, following Kirsten virus transformation of the cells (Mason *et al.*, 1987). Secretion of MEP is primarily due to its low affinity for the M-6-P receptor, caused by an alteration in the glycosylation of the M-6-P recognition marker. Upon transformation by the *ras* oncogene, murine NIH-3T3 cells become invasive (Barbacid, 1987). Again, plausible links can thus be discerned between ATP depletion and cell invasion.

Proteinases associated with malignant tumours are not always secreted, but may also be bound to the plasma membrane (Krepela *et al.*, 1987; Sloane *et al.*, 1986; 1990; Rozhin *et al.*, 1987; Keren and Le Grue, 1988). Some of these may normally be membrane-bound enzymes, but which are mis-trafficked in tumours. Examples are the lysosome associated membrane proteinase (LAMPS) and the CD63 lysosomal proteinase, which are expressed on the cell surface of tumours, especially those exhibiting the greatest propensity for metastasis (Heffernan *et al.*, 1989). This suggests that there may be a mechanism whereby "lysosomes" and their membranes may, under some conditions, become associated with the surface of invasive cells. Evidence will be provided in Chapter 4 of this thesis that, in neutrophils, which are normally invasive cells, this mechanism might involve fusion of the membrane of proteinase-containing organelles with the plasma membrane of the cell (Kobayashi and Robinson, 1991). There may be some advantage to an invasive cell in having membrane-bound proteinases and this disposition of the proteinases may also explain why intimate contact is required between the invading cell and the barrier being invaded.

Most biochemical evidence indicating the involvement of proteinases in tumour invasion has been gained from classical biochemical protein isolation techniques, in which the tumour mass is homogenised prior to protein isolation or

membrane or organelle fractionation (Strauli, 1980; Sloane *et al.*, 1981). Such techniques can only give an indication of the total quantity of proteinases present in fractions of the tumour mass, relative to that in an equivalent mass of the normal tissue. No indication of the source of the isolated proteinases is given; whether these arise from infiltrating leucocytes, or from the tumour cells themselves. The form of the protease isolated may also not be that which is involved in the invasion process, due to exposure to proteases during the homogenisation and isolation procedure. Care should, therefore, be taken in the interpretation of data from tissue homogenates. Similarly, information gained from tumour cell cultures, should be interpreted with some care, as cells in culture are extremely sensitive to their environment and particularly to the pH of the culture media (Krepela *et al.*, 1989).

Consequently, in the studies reported here, it was decided to adopt a different approach, using the technique of immunocytochemistry. The somewhat ambitious original aim of the present project was to establish the precise source and state of processing of secreted or membrane-associated proteases and to demonstrate where these proteinases occur in invasive cells. Such information, it seemed, would be best gained by an immunocytochemical study, at the electron microscope level, of tumour tissues and invasive cells. Direct observation, using immunolabelling techniques, was considered the most desirable approach, subject to the least error in interpretation.

1.3 Immuno-electron microscopy

In principle, immuno-electron microscopy (I.E.M.) is simple and the object of the immunolabelling technique is to have the biologically specific reaction, between an antibody and its specific antigen, expressed in the form of an electron-dense material, which can be detected in the electron microscope - fixed at the same place in the sample as the antigen is located.

The first requirement, before an immunocytochemical study can be undertaken, therefore, is the availability of suitable antibodies which specifically target the antigen(s) of interest (Elliott, 1989). In the present study, human tissues were of principle interest, so antibodies targeting human antigens were required. A tissue, containing the antigen of interest is also required. In the first instance this may be as a potential source from which the antigen might be isolated, for the raising of antibodies. It is not always necessary, however, to isolate human antigens in order to raise anti-human antibodies. Alternative strategies are to exploit cross-species

immuno-reactivity, whereby antibodies raised against an animal immunogen may be used to target the equivalent antigen in human tissue, or to use synthetic peptides as the immunogen. Human tissue is unavoidably required for the I.E.M. studies themselves but, fortunately, very small amounts of tissue, such as might be available from biopsies, are sufficient for this purpose.

As mentioned previously, the present study is part of a larger study on the relevance of proteinases in tumour invasion. In the first few years of this project, workers in this laboratory, including the present author, were involved in assembling and characterising a portfolio of the relevant anti-proteinase antibodies. Three different approaches were adopted. Sometimes the proteinase antigen was isolated, mostly from animal tissues (Jacobs *et al.*, 1989; Pike and Dennison, 1989; Pike, 1991), but also from human tissue, where possible (Fortgens, 1991; Coetzer, 1993). An efficient second approach was, where possible, to collaborate with workers who had already isolated the antigen of interest. In this way, polyclonal anti-human cathepsin B antibodies were obtained from Dr D Buttle of the Strangeways Laboratory, Cambridge, UK. and polyclonal anti-human elastase was obtained from Dr J Travis of the Department of Biochemistry, University of Georgia, Athens, USA.

A third approach was to prepare peptide antibodies, which are useful in a number of unique ways. Peptide antibodies were raised for studies on the processing of cathepsin B. In this case, target peptide sequences were chosen from positions in the precursor sequence of human procathepsin B. Peptide antibodies were also used as a way of obviating the necessity to isolate the target antigen from scarce human tissues. By selecting sequences in the active site of the target enzyme, it was hoped that these antibodies might also be immunoinhibitory, and might, because of this, also have some potential utility in tumour immunotherapy (Dennison, 1989; Coetzer *et al.*, 1991). This aspect will be discussed further in Chapter 3.

The next requirement for this study was the production of various sizes of colloidal gold labels. Colloidal gold labels have become the most popular for electron microscopy, due to their regular, unmistakable shape, their electron density and the fact that colloids can be produced in various sizes. Gold colloids adsorb proteins, such as antibodies or protein A, to their surface by non-covalent electrostatic forces (Horisberger and Rosset, 1977), in a form in which they remain biologically active. Recognition between the immunoglobulin or protein A, bound to the gold probe, and the primary antibody, used to probe a tissue section for a particular antigen, is made visible in the electron microscope by the presence of the electron-dense gold particle.

Different sizes of colloids can be produced by varying the reducing agent used to reduce a chlorauric acid solution, and their distinct sizes allow the simultaneous detection (Horisberger, 1981; Slot and Geuze, 1985) and quantitation (Slot *et al.*, 1989) of a number of antigens. Gold probes may also be used for light microscopy and as a sensitive detection system for antigens blotted on nitrocellulose membranes. For this purpose, a process of "silver amplification" is used to render the gold label visible to the naked eye (Danscher and Nörsgaard, 1983). Silver is displaced from a solution of silver lactate by the metallic gold probe colloid, the displaced silver effectively increasing the size of the gold particle to a visible dimension. This provides a sensitive alternative to the popular horseradish peroxidase enzyme labelling systems.

Due to financial constraints, it was necessary to produce and characterise the colloidal gold probes in this laboratory (see Chapter 2) and, as this had not previously been done here, it was necessary to develop the technique from scratch. The method of gold probe production, the particle size and the way in which residual protein binding sites on the gold probe are quenched, are reported to have an effect on the labelling characteristics of the gold probe and on non-specific interactions between tissue components and the gold probe (Birrell *et al.*, 1987). The effect of these various factors on the surface charge of the resulting gold probes was investigated as part of a study of gold probe production (Elliott and Dennison, 1990), but will not be reported in this thesis. Although this study revealed that the production and quenching procedures can markedly influence the surface charge of gold probes, the exact nature of the interactions between gold particles and proteins remains largely unknown and so the origin of non-specific binding is also not properly understood.

With the antibodies, tissues and gold labels to hand, it becomes necessary to optimise the labelling systems, to determine the optimal concentrations and proportions of reagents. In the present study it was considered most practicable to optimise the labelling systems on normal human tissues, before progressing to studies on tumour tissues. The tissues used for this purpose were from human blood or cell cultures or were obtained from patients undergoing therapeutic splenectomy. As normal human tissues are at an even higher premium than tumour tissues, it is perhaps fortunate that only small amounts of tissues were required. In the event, it was found that a great deal of methods-development, using normal tissue, was required to get the "cryo" immunolabelling system optimised and properly operational in this laboratory. This methods-development, a necessary preface to

studies on tumour tissues, constitutes the main thrust of the work reported in this thesis.

In any immunolabelling procedure, the target antigen must be preserved through the many processing steps, necessary to get the tissue into a physical form suitable for viewing in the electron microscope. Fixation, for example, is aimed at preserving the integrity and ultrastructure of tissue, but may result in the antigen becoming cross-linked (Hayat, 1986) or denatured (Tokuyasu, 1986), so that it is unrecognizable by the antibody. In I.E.M., therefore, preservation of tissue ultrastructure becomes of secondary importance and may often have to be sacrificed to preserve antigenicity. Various antigens have differing sensitivities to different fixation regimes and as a first approach the mildest, least denaturing, conditions are sought. Often these are not optimal for the preservation of tissue structure, however, and may also not fix all antigens adequately. In this study, preserving the best possible ultrastructure, while maintaining antigenicity, proved one of the major problem areas.

After fixation, steps are required to render the tissue rigid enough to allow ultramicrotomy sectioning. The conventional approach to this is to embed the tissue in a plastic resin. This involves dehydration of the tissue, by solvent substitution, infiltration with resin monomer and subsequent polymerisation which, for hydrophobic resins, involves heating. Clearly, this is potentially destructive to the antigen. So-called "cold-cure" hydrophilic (acrylic) resins (Lowicryl and LR White) have been developed, to avoid the denaturing effects of the high temperatures needed to cure epoxy resins. Special low temperature refrigerators and ultraviolet globes are required to polymerise Lowicryl resins (Glauert and Young, 1989).

Various other systems have been introduced to overcome the antigen-denaturing effect of tissue embedding procedures. These include the cryo-fixation and freeze-substitution methods in which specimens are ultra-rapidly frozen to the vitreous state (Dubochet *et al.*, 1988), and the cell water slowly replaced by solvents and resins at low temperatures (Livesey *et al.*, 1990; Jestaitis *et al.*, 1990). These methods require expensive equipment, however, which was not available in this laboratory.

Alternatively, fixed thick sections of tissues may be immunolabelled before being resin embedded and sectioned (Priestley and Cuello, 1983). In these so-called "pre-embedding" immunolabelling procedures, labelling takes place before the damaging effects of tissue processing. These procedures are beset with many

problems, however. Penetration of immunoreagents into the thick tissue sections, is one such problem (Priestley and Cuello, 1983). Tissues may be permeabilised, in an attempt to promote penetration, but this may cause further antigen-denaturation (Robinson, 1985). The subsequent thin sectioning of the pre-embedding-labelled thick sections is also problematic.

A far better option is the use of cryoultramicrotomy, in which ultra-thin frozen sections of fixed tissue are cut on an ultramicrotome to which a freezing apparatus has been fitted. In this "ultracryo"-technique, specimens are fixed, cryoprotected against ice crystal damage, with sucrose infiltration, and mounted on a stub that is inserted into the microtome. The tissue block is rapidly frozen, by plunge-freezing into liquid nitrogen, ultrathin sectioned, and the section is collected from the knife-edge in the cryochamber on a droplet of sucrose, thawed and immunolabelled in the fully hydrated form. The sections are then "sealed" and contrasted with a film of methyl cellulose, containing uranyl acetate. This prevents the section from dehydrating in the electron beam, and allows the specimen to be viewed in the electron microscope in a fully hydrated state (Griffiths *et al.*, 1983; Tokuyasu, 1980). This so-called "Tokuyasu technique" was the main labelling technique used in this study.

The Tokuyasu technique was chosen as it has two main advantages. The only potentially antigen-denaturing step is the fixation and it is, therefore, the method of choice for sensitive antigens. It is also rapid. In an optimised system, it is possible to fix and process the tissue, section, label and view all in one day. By contrast, alternative resin embedding techniques take at least three days before they may be labelled and viewed. However, at the outset of this study, the cryoultramicrotomy technique was newly-developed and this University did not have a cryoultramicrotome, nor any of the ancillary equipment necessary for producing the special glass knives and grids required. The technique was also not well-established elsewhere in South Africa. Consequently, much preliminary equipment- and methods-development was required.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

In this chapter, some of the basic techniques used to prepare and evaluate reagents will be described, in general terms. More specific methods will be described in the relevant sections of the text.

Most of the labelling studies reported in this thesis were performed using the so-called "Tokuyasu technique" of cryoultramicrotomy and immunolabelling (reviewed by Tokuyasu, 1986), which is, perhaps, the mildest, most rapid, means of tissue preparation, facilitating the preservation of maximal tissue antigenicity. In this technique, fixation of the tissue is the only potentially antigen-denaturing step, and, since tissues do not have to withstand the rigours of resin embedding, relatively mild fixatives and short fixation regimes may be used. With the Tokuyasu technique there is a minimal chance of antigen denaturation, which is especially advantageous in the simultaneous study of a number of antigens in the same tissue.

A necessary preface to immunolabelling studies, is the preparation of knives, grids, colloidal gold labels, and tissues. At the outset of the present study, the immunolabelling technique was not established in this laboratory and the equipment available was not suitable for a study of this nature. Consequently, some of the modifications of existing equipment, that were necessary in order to undertake this study, will also be described in this chapter. Gold labels are an expensive commodity, with a limited shelf life, so techniques for the "in house" production and characterisation of gold probes were also established and will also be described here.

2.2 Antibody characterisation

2.2.1 SDS-PAGE

SDS-PAGE of protein fractions was used in this study to analyse the purity of antigens, and as a preface to western blotting of antigens for determination of antibody specificities.

SDS binds strongly to most proteins in a fixed mass ratio of 1.4:1.0 (Reynolds and Tanford, 1970), converting them from globular native proteins to highly negatively charged rods, the length of which is dependent on their MW. The negative charges of the SDS effectively mask the charges of the proteins, giving them all similar charge-to-mass ratios. Because of the sieving effect of the gel, the mobility of a protein/SDS complex on SDS-PAGE depends on its size only, and hence relates to the size of the protein. A standard curve may be constructed by plotting the mobility of the protein/SDS complex against the log of the MW of several standard proteins. This curve may be used to estimate the MW of an unknown protein from its mobility in SDS-PAGE.

The linear relationship between log MW and mobility breaks down in the case of glycoproteins and in proteins constrained by disulfide bridges. It is common practice, therefore, to reduce the proteins before SDS-PAGE separation. This results in the MW of sub-units (if any) being determined.

For this study the discontinuous SDS-PAGE system of Laemmli (1970) was used on the Hoefer SE250 Mighty Small slab gel unit.

2.2.1.1 Reagents

Solution A: Monomer Solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]. Acrylamide (58.4 g) and bis-acrylamide (1.6 g) were dissolved and made up to 200 ml with dist.H₂O.

Solution B: 4 x Running Gel Buffer (1.5M Tris-HCl, pH 8.8). Tris (36.3 g) was dissolved in approximately 150 ml of dist.H₂O, titrated to pH 8.8 with HCl and made up to 200 ml.

Solution C: 4 x Stacking Gel Buffer (500mM Tris-HCl, pH 6.8). Tris (12 g) was dissolved in 150 ml dist.H₂O, titrated with HCl to pH 6.8 and made up to 200 ml.

Solutions **A**, **B**, and **C** were filtered through Whatman No. 1 filter paper before use.

Solution D: 10% (w/v) SDS. SDS (10 g) was dissolved in dist.H₂O with gentle heating, if necessary, and made up to 100 ml.

Solution E: Initiator [10% (w/v) ammonium persulfate].
Ammonium persulfate (0.5 g) was made up to 5 ml just before use.

Solution F: Tank Buffer [250 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3]. Tris (12 g) and glycine (57.6 g) were dissolved and made up to 4 litres with dist.H₂O. Prior to use, 2.5 ml of SDS stock (solution E) was added to 250 ml for use in the Mighty Small apparatus.

Solution G: Treatment Buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution E), glycerol (2 ml) and β -mercaptoethanol (1 ml) were made up to 10 ml with dist.H₂O.

Stain stock solution [1% (w/v) Coomassie Blue R-250].
Coomassie Blue R-250 (1 g) was dissolved in dist.H₂O by magnetic stirring for 1 h at room temperature and made up to 100 ml. The solution was filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (w/v) Coomassie 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 the volume was brought to 500 ml with dist.H₂O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]
Methanol (500 ml) was mixed with acetic acid (100 ml) and the volume was made up to 1 litre with dist.H₂O.

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 dist.H₂O.

Running and stacking gels. Running and stacking gels were made up as indicated in Table 1.

Table 1. Composition of 12.5% running and stacking gel

	Running Gel (ml)	Stacking Gel (ml)
Monomer solution	6.25	0.95
Running gel buffer	3.75	
Stacking gel buffer		1.75
10% SDS solution	0.15	0.07
Amm. persulfate	0.075	0.035
TEMED	0.015	0.015
dist.H ₂ O	4.75	4.2

2.2.1.2 Procedure

For SDS-PAGE, a Hoefer SE250 Mighty Small apparatus was assembled as described by the manufacturer. Aluminium and glass plates, separated by two 1.5 mm spacers along each outer edge, were clamped to the electrophoresis pod and the apparatus was placed on a clean glass plate. The lower edges of the troughs, formed between the aluminium plates and the glass plates, were sealed with molten agarose [1% (w/v), in dist.H₂O], which was allowed to solidify. The running gel, prepared immediately before use, was loaded into the troughs, to within 3.5 cm from the top of the glass plate, overlaid with dist.H₂O and allowed to solidify (approx. 1 h). The water was removed and the troughs were filled with stacking gel, into which 10- or 15-well combs were inserted, to form sample application wells. Once the gel had set (about 30 min), the combs were removed, the wells rinsed with dist.H₂O and shaken dry.

Tank buffer, containing SDS, was poured into the upper and lower electrode compartments. Samples and molecular weight reference standard preparations, first diluted in stacking gel buffer (if necessary), and further diluted 1:1 with treatment buffer, to give a minimum concentration of 0.5 µg of protein in the volume loaded, were boiled (90 sec), cooled, and bromophenol blue tracker dye added. Treated samples and reference proteins were loaded into the wells and electrophoresed at 18 mA per gel, until the bromophenol blue reached the bottom of the running gel. One gel was used for western blotting, and the other was stained as a reference.

The reference gel was stained using Coomassie Blue R-250 stain (4 h), destained in destain I overnight and in changes of destain II until the background was decolourised, and stored in dist.H₂O or destain II in zip-sealed bags until photographed.

2.2.2 Western blotting of SDS-PAGE gels

The specific recognition of KLH-adsorbed peptide-antibodies (Chapter 3) and other antibodies to SDS-denatured, whole target enzyme or precursor sequences, and potential non-specific reactivity, was assessed by western blotting (Towbin *et al.*, 1979; Burnette, 1981) of pure and crude fractions of the target proteins, separated by SDS-PAGE (Laemmli, 1970).

Binding of the primary antibodies to the target antigen, was demonstrated using either a peroxidase labelled anti-primary antibody or, if greater sensitivity of detection was required, via a biotinylated, anti-primary, secondary antibody and the streptavidin-biotin-peroxidase complex. The primary antibody was also detected using the more sensitive immunogold or protein A gold and silver amplification system (Moeremans *et al.*, 1984) (see Section 2.3.7), where this procedure was deemed necessary.

2.2.2.1 Reagents

Tris-glycine blotting buffer. Tris-HCl (27.23 g) and glycine (64.8 g) were dissolved in approx. 3.5 litres of dist.H₂O, AR methanol (900 ml) added and the volume made up to 4.5 litres. Just before use, 4.5 ml of 10% SDS was added.

Tris buffered saline (TBS) [0.02 M Tris, 0.2 M NaCl, pH 7.4]. Tris (2.42 g) and NaCl (11.69 g) were dissolved in 950 ml of dist.H₂O, titrated to pH 7.4 with HCl and made up to 1 litre.

Tris wash (TBS-Tween) [0.02 M Tris, 0.2 M NaCl, 0.1% (v/v) Tween 20, pH 7.4]. Tris (2.42 g), NaCl (11.69 g) and Tween 20 (1 ml) were dissolved in 950 ml of dist.H₂O, titrated to pH 7.4 with HCl and made up to 1 litre.

Secondary antibodies, antigens and locating agents. Sheep IgG was isolated (see Section 3.7.2), and conjugated to HRPO according to the method of Hudson and Hay (1980). Biotinylated goat-anti-rabbit IgG and donkey anti-sheep IgG

were from Sigma. Streptavidin-biotin-peroxidase complex was from Amersham and the Protein A gold and immunogold probes were prepared as described in Section 2.3.6.

Substrate [0.06% (w/v) 4-chloro-1-naphthol in Tris-methanol, 0.0015% (v/v) H₂O₂]. 4-Chloro-1-naphthol (0.03 g) dissolved in methanol (10 ml) was diluted in Tris buffered saline (2 ml to 8 ml TBS), and H₂O₂ [4 µl of 35% (v/v), solution] was added immediately before use.

2.2.2.2 Procedure

Gloves were worn throughout this procedure, to prevent keratin contamination of the reagents and consequent non-specific binding of antibodies (Ochs, 1983; Shapiro, 1987).

SDS-PAGE was carried out in a Hoefer SE250 Mighty Small apparatus as described in Section 2.5, separation being effected at 18 mA per gel. For western blots with anti-peptide antisera, up to 15 µg of enzyme or crude extract was applied per lane. Normally, however, approximately 1 to 5 µg of protein was loaded. Duplicate gels were run, one gel being used for western blotting, and the other being stained as a reference.

The blotting apparatus was assembled with the buffer-moistened nitrocellulose sheet, cut to the size of the gel, placed on the anode side of the gel, with both sandwiched between sheets of Whatman No. 1 blotting paper and ScotchBrite pads. Electrotransfer was effected at 200 mA for 16 h. The gel was stained (see Section 2.2.1.2), to check the efficiency of the electrotransfer of protein to the nitrocellulose membrane, and the membrane was removed and allowed to dry.

The strip of nitrocellulose, containing the blotted molecular weight marker proteins, was cut from the membrane and stained with 1% aqueous Sudan Black. The rest of the membrane was blocked (1 h) using either 5% foetal calf serum (Gibco), 10% horse serum or 5% low fat milk powder, each in TBS. The membrane was washed in TBS-Tween (3 x 5 min) and the primary antibody, diluted to the desired concentration (2 - 10 µg/ml in 0.5% BSA in TBS, unless otherwise indicated) was applied for 1 h at room temperature. A control strip incubated with a preimmune antiserum or IgG preparation, of the same species, was run in parallel at the same concentration as the test.

Binding of the primary antibody to the target antigen was visualised using a sheep anti-rabbit horseradish peroxidase conjugate (1 in 400 in 0.5% BSA in TBS) or a biotinylated goat anti-rabbit linker (1 in 500 dilution in 0.5% BSA in TBS), followed by an avidin-biotin peroxidase complex (1 in 400 in 0.5% BSA in TBS). The strips were washed and substrate applied, until the bands became clearly visible. When a sheep primary antibody was used, an anti-sheep linker antibody was additionally used between the primary antibody and detection systems.

23 Tissue preparation and gold labelling reagents

2.3.1 Buffers

Phosphate buffered saline (PBS) or Tris buffered saline buffer (TBS) were used in immunolabelling protocols and PBS was also used in some fixatives.

Two versions of TBS were used in labellings in which background or non-specific labelling seemed problematic. One, designated IGSS I, had a high salt content and contained a detergent, to overcome charge and hydrophobic interactions between tissues and labelling reagents. In the second, designated IGSS II, a different pH was used to overcome non-specificity. The nomenclature originates from their original use in immunogold and silver amplification reactions for light microscopy (De Mey, 1986).

For fixation, a HEPES buffer was most often used to avoid the potential extraction of cytoplasmic components and some lipids, by phosphate and cacodylate buffers (Griffiths, 1993).

2.3.1.1 Reagents

1 M sodium cacodylate stock solution. Sodium cacodylate (10.7 g) was dissolved in 45 ml of dist.H₂O, titrated to pH 7.2 with sodium hydroxide, and made up to 50 ml. For use, the stock solution was diluted to 100 mM, and the pH rechecked.

200 mM (133 mM) sodium cacodylate. A 200 mM (133 mM) sodium cacodylate solution was prepared by diluting 10 ml (6.65 ml) of the 1 M

sodium cacodylate stock solution to 50 ml with dist.H₂O. The pH was adjusted if necessary.

400 mM HEPES stock solution. HEPES (100.92 g) was dissolved in 900 ml of dist.H₂O, adjusted to the required pH (7.2 or 8.0) with NaOH and made up to 1 litre. The solution was stored frozen. For use, the buffer was diluted to 200 mM and the pH adjusted, if necessary.

267 mM (200 mM) HEPES. A 267 mM (200 mM) solution of HEPES was prepared by diluting 33 ml (25 ml) of 400 mM stock HEPES solution to 50 ml with dist.H₂O. The pH was adjusted, if necessary.

200 mM phosphate buffered saline stock solution, pH 7.2 (10X PBS). NaH₂PO₄·2H₂O (13.8 g), NaCl (87.6 g) and KCl (1.86 g) were dissolved in 950 ml dist.H₂O, adjusted to the required pH (7.2 or 8.0) with NaOH, and made up to 1 litre.

54 mM (20 mM) phosphate buffered saline (PBS). A 54 mM (20 mM) solution of PBS (50 ml) was prepared by diluting 13.5 ml (5 ml) of 10X stock solution to 50 ml with dist.H₂O. The pH was adjusted, if necessary.

10 mM phosphate buffered saline (PBS). A 10 mM solution of PBS was prepared by diluting the 10X stock buffer 1 part to 19 parts with dist.H₂O (or, when making up fixatives from stock solutions, with the liquid fixatives and dist.H₂O) and the pH adjusted. Unless otherwise stated, when "PBS" is referred to in this study, this will be 10 mM PBS, pH 7.2.

200 mM borate buffer, pH 9.0. Boric acid (12.36 g) was dissolved in 950 ml dist.H₂O, the pH was adjusted to pH 9.0 with NaOH, and the volume made up to 1 litre.

Buffer IGSS I. Tris base (7.9 g), sodium chloride (25 g) and Tween-20 (5 ml) were dissolved in approx. 800 ml dist.H₂O, titrated to pH 7.4 with 1 M HCl and made up to 1 litre.

Buffer IGSS II. Tris base (7.9 g) and sodium chloride (9 g) were dissolved in approx. 800 ml dist.H₂O, titrated to pH 8.2 with 1 M HCl and made up to 1 litre.

2.3.2 Fixatives

In this study 8% paraformaldehyde, in HEPES buffer, was mainly used to fix tissues as many antigens are sensitive to exposure to even low concentrations of glutaraldehyde (Tokuyasu, 1986). Paraformaldehyde is a less cross-linking fixative than glutaraldehyde, giving better antigen survival, though generally poorer preservation of tissue ultrastructure, except at higher concentrations (Baker and McCrae, 1966). Paraformaldehyde, as opposed to commercially prepared formalin or formaldehyde, is used as the commercial products contain undetermined amounts of impurities, such as methanol and formic acid (Hayat, 1981).

2.3.2.1 Reagents

16% Paraformaldehyde stock solution. Paraformaldehyde (BDH, Poole, England) (16 g) was dissolved in dist.H₂O (100 ml), warmed to 60°C and cleared with a minimum amount of 1 M NaOH. The solution was stored at -10°C until required.

8% Paraformaldehyde. 8% solutions of paraformaldehyde, in different buffers, were prepared from the 16% paraformaldehyde stock solution, by diluting the stock solution with an equal volume of 400 mM HEPES buffer, 200 mM sodium cacodylate, or 20 mM PBS (see Section 2.3.1.1). The final pH was checked and adjusted, if necessary.

4% Paraformaldehyde. 4% solutions of paraformaldehyde, in different buffers, were prepared from the 16% paraformaldehyde stock solution, by diluting one volume of the stock solution with 3 volumes of 267 mM HEPES buffer, 133 mM sodium cacodylate, or 54 mM PBS (see Section 2.3.1.1). The final pH was checked and adjusted, if necessary.

1% Glutaraldehyde. 25% Glutaraldehyde (Merck) (1 ml) was diluted to 25 ml with PBS, pH 7.2.

2% (or 4%) Paraformaldehyde/0.2% glutaraldehyde. 16% stock para-formaldehyde [1.25 ml (or 2.5 ml)] and 25% glutaraldehyde (20 μ l) were made up with 1 ml of 10X stock PBS or 1 M sodium cacodylate stock solution, and 8 ml of dist.H₂O, the pH adjusted with NaOH or HCl, and a further 1 ml of dist.H₂O added.

2.3.3 "Blocking" agents.

Attempts to prevent non-specific antibody attachment to the tissue, via free aldehyde groups or by charge or hydrophobic interactions, were made using gelatin or other "blocking" proteins (Griffiths *et al.*, 1983; Birrell *et al.*, 1987; Behnke, 1986). These extinguish potential non-specific binding sites.

All blocking reagents were dispensed in aliquots, sufficient for an immunolabelling procedure, and stored frozen (-10°C) until required.

2.3.3.1 Reagents

1% and 5% Bovine serum albumin (BSA). BSA (Boehringer, Fraction V) (1 g or 5 g) was dissolved in PBS and made up to 100 ml.

5% Foetal calf serum. Sterile foetal calf serum (GIBCO) (5 ml) was made up to 100 ml with 200 mM HEPES buffer, pH 7.2.

0.02M glycine in PBS, pH 7.2. Glycine (0.15 g) was dissolved in PBS and made up to 100 ml.

1% Fish skin gelatin. Fish skin gelatin (Sigma) (0.1 ml) was dissolved and made up to 10 ml with PBS.

2.3.4 Production of gold particles by citrate and tannic acid-citrate

Gold labels may be produced from colloidal gold particles of different sizes, prepared by reducing gold chlorides with various reducing agents (Horisberger, 1981). Two types of gold labels, "immunogold" and protein A gold probes have become the most commonly used, for immunocytochemistry at the electron microscope level. The probes are made by adsorption of protein A or immunoglobulins to the surface of gold particles, by non-covalent electrostatic forces, forming a protein A gold or "immunogold" probe (Horisberger and Rosset, 1977).

Since these two proteins are adsorbed to the gold particles in an undenatured form, they continue to bind the primary antibody and act as a bridge between the primary antibody and the colloidal gold particle marker.

Gold probes have many advantages over the potentially diffusable reaction products of enzyme labels. The characteristic spherical shape and high electron density of gold probes, makes them easy to distinguish from biological structures. Their discrete nature and the fact that gold labels or "probes" may be made from colloids of different particle sizes, makes gold probes useful for simultaneous multiple antigen labelling, and quantitation (Slot and Geuze, 1985).

Protein A is a staphylococcal protein, of 42 kDa, that binds to the Fc portion of IgG molecules of many animal species. This binding is strongest to human, rabbit, guinea-pig, swine and dog IgGs (Langone *et al.*, 1978). Binding to IgG from cow, mouse and horse is weaker and to IgG from sheep, goat, rat and chickens is considerably weaker (Langone, 1982). These IgG species are listed in order of decreasing affinity. The present study was performed largely with rabbit antibodies and protein A gold probes, the latter being a convenient, universal, reagent. Where primary antibodies from other species, such as sheep (which do not react well with protein A) were used, a rabbit anti-species specific linker antibody, followed by a protein A gold probe was used.

Protein A, binds to the Fc fragment of a tissue-bound primary antibody, in a one-to-one ratio (Slot *et al.*, 1989), whereas more than one immunogold probe may bind to a single tissue-bound primary antibody. Immunogold probes, therefore, generally give higher labelling signals than protein A gold probes. The inclusion of an extra "linker antibody" in the protein A gold labelling system, when, for example, a sheep primary antibody is used, has the additional effect of amplifying the protein A gold labelling signal, as more than one secondary antibody can bind to the primary tissue-bound antibody.

Many other factors complicate the direct quantitation of antigens by counting the number of tissue-bound gold labels. These may be described under the heading, "factors affecting labelling efficiency". Labelling efficiency, the number of gold particles observed, divided by the number of antigens known to be present on the surface labelled, is usually about 10%, at best (Griffiths, 1993).

Labelling efficiency is affected by a number of probe and tissue antigen-related variables. The size of the gold particle chosen should not be so large that the binding of one gold particle precludes the binding of subsequent gold particles in its vicinity, a phenomenon known as "steric hindrance". In a similar fashion penetration of gold probes into tissues, to bind antigen-bound primary antibodies, may be more of a problem with larger gold probes. Smaller gold probes (2-5 nm), generally give a greater labelling density and sensitivity than larger probes (20-30 nm). Smaller gold probes (5 to 10 nm) were, therefore, generally used in this study.

The size and size variation of the colloidal gold particles, used in label production, is important when multiple simultaneous labellings, using different sizes of gold labels, are carried out. For this reason a method of colloid production which produces uniformly sized colloidal particles was sought. The tannic acid-citrate method of Slot and Geuze (1985) was used in this study as it produces a uniform colloid with a size range from 5 nm to approx. 15 nm, depending on the amount of tannic acid added.

During the formation of colloidal gold particles, nucleation and growth of colloidal gold particles are two opposing phenomena. The rate of reduction of the chloroauric acid solution, from Au^{3+} to metallic Au, determines the number of nucleation points formed. Particle growth, however, occurs by further condensation of Au on the surface of the nuclei. The size or average particle diameter (APD) of the particles formed, is, however, inversely proportional to the cube root of the number of nuclei formed. Rapid reduction, therefore, gives rise to a larger number of nucleation points and, consequently, to smaller colloidal particles.

Tannic acid causes fast reduction, while citrate reacts slowly. In the citrate-tannic acid method, various sizes of colloids are produced by varying the amount of tannic acid added. At the higher concentration of tannic acid, used for the production of the smaller gold probes, reduction is almost exclusively due to the tannic acid. Nevertheless, even at lower concentrations of tannic acid, the size of the particle appears to be regulated by tannic acid, and the variation in the size of the colloid is generally less than 10%, producing a desirably homodisperse colloid, suitable for the production of probes for multiple simultaneous immunolabelling regimes (Slot and Geuze, 1985).

The production of colloidal gold probes may be divided into 3 sections: production of the colloidal gold particles, determination of the minimum amount of

protein required for stabilisation of the particles and finally the production, purification, sizing, and testing of the colloidal gold probe.

2.3.4.1 Reagents

All reagents must be made in scrupulously cleaned glassware, with AR chemicals and highly purified water, preferably produced by reverse osmosis. The containers used for the final production of the gold colloid must also be scratch-free.

1% Chloroauric acid solution (HAuCl₄). HAuCl₄ (Merck, Darmstadt) (1g) was dissolved in dist.H₂O, made up to 100 ml, and stored in the dark at 4°C, until used.

1% Trisodium citrate. Trisodium citrate.2H₂O (Merck, Darmstadt) (1.14 g) dissolved in dist.H₂O and made up to 100 ml, was freshly prepared daily.

1% Tannic Acid. Tannic acid (Mallinckrodt #1764) (0.1 g), dissolved in dist.H₂O and made up to 10 ml, was freshly prepared daily.

25 mM K₂CO₃. K₂CO₃ (3.45 g) was diluted with dist.H₂O (10 ml) and filtered through a 0.22 µm filter before use.

2.3.4.2 Procedure

To make 200 ml of gold sol, two solutions were prepared: solution A consisted of dist.H₂O (160 ml) and 1% HAuCl₄ (2 ml) and Solution B consisted of 1% trisodium citrate (8 ml), dist.H₂O (32 ml) and a variable amount of tannic acid, depending on the size of colloid required (see Table 2). An equal amount of K₂CO₃ is added when 1 ml or more tannic acid is used. Solutions A and B were separately warmed to 60°C and mixed rapidly, while stirring. Once a red colour is formed the solution was heated to 95°C and cooled on ice. Larger particles (using lower concentrations of tannic acid) take longer to form and the red colour can take up to 1 h to develop.

Table 2. Amount of tannic acid to be added per 200 ml of combined solutions A and B, to produce the desired size of gold particles.

Size of gold particle (nm)	1% tannic acid (ml)
3.5	10.0
4.0	5.0
5.0	2.0
6.0	1.0
7.5	0.5
9.5	0.2
10.0	0.16
11.5	0.1
14.0	0.05

2.3.5 Determination of minimum amount of protein for colloid stabilisation

Optimal adsorption of macromolecules, to the surface of the hydrophobic, negatively charged colloidal gold particles, occurs under conditions allowing maximal multi-point contact between the macromolecule and the "edge" surface of a gold particle, which are thought to contain increased Van der Waal's forces. Such multipoint contact is favoured by adsorption of minimal amounts of stabilising protein, in low ionic strength buffers, at the pI of the protein or at pI + 0.5 pH units) (De Mey, 1986).

At the pI of the protein, the protein is predominantly in the zwitterionic form, and the interfacial tension maximal. This allows stronger, more stable adsorption to the hydrophobic surface of the gold particles. The adsorbed protein stabilises the colloid against an electrolyte challenge; at electrolyte levels at which the colloid becomes unstable, its colour changes from pink to blue (Geoghegan and Ackerman, 1977). The minimum amount of protein required to stabilise a colloid, at a particular pH, may be established by titration and electrolyte challenge. The lowest amount of protein required to stabilise a set volume of the colloid against salt-induced aggregation, at the pI, or slightly to the basic side of the pI of the protein, is known as the "minimum stabilisation concentration" for the colloid and the particular protein. The "minimum stabilisation concentration" of protein (+ 10 %) is then used in the production of the gold probe.

2.3.5.1 Reagents

10% NaCl. NaCl (1 g) was dissolved in dist.H₂O (10 ml).

2 mM Borate buffer. Boric acid (0.123 g) was dissolved in 950 ml dist.H₂O, adjusted to pH 9.0 with NaOH, and made up to 1 litre.

Donkey or rabbit anti-sheep IgG. Affinity purified donkey- and rabbit anti-sheep IgG (Sigma Chemical Co.) was reconstituted to 0.5 mg/ml and dialysed against 2 mM borate buffer.

Protein A. Protein A (Sigma Chemical Co.) was diluted to 1 mg/ml with dist.H₂O.

2.3.5.2 Procedure

After high speed centrifugation of the proteins, the dialysed IgG or protein A, was diluted to 0.2 mg/ml for the 20 nm and 0.75 mg/ml for the 5 nm gold probes, and a dilution series set up, in a Nunc flat-bottomed, high-bond microtitre plate, as indicated in Table 3, and mixed by manual agitation.

The colloidal gold sol was adjusted, with K₂CO₃, to the pI (or just above the pI) of the protein and 100 µl was added to each well of the microtitre tray and mixed by agitation. [For IgGs, pH 8.0 was used (De Mey, 1986) whereas, for protein A, pH 6.0 is recommended (Horisberger and Clerc, 1985)]. The adsorption was allowed to proceed for 2 min before challenge with 10% NaCl (10 µl). The plate was again agitated, to mix the solutions, and allowed to stand 5 min before the colour change was assessed visually. The end point, the highest dilution giving no colour change (pink to blue) was assessed and the minimum amount of protein stabilising 100 µl of gold was used to calculate the amount of protein required to stabilise larger volumes of sol.

Table 3. Dilution series for determination of minimum stabilisation concentration.

Protein (μ l)	Solvent* (μ l)
10	0
9	1
8	2
7	3
6	4
5	5
4	6
3	7
2	8
1	9

* The solvent is preferably dist.H₂O. If the protein is insoluble in this, a low ionic strength buffer (less than 10 mM) is used.

2.3.6 Production and purification of gold probes

After the addition of the minimal stabilisation concentration of protein, residual protein-binding sites on the gold probe may be quenched by the addition of excess quenching protein, or polyethylene glycol (PEG). In this study, BSA was exclusively used as a quenching agent as PEG was found to be less effective at preventing probe aggregation upon centrifugation, leading to a lower yield of probe. For the production of protein A gold probes, it is necessary to use globulin-free BSA.

A one-step glycerol gradient centrifugation procedure is favoured for the purification of the probe. The alternative non-gradient method involves washing, with buffer, by serial cycles of centrifugation, aspiration of the supernatant and resuspension of the pellet. In such a system, the aggregates form a non-mobile pellet at the bottom of the tube and the unaggregated, mobile pellet has to be removed carefully and rewashed. In the continuous gradient system, the colloid fractionates in the gradient on the basis of size, the aggregates passing right through, and the unbound proteins are simultaneously removed.

2.3.6.1 Reagents

10% BSA quenching agent for immunogold probes. BSA (Boehringer, FractionV) (1 g) was dissolved in about 8 ml of dist.H₂O, adjusted to pH 8.0 with NaOH and made up to 10 ml. The solution was micro-filtered just before use, taking care to avoid frothing.

10% BSA quenching agent for protein A gold probes. BSA (Boehringer, FractionV) (1 g) was dissolved in 10 ml of dist.H₂O, giving a pH of approximately 6.8. The solution was micro-filtered just before use, taking care to avoid frothing.

20 mM Tris buffered saline (TBS), pH 8.0. Tris base (2.42 g) and sodium chloride (9 g) were dissolved in approx. 800 ml dist.H₂O, titrated to pH 8.0 with 1M HCl and made up to 1 litre.

20 mM Tris buffered saline containing 0.1% BSA (TBS-BSA), pH 8.0. BSA (0.1 g, Boehringer, Fraction V) was dissolved in 100 ml of TBS.

10% and 30% glycerol. Glycerol (10 or 30 ml) was diluted with 0.1 % BSA in PBS or TBS-BSA (90 or 70 ml) to make a 10 or 30% glycerol solution, for gradient separation of protein A gold probes or immunogold probes, respectively.

2.3.6.2 Procedure

The calculated minimal stabilising amount of protein is added slowly, while continually and rapidly stirring the pH-adjusted sol. After 2 min, residual protein-binding sites are quenched by the addition of the appropriate 10% BSA solution, to a final concentration of 1% (globulin-free BSA, in the case of protein A gold probes, and pH 8 BSA for the immunogold probes).

The colloidal gold particles are centrifuged (30 min, 4°C) at the speed required to pellet that particular size of colloidal gold particle (Table 4). The mobile pellet is suspended in approx. 1 ml of the supernatant and loaded onto a 15 ml 10→30% glycerol gradient, centrifuged (45 min, 4°C, at the speed indicated in Table 5), and the dark red band near the middle of the gradient is collected and stored at 4°C. Alternatively the protein A gold or immunogold probes are washed twice in 0.1%

globulin-free BSA in PBS or 0.1% BSA, respectively, by centrifugation at the speeds indicated in Table 4.

Table 4. Speeds for centrifugation of colloid after minimal stabilisation and quenching, for removal of unbound proteins.*

Size of particle (nm)	Rotor 70 Ti (RPM)	X g (r_{max})
4	47 000	225 000
5	45 000	210 000
6	42 000	185 000
7	38 000	155 000
8	33 000	105 000
9	30 000	92 000
10	27 000	75 000
12	18 000	37 000
14	10 000	7 000

* If gradient centrifugation is not to follow this step, the pellet is resuspended in 0.1% BSA in PBS or TBS, as indicated above, and recentrifuged as above. Centrifugation conditions, 30 min, 4°C.

Table 5. Gradient centrifugation purification of colloidal gold probe pellet.

Size of particle (nm)	Rotor SW 40 (RPM)	X g (r_{max})
4	40 000	284 000
5	37 000	240 000
6	32 000	170 000
7	30 000	150 000
8	26 000	115 000
9	22 000	85 000
10	19 000	65 000
12	11 000	20 000
14	5 000	5 000

2.3.7 Characterisation of colloidal gold probes

Characterisation of the gold probe involves checks on the specific binding activity, and the size and size distribution of the harvested probe. Binding of the colloidal gold probe to a target antigen can be visualised using the silver amplification procedure (Danscher and Nørsgaard, 1983). This produces an intensely black labelling product that makes gold labelling extremely sensitive. Gold labels, bound to the antigen, displace metallic silver from soluble silver solutions (e.g. silver lactate). In this way, growing shells of precipitated silver surround the gold particles, increasing their effective size, and give a visible black-brown colour at the site of binding of the gold label. The intensity of the enhancement is controlled by the time for which the gold is allowed to displace the metallic silver.

2.3.7.1 Reagents

Target and non-target immunoglobulin species. Both target and non-target immunoglobulins were diluted in 1% BSA in PBS to give a final range of 1 µg to approx. 10 ng, in 2 µl of blotted target immunoglobulin.

Membrane blocking solution. BSA (1 g, Boehringer, Fraction V,) was dissolved in PBS (100 ml).

Protein A gold probe diluent. Globulin-free BSA (Sigma) (0.1 g) was dissolved in PBS (100 ml).

Immunogold probe diluent. BSA (Boehringer, Fraction V) (0.1 g) was dissolved in PBS (10 ml).

20 mM Tris buffered saline containing 0.1% BSA (TBS-BSA), pH 8.0. BSA (Boehringer, Fraction V) (0.1 g) was dissolved in TBS (100 ml, Section 2.3.1.1).

Citrate buffer, pH 3.9. Trisodium citrate (2.35 g) and citric acid (2.55 g) were dissolved in 85 ml of dist.H₂O.

Silver amplification solutions.

- A. Hydroquinone (850 mg) was added to 85 ml of citrate buffer, pH 3.9.
- B. Silver lactate (110 mg) was added to 15 ml of dist.H₂O and protected from light.

When required, the reagent was prepared by adding A to B (in the dark). The silver amplification solution was made immediately before use, in scrupulously clean glassware and with high grade dist.H₂O.

5% Sodium thiosulphate. Sodium thiosulphate (5 g) was made up in 100 ml of dist.H₂O.

2.3.7.2 Procedure

The colloid was photographed in the electron microscope and the size of the particles of the probe measured, to establish the size and size variation of the harvested probe. This was carried out by coating a grid with a formvar support membrane (Hayat, 1986), and further coating the membrane with 0.1% polylysine in dist.H₂O. A thin layer of gold sol was allowed to adhere, and was photographed and the particles were assessed for size. A size variation of less than 10% was considered acceptable for multiple labelling procedures.

The specific binding activity of the probes was also established. A 0.22 μm nitrocellulose membrane was cut to fit a Hoefer Decaprobe apparatus. The gasket was laid in position on top of the membrane, on the base of the assembly. A dilution series (2 μl) giving 1 μg to approx. 10 ng of target and non-target immunoglobulin species was dot-blotting on the membrane and the Decaprobe apparatus was assembled. Residual protein-binding sites on the membrane were blocked with the membrane blocking solution.

The gold probes were diluted with protein A gold or immunogold probe diluent to a dilution at which the gold probe gives an absorbance at A₅₂₀ of between 0.05 and 0.1. The relevant diluted gold probe (150 μl) was added to the relevant channel in the Decaprobe, containing the target and non-target dilution series. The Decaprobe was rocked gently for 30 min and the unbound gold probe washed away with PBS or TBS (for protein A and immunogold probes, respectively), with 5 changes of 0.5 ml of buffer over 20 min. The Decaprobe was dismantled and

the entire membrane placed in a clean dish and washed thoroughly in 5 changes of dist.H₂O, in 10 min.

The membrane was placed in the dark, the silver amplification reagents applied and the blot developed until the membrane-bound gold is evident (approx. 5 min). The silver amplification reaction was terminated by the addition of 5% sodium thiosulphate (5 min) and the membrane was washed, dried and stored in the dark.

A satisfactory binding activity was given by any probe specifically detecting at least 50 ng of the correct target immunoglobulin.

2.4 The cryoultramicrotomy technique

The thawed cryosection immunolabelling technique, used in this study, embodies cryoultramicrotomy, for embedding and sectioning, and the so-called thawed, frozen, ultrathin, section method of Tokuyasu (Tokuyasu, 1986), for immunolabelling and viewing in the electron microscope. Together, these constitute one of the mildest, quickest, but one of the most technically-demanding of the techniques available for immunolabelling. Cryoultramicrotomy demands excellently sharp glass knives and special tungsten coating of the knife edge to preserve its keenness. Grids need to be specially prepared; formvar and carbon coating has to be followed by a cleaning and charge- or hydrophilicity-inducing treatment, to enable thawed frozen ultrathin sections to adhere, during the subsequent washing and labelling steps. These technical procedures and modification of equipment will be described before the treatment and handling of the specimen is described.

For the thawed cryosection technique, the specimen requires fixation, cryoprotection and rapid freezing, before sectioning, immunolabelling and sealing for viewing in the electron microscope. Fixation may be slight, as the tissue does not have to withstand harsh treatment, as in resin embedding processes. Fixation (which will be discussed in Chapter 4) is the only potentially tissue-damaging technique in the Tokuyasu procedure, as labelling and viewing is performed on a fully hydrated specimen. Cryoprotection of the tissue is necessary, in order to prevent the damaging effects of ice crystal formation, during the freezing process. The tissue blocks should be small and frozen very rapidly to the structureless, vitreous state, rather than to a hexagonal or cubic crystalline state. This requires cooling at a rate $> 10^5$ °C/sec, to

below -140°C (Dubochet *et al.*, 1988). Plunge-freezing in liquid nitrogen is a convenient method for achieving such cooling rates. The frozen tissue block may be stored indefinitely in liquid nitrogen for subsequent sectioning.

In this section, the technicalities of cryoultrasectioning will be considered first; knifemaking and the modifications made to an existing commercial knifemaker, will be described, followed by a description of the preparation of grids and the modification of a sputter-coater, used to render grids hydrophilic, before details of the Tokuyasu technique are given.

2.4.1 Glass knife making and preparation

A far sharper glass knife than is required for resin sectioning, is a crucial requirement for successful cryoultramicrotomy, and this puts more stringent demands upon the knife-making instrument. Most existing knifemakers were designed to make glass knives for resin sectioning and, without modification, have proved to be inadequate for making knives for cryoultramicrotomy.

The principles of knife making for cryoultramicrotomy have been discussed by Griffiths *et al.* (1983). A glass knife is made by first cutting 25 x 50 mm rectangles from a strip of glass (5 x 25 x 400 mm) and then bisecting the rectangles to make two squares from each. The reason for adopting this approach is so that, when the final break is made, there will be equal weights of glass on either side of the fracture, resulting in accurate breaks, with the fracture plane at right-angles to the surface of the glass. Finally, the cutting edge is produced by making a diagonal score line across the 25 mm squares and applying a tensile stress across the score.

The sharpness of the cutting edge is determined by how close the break comes to passing through the two opposite corners of the glass squares - a theoretical ideal would be for the fracture to pass accurately through the corners, bisecting them at 45° . In practise, the fracture veers away from the corner. How close it comes to the corner may be judged by measurement of the width (w) of the counter-piece, which should be less than 0.1 mm in a satisfactory glass knife (Griffiths *et al.*, 1983) (Fig. 1).

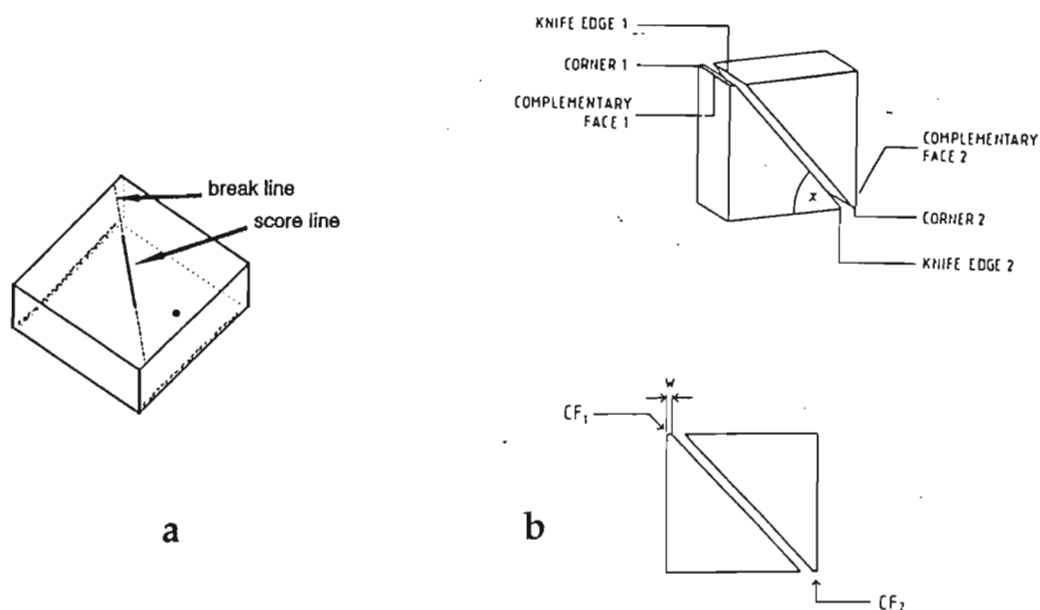


Figure 1. Making glass knives by fracture of a 25 mm square of glass.
 a, the score line and a theoretical break line.
 b, the nearness of the break to bisecting the opposite corners of the glass and hence the sharpness of the knife, is indicated by the thickness (w) of the counter-piece edge, or complementary face (CF) in an actual break line (from Griffiths *et al.*, 1983).

Without modification, our LKB 7801B KnifeMaker was found to be incapable of reproducibly making knives acceptable for cryoultramicrotomy. The reason for the irreproducibility was found to be largely the accumulative effect of a number of manufacturing tolerances, and/or wear, in the clamping head and cutter wheel holder assembly (see Fig. 4) which, together, allowed the cutter to move sideways over a distance of 0.34 mm. With this amount of play in the mechanism, cutting to within 0.1 mm of the corner was largely a matter of chance. The modifications, described below, made to improve the accuracy of the mechanism, as well as other modifications, led to a marked improvement in the reproducibility of knifemaking, and a corresponding increase in the proportion of acceptable knives produced (at least a 100% improvement in the success rate).

2.4.1.1 Modification of LKB 7800 series KnifeMaker

The KnifeMaker was first modified according to Stang (1987) (Fig. 2). This involved manufacture of the aluminium T-shaped holder, and machining of the "38" cam lobes, of the scoring selector (Fig. 3) to reduce the score length to 12 mm. This 12 mm score was used for all glass pieces, including long pieces. In addition, the following modifications were made:-



Figure 2. An LKB 7800 series KnifeMaker incorporating the T-piece modification of Stang (1987). The front glass holder, guide plate, guide rings, damper lever and pads shown in Fig. 3a are replaced by the T-piece.

- a) The play between the cutter wheel and the cutter wheel holder bracket was eliminated by removing the cutter wheel and bending the side plates of the cutter wheel holder bracket inwards, by about 0.01 mm at a time, until only a very slight interference fit was perceptible, between the cutter wheel and the holder bracket, but which nevertheless still allowed free rotation of the cutter wheel.
- b) Similarly, the play between the cutter wheel holder and the cutter wheel holder bracket (LKB part No. 24-10 688) (originally 0.06 mm) was eliminated by bending the side plates towards each other, by about 0.01 mm at a time, until only a very slight interference fit was perceptible (Fig. 3).
- c) The play (originally 0.11 mm) between the cutter wheel holder bracket and the scoring shaft was eliminated by machining a recess 1.3 mm (deep) x 6.5 mm x 15 mm into the cutter wheel holder bracket and inserting a beryllium copper spring, 0.5 mm thick, bent to form two lines of contact with the scoring shaft, for extra stability (Fig. 4a). This spring must not press too firmly against the scoring shaft, or the cutter wheel holder bracket will not retract after leaving the cam.

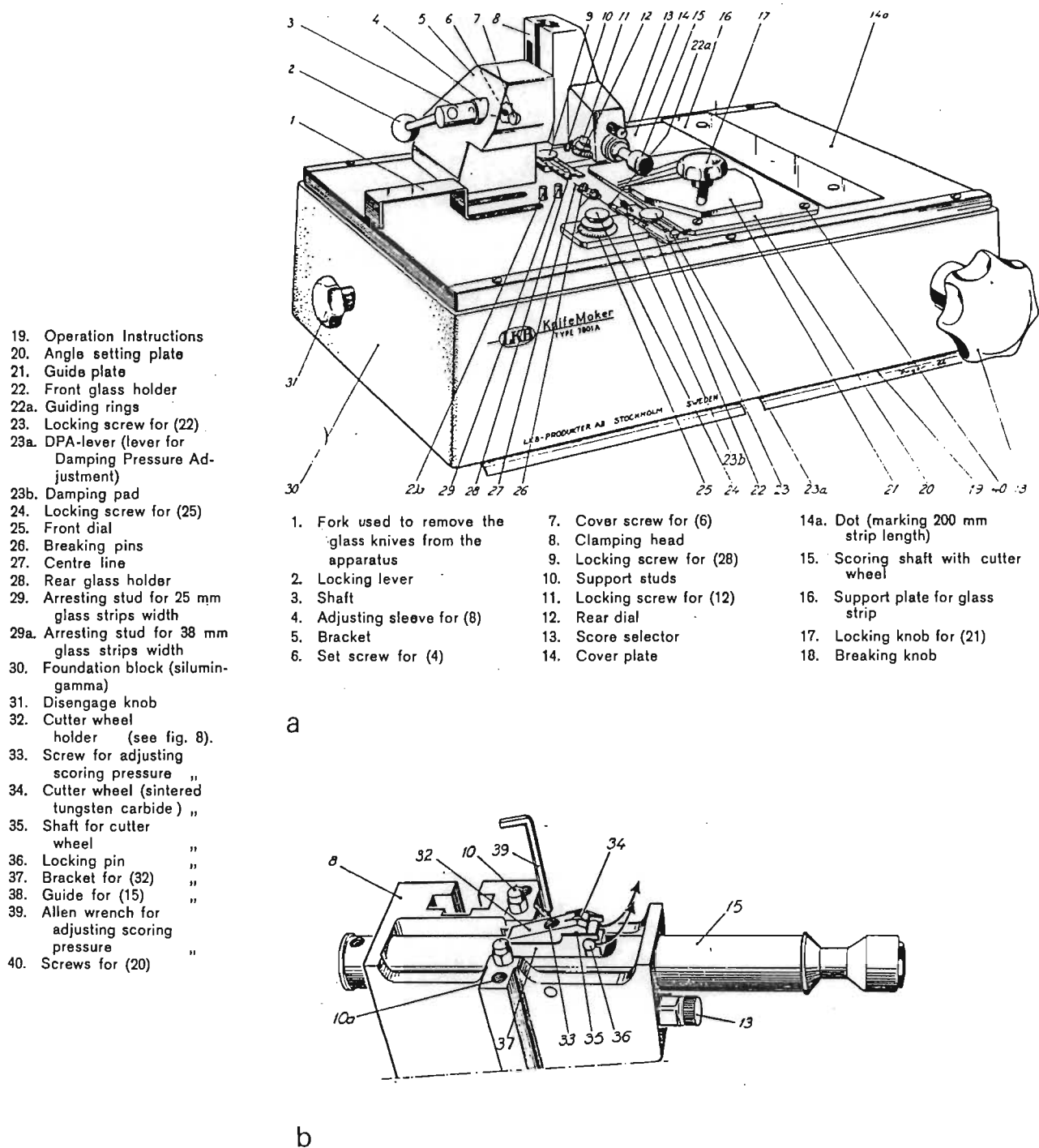


Figure 3. Components of LKB 7800 series KnifeMaker (from manufacturer's pamphlet).
 a shows the components of the unmodified knifemaker
 b shows a view of the underside of the clamping head, showing the cutter wheel and the scoring shaft, and adjustment screw for guide for scoring shaft.

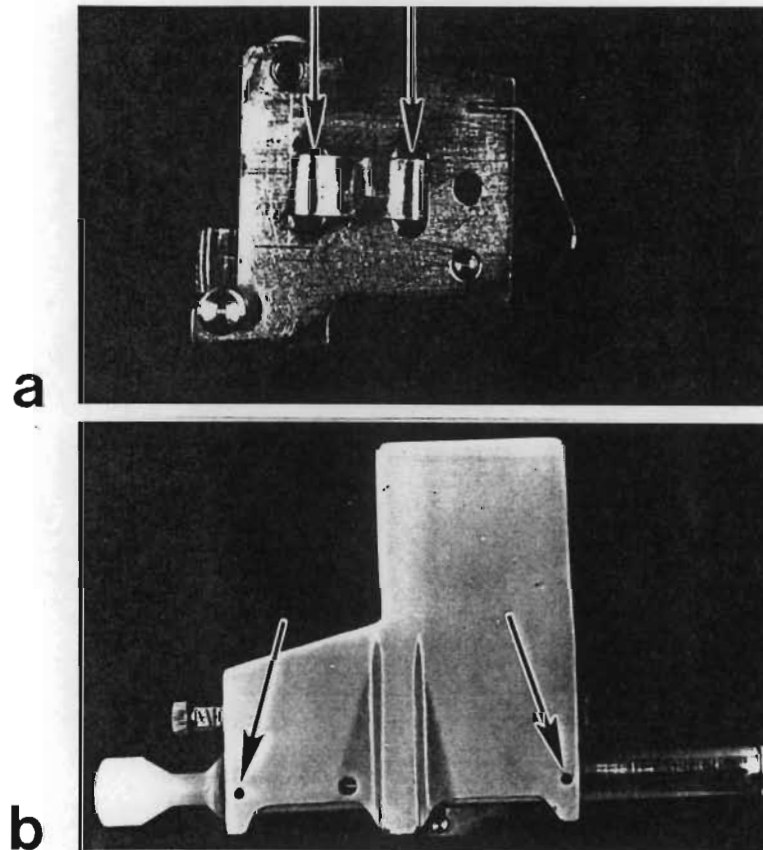


Figure 4.

Modifications to the LKB 7800 series KnifeMaker.

- a, The beryllium copper spring inserted to eliminate play between the cutter wheel holder bracket (LKB Part No. 24-10 688) and the scoring shaft . The arrows indicate the two lines of contact between the spring and the scoring shaft.
- b, The holes drilled and tapped into the clamping head to accommodate grub screws . The arrows indicate the position of the holes accommodating teflon plugs and grub screws that eliminate play between the scoring shaft and the clamping head.

d) The play (originally 0.06 mm) between the scoring shaft and the clamping head was eliminated by drilling two 2.5 mm holes, into the clamping head (Fig. 4b), and tapping these with a 3 mm I.S.O. thread. Small teflon plugs were inserted into the holes, followed by grub screws that were tightened to an extent which just allowed free travel of the scoring shaft.

- e) The breaking pins, which apply the breaking force to the glass, were removed, smoothed in a lathe with fine water paper and buffed to a polished finish.
- f) The play between the scoring shaft guide (Fig. 3, item #38) and the scoring shaft itself (Fig. 3, item #15) was eliminated by bending the outer “forks” of the scoring shaft guide outwards, a small amount, until a slight interference fit was achieved in the scoring shaft groove.

The angle at which the glass breaks, which should be 90° to the glass surface, is determined by the exact positioning of the cutter wheel scoring edge over the domed breaking pins. Consequently, it is necessary that the the cutter wheel is accurately centralised and locked between the support studs to ensure that the score is directly over a line joining the axes of the breaking pins. The holes in the scoring shaft guide are larger than the screw diameters and so the position of the scoring shaft guide can be altered. Movement of the scoring shaft guide causes rotation of the scoring shaft and consequential lateral displacement of the cutter wheel. The necessary adjustment can be made, without waste, by test breaks while cutting the glass strips into 50 x 25 mm rectangles. If the cutter wheel is off centre, the glass will not fracture at right angles to the plane of the glass. If this is so, the shaft guide screws may be loosened slightly, the shaft guide plate moved in the appropriate direction to compensate, and the screws retightened. Further test cuts may be made and small corrections made by loosening the shaft guide screws, tapping the plate left or right and retightening the screws. This procedure is repeated until the break in the test piece is true and vertical. When the cutter wheel is properly centred, the KnifeMaker will break optimal knives, with cutting edges at right angles to the plane of the glass. Conversely, if the cutter wheel is off-centre, the resulting knives will have sub-optimal, out-of-square cutting edges. Proper adjustment in this respect greatly improves the accuracy of knife breaking.

Accurate and reproducible positioning of the glass pieces is also important. The rear and front glass holders have tongues which slide in slots in the base of the KnifeMaker. On our KnifeMaker the clearance, between the tongues and the slots, was about 0.11 mm, which enabled each glass holder to move left or right by about 0.08 mm, and this affected the reproducible positioning of the glass. The play was consequently eliminated, by peening the end of each slide tongue on the inner side and filing the raised section to a slight interference fit, using a #4 needle file. While this approach proved adequate, the same effect might be better achieved by the use of appropriately inserted captive balls and springs.

In addition to the modifications and adjustments to the KnifeMaker itself, it was found to be necessary to orientate the glass strips and glass pieces in the KnifeMaker in a specific manner. The glass strip should be orientated on the KnifeMaker with the scored long edge break downwards and the clean break edge upwards. When using 25 mm strips, the strip is first cut into 25 x 50 mm rectangles, marked as shown in Fig. 5a with a felt-tipped pen and cut into two 25 mm squares. The resulting squares are orientated in the KnifeMaker, for breaking, as shown in Fig. 5b. It is important to check that the glass strips used have consistent dimensions: this was not true of glass strips from all manufacturers.

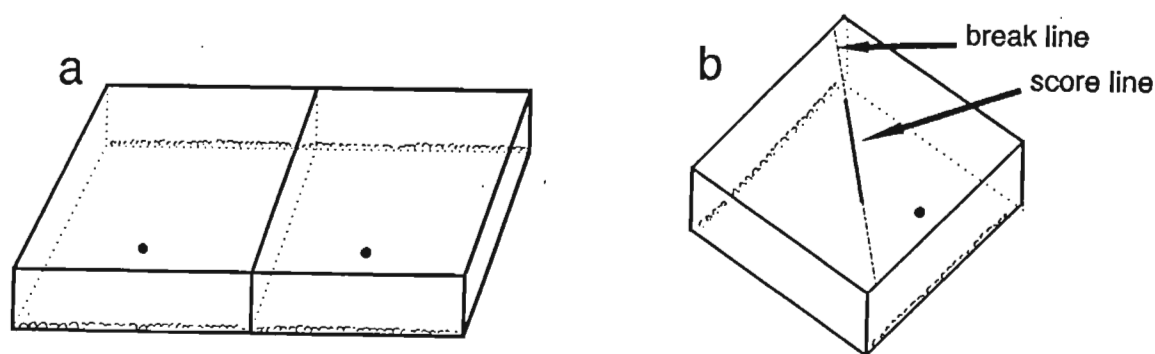


Figure 5. Orientation of glass blocks for cutting 45° knives, from 25 mm strip.
 a Marks are made on the glass rectangle as shown, using a felt-tipped pen, before it is cut into 25 mm squares.
 b The 25 mm squares are orientated as shown, before being broken into knives.

2.4.1.2 Tungsten coating of glass knives

The keenness of the knife edge is maintained indefinitely in storage and the life of the edge considerably prolonged by coating the knife with a thin layer of tungsten metal (Roberts, 1975).

Freshly made knives were placed in an Edwards E306A high vacuum coater and tilted such that the line from the filament source to a knife edge bisected the cutting angle of the knife, allowing deposition of the metal film on both faces of the knife simultaneously. A 25 mm length of 0.5 mm pure tungsten wire,

bent to form a "v"-shaped filament, was mounted at a distance of 8 - 10 cm from the cutting edge of the glass knife. The pressure in the vacuum coater was reduced to 5×10^{-5} Torr, and the filament was cleaned by briefly heating, with a guard in place between the knives and the filament. The guard was removed and the tungsten was evaporated onto the cutting surface of the knives, by increasing the current through the filament to 80 - 90% of the value of Z, for 3-5 min (Z being the current which melts a filament of the same length). Coated knives were removed and stored in a container, stuck down at the base with double-sided tape.

2.4.2 Preparation of grids for cryoultramicrotomy sectioning

Before use, the 100 mesh hexagonal grids are coated with a support layer of formvar which, in turn, is coated with a thin layer of carbon (see Section 2.4.2.3). The carbon layer is hydrophobic in character and requires "activation", by the process of glow-discharging, to render it hydrophilic so that hydrated sections will easily stick to the grid.

In the process of glow-discharging, the grid is placed in a vacuum, between two electrodes, in a position corresponding to the so-called Crookes' space, a dark zone in the purplish glow that occurs when a high voltage DC glow-discharge is set up between the electrodes (Benada and Pokorny, 1990). In the Crooke's dark space the high velocity electrons have a cleaning effect, decomposing adsorbed hydrocarbon molecules (Holland, 1960) and imparting a negative charge to the surface of the carbon layer, to which proteins will adhere (Benada and Pokorny, 1990).

In order to effect activation of grids by glow-discharging, a Polaron sputter-coater was modified, as described below, using the information provided by Benada and Pokorny (1990) as a guide.

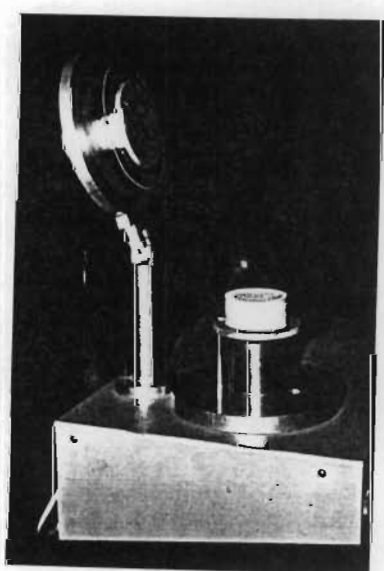
2.4.2.1 Modification of sputter coater

In both the original modification of Benada and Pokorny (1990) and our modification, the top plate assembly, with annular target, and the specimen support, of the Polaron E 15000 series sputter-coater unit, were replaced. In the modification of Benada and Pokorny (1990) the aluminium lid was replaced by a Perspex (Plexiglass) lid, a stainless steel spacer and an aluminium disc anode. In our model the aluminium lid was retained, and insulation was achieved by the use of a PTFE spacer (20 x 40 mm o. d.) and a Perspex insulating shield. The insulating shield

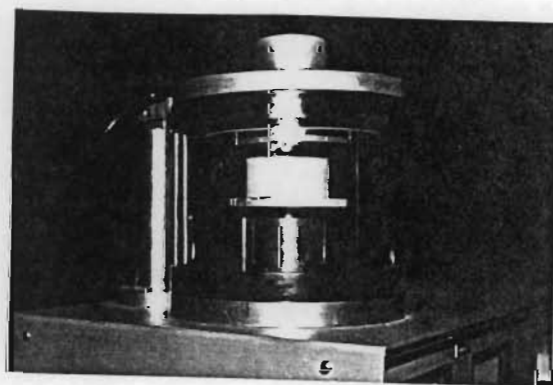
(2 mm thick x 130 mm diameter) was placed behind the cathode (6 mm thick x 90 mm diameter), between it and the aluminium lid, to obviate flash-over (Fig. 6).

In our modification the original lower plate was replaced with a larger anode (6 mm thick x 89 mm diameter) and a PVC support made of 3 mm wall-thickness tubing (63 mm o. d. x 28 mm) covered with a nylon (20 mesh) support grid, at one end. The support is placed on top of the anode electrode, to lift the grids into the Crooke's dark space. The electrodes, located 40 mm apart, were attached to the built-in power supply, capable of delivering at least 800 kV, the lowest voltage required to strike the plasma when the chamber is evacuated. The chamber (originally filled with air) requires evacuation to at least 1.5 Torr before the plasma will strike.

The higher the voltage and amperage used in the glow-discharging process, the more damaging the action of the electrons to the formvar film. Optimisation procedures, varying these parameters and checking the induced hydrophilicity, by checking the water-wettability of the surface of the grids, and examination of the formvar grid surface for damage, using scanning electron microscopy, indicated that adequate glow-discharging may be achieved at 800kV, 16 mA for 10 sec. Newly prepared, carbon-coated formvar grids were treated in this way before the grids were used as supports for thawed cryoultramicrotomy sections.



a



b

Figure 6

Diagram showing the key modifications of the Polaron sputter coater.
 a Instrument with glass chamber removed and lid open.
 b Lid closed and glass chamber in position.

2.4.2.2 Formvar grid preparation

Formvar (0.2 g) was dissolved in absolute alcohol (100 ml) and stored in an air-tight container. A dish (approx. 20 cm diameter x 40 mm deep) was completely filled with dist.H₂O. A cleaned glass slide is completely dipped into the formvar, drained briefly and allowed to dry. The dried formvar film was loosened around the edges of the slide, with a blade, and floated off onto the surface of the water. Copper grids (100 mesh, hexagonal) were placed, shiny side up, on the floating film. The film was recovered from the surface of the water, using a circular, downward scooping action, through the water, bringing the grids up out of the water with the film, covering the dull surface, uppermost on a square piece of wire mesh or a glass slide. The film-covered grids were allowed to dry before being carbon-coated and glow-discharged.

2.4.2.3 Carbon-coating and glow-discharging of grids

Grids were coated with a thin layer of carbon from a graphite source rod in an Edwards E306A high vacuum coater. At a vacuum of 10^{-5} Torr, the variable transformer is adjusted until the evaporation point of carbon is reached, and evaporation is maintained for 10 secs. The thickness of the carbon deposit may be judged from a piece of white paper inserted under the grids before the coating procedure. This should show a faint shadow around the wire mesh or glass supporting the grids.

The grids were rendered hydrophilic by placing them in the modified sputter coater (see Section 2.4.2.1), under a vacuum of approx. 0.5 Torr and discharging a current of 16 mAmps for 10 secs. The vacuum was broken and the grids removed and stored in a petri dish for use.

2.4.3 The "Tokuyasu" thawed cryosection technique

The Tokuyasu thawed frozen hydrated section technique, involves tissue fixation, trimming, cryoprotection, mounting on a stub for insertion into the cryoultramicrotome, rapid freezing in liquid nitrogen and sectioning before immunolabelling (Griffiths *et al.*, 1983; Tokuyasu, 1986; Griffiths, 1993). The related technique of embedding of cells in gelatin, before trimming, cryoprotection, freezing-and-sectioning of tissue blocks, immunolabelling, and contrasting-and-sealing of the section for viewing in the electron microscope, is also described in this Section.

The fixatives used in this study are described in Section 2.3.2 and the fixation protocols, being antigen-specific, are described in Chapter 4.

Infusion of cryoprotectants such as sucrose, containing polyvinyl pyrrolidone (PVP) or polyvinyl alcohol, into chemically fixed specimens, before freezing, has been claimed to give superior sectioning plasticity to frozen tissue (Tokuyasu, 1989). Infusion with sucrose (2.1 - 2.3 M), however, generally provides adequate sectioning plasticity for ultrathin sectioning, at temperatures of between -80° to -110°C. Higher concentrations of sucrose make the block relatively brittle at sectioning temperatures below -120°C, whereas infusion with lower concentrations of sucrose causes the blocks to be harder but more brittle. In this study 2.1M sucrose was used as a cryoprotectant and the cutting temperature was -100°C.

2.4.3.1 Reagents

2.1 M Sucrose in PBS, pH 7.2. AR grade sucrose (71.8 g) was dissolved and made up in 100 ml PBS, pH 7.2.

2.3 M Sucrose in PBS, pH 7.2. AR grade sucrose (78.7 g) was dissolved and made up in 100 ml PBS, pH 7.2.

10% Gelatin for embedding cells. Microbiological grade gelatin (Merck, Darmstadt) (10 g) was added to 100 ml boiling dist.H₂O, heated to dissolve and chilled rapidly on ice. Chilled dist.H₂O was poured on top of the gelatin and left overnight at 4°C. The dist.H₂O was decanted and the gelatin aliquotted and stored at 4°C.

3% Aqueous uranyl acetate. Uranyl acetate (3 g) was dissolved in dist.H₂O, made up to 100 ml and stored in the dark. This solution was made up freshly every 2 months.

2% Methyl cellulose. Methyl cellulose (25 centipoises, Sigma M6385) (2 g) was added to dist.H₂O (100 ml) and left to dissolve for a few days at 4°C before centrifugation at high speed (185 000 x g, 1 h, 4°C). The supernatant was stored at 4°C for up to 4 weeks.

0.3% Uranyl acetate/Methyl cellulose contrasting-and-sealing reagent. Immediately before use (within 15-30 min of use) methyl cellulose (9 parts) was mixed with 3% uranyl acetate (1 part) and stored on ice until required.

2.4.3.2 Procedure

After fixation (see Chapter 4), tissues were trimmed to approx. 1 x 1 x 1 mm, to give a trapezoid shaped surface to be sectioned, cryoprotected by infiltration with 2.1 M sucrose (15 - 30 min, depending on the size of the tissue block) and mounted on a stub, for insertion into the microtome. Excess sucrose was removed from around the block, and the block was frozen by plunge-freezing. The block was also agitated in liquid nitrogen (-190°C) to ensure a maximal rate of heat exchange and cooling, and was stored in liquid nitrogen for use.

Non-adherent cells, such as leucocytes, require embedding to keep them together during cryoprotection. In such cases, residual reactive aldehyde fixative was quenched by washing the cells in 0.02 M glycine in PBS (PBS-glycine), in an Eppendorf tube. Excess PBS-glycine was removed and a few drops of warm, liquid gelatin (at approx. 30°C) was added and allowed to infiltrate for up to 15 min at the lowest temperature at which the gelatin remained liquid. Excess gelatin was removed and the gelatin-embedded block was set by cooling on ice. The gelatin block was trimmed and cryoprotected, mounted, frozen and stored as before. Inadequate infiltration of sucrose, during cryoprotection, results in an opaque block, indicative of the presence of cubic or hexagonal ice. Such a block crumbles upon sectioning and is thus impossible to section.

Each specimen for sectioning was transferred to a pre-cooled (-100°C) cryoultramicrotome chamber of an RMC MT6000XL ultramicrotome, fitted with a CR2000 cryo attachment, and allowed to equilibrate. Sectioning was performed, using a tungsten-coated knife, at a block and knife temperature of -100°C and a cutting speed of less than 1 mm/sec. Sections are manipulated off the knife-edge with an eyelash attached to the end of a wooden applicator stick, and collected on a droplet of 2.3 M sucrose, in a 1 mm nichrome wire loop. The droplet is brought in to the chamber quickly enough for the sucrose still to be liquid, when the loop approaches the knife, from which the sections are gathered. As the loop is brought within 1 mm of the sections on the knife, the sections "jump" onto the sucrose and adhere to the underside of the drop. If the droplet, used to retrieve the sections, freezes before the loop is withdrawn from the chamber, the sections do not flatten out, and remain

wrinkled. If the loop is withdrawn quickly enough, the section and sucrose thaw, and the section flattens out on the droplet of sucrose.

As the droplet of sucrose is brought out of the chamber, carrying the section(s), and is placed in contact with a glow-discharged grid, the section(s) adhere(s) avidly to the surface of the grid. The grid and section are lifted, with the loop, on the drop of sucrose, and the grid is floated off, section-side-down, onto PBS in a 35 mm Petri dish. Sections are kept on PBS until labelled.

Immunolabelling was performed by incubation of the grid, section-side-down on droplets of reagents on Parafilm. Grids were transferred from reagent to reagent with a 1 mm nichrome wire loop. (Loop carryover causes a dilution factor in the final concentration of antibody, used in labelling, therefore, the same loop should be used in all labellings subsequent to labelling optimisation). Various blocking procedures were used, before immunolabelling. Sections were blocked in 0.02 M glycine in PBS, 10% foetal calf serum, 1% BSA in PBS or 1% fish skin gelatin (Section 2.3.3) for between 15 to 30 min, and unless stated otherwise, exposed (1 h, RT) to primary antibody (10 μ l, diluted in 1% BSA in PBS or 5% foetal calf serum), washed in 50 μ l changes of PBS (6 x 20 min), treated for 30 min with secondary antibody or protein A gold probe (10 μ l), washed as before, and fixed in 1% glutaraldehyde in PBS (5 min). The sections were washed on dist.H₂O (4 changes, total time, 5 min) and counterstained in 0.3% uranyl acetate/methyl cellulose, by transferring quickly through two changes of this reagent and incubating on a further 50 μ l drop, on ice (10 min).

Finally, the grids were removed from the uranyl acetate/methyl cellulose medium, on drops of this reagent suspended from a nichrome wire loop (of 3-3.5 mm diameter, just slightly larger than the grid). Excess medium was removed with fiber-free filter paper, leaving the grid supported in the loop by a thin layer of medium. The final residual support film should dry to give a gold-blue interference colour. The thickness of the film is critical with respect to both contrast and fine structure preservation. Should the film break or be too thin, the section will be exposed and irrevocably damaged due to drying artefacts. If too thick, depending on the final thickness of the film, illumination problems will be experienced in the electron microscope, as the beam passes through the film less easily.

Once the uranyl acetate/methyl cellulose film dries, the grid must be carefully removed, without tearing away the sealing layer covering the

section, by piercing the film between the loop and the outer periphery of the grid. The grid was viewed in a Jeol 100CX transmission electron microscope, at 100 kV.

2.5 LR White resin embedding

Recognition of the cathepsin B antigen, by the anti-human liver cathepsin B antibody, appears to require a harsh tissue embedding procedure, to denature the antigen. The dehydration and embedding procedure involved in processing tissues for LR White resin embedding, seemed to promote denaturation sufficiently to permit labelling for cathepsin B.

LR White is a hydrophilic, methacrylate resin which polymerises at 50°C, in the absence of oxygen. It can tolerate dehydration to only 70% and generally is preferable to epoxide and polyester resins, as it is less damaging to tissue antigens. The hydrophobic epoxide and polyester resins require "etching" with strong alkalis or H₂O₂, to introduce hydrophilic groups into the resin. They also require heat polymerisation at 70°C for 48 h and absolute dehydration; features that would be potentially damaging to the survival of many antigens. The LR White resin offers a compromise. It is inferior to the hydrophilic Lowicryl resins, in that it tends to be unstable in the electron microscope beam and gives rise to polymerisation artefacts, but it requires none of the specialised equipment required for Lowicryl resin embedding, such as low temperature refrigeration and UV light sources, to effect polymerisation.

LR White polymerisation can be promoted by the addition of a catalyst, or allowed to occur spontaneously at 50°C. Catalyst-induced polymerisation, is exothermic and gives rise to marked tissue distortion due to uneven heating. When polymerisation is allowed to occur spontaneously in gelatin capsules (an oxygen-free environment) at 50°C, however, tissue morphology is adequately preserved. This procedure was, therefore, used in preference.

2.5.1 Fixation

Tissue blocks were variously fixed (see Section 2.3.2 and Chapter 4).

2.5.2 Dehydration and embedding

2.5.2.1 Reagents

25%, 50% and 70% alcohol. Absolute alcohol (25, 50 and 70 ml) was made up to 100 ml with dist.H₂O, in each case.

LR White resin. LR White resin was obtained from Wirsam Scientific, South Africa.

Uranyl acetate. Uranyl acetate (1 g) was dissolved in dist.H₂O (50 ml), solubility being assisted by the addition of 95% ethanol (1 ml). Uranyl acetate is light sensitive and should be kept out of the light, and stored at 4°C.

Lead citrate. Lead nitrate (1.33 g) and trisodium citrate.2H₂O (1.76 g) were added to freshly boiled and cooled double dist.H₂O and shaken continuously for 1 min, then intermittently for 30 min. NaOH (8 ml of freshly made 1 M solution) was added to clear the milky solution. The solution was adjusted to 50 ml with freshly boiled and cooled double dist.H₂O and stored in the refrigerator in an air-tight container, in a foil wrapper.

2.5.2.2 Procedure

Fixed (see Chapter 4) human splenic tissue blocks (approx. 2 x 2 x 2 mm) were dehydrated through 25% and 50% alcohol (15 min each), followed by two changes of 70% alcohol (1 h each). Tissue blocks were transferred to increasing amounts of LR White in ethanol (1:1 for 30 min; 2:1 for 20 min) and into pure LR White (60 min) followed by overnight in a further change of resin (Newman *et al.*, 1983).

Each tissue block was transferred to a gelatin capsule. The capsule was completely filled with resin and polymerisation effected at 50°C for 24 h. Blocks showing any sign of softness were reincubated for a further 24 h. The gelatin capsule was removed and the block trimmed and mounted on a stub and sectioned on an RMC MT 6000XL ultramicrotome. Sections with a gold (60-70 nm thick) or silver-gold (50-60 nm) interference colour were collected on 200 mesh copper or nickel grids, and allowed to dry overnight before immunolabelling.

Immunolabelling was performed by incubation of the grid, section-side-down on droplets of reagents on Parafilm. Transfer of grids from reagent to reagent was effected with a 1 mm nichrome wire loop. Sections were blocked in 0.02 M glycine in PBS, 10% foetal calf serum, 1% BSA in PBS or 1% fish skin gelatin (Section 2.3.3) for between 15 to 30 min, and unless stated otherwise, exposed (1 h, RT) to primary antibody (10 μ l, diluted in 1% BSA in PBS or 5% foetal calf serum), washed in 50 μ l changes of PBS (6 x 20 min), treated for 1 h with secondary antibody or protein A gold probe (10 μ l) and washed as before. The sections were washed in a jet of dist.H₂O and counterstained on a droplet of uranyl acetate (10 min). This was followed by jet washing in dist.H₂O and incubation on droplets of lead citrate (10 min) in a closed petri dish, into which pellets of NaOH have been placed to remove CO₂. The grids were finally jet washed in a stream of dist.H₂O, dried and viewed in a Jeol 100CX transmission electron microscope, at 80 kV. Specific details of immunolabelling procedures are given in Chapters 4 and 5.

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF PEPTIDE AND POLYCLONAL ANTIBODIES

3.1 Introduction

At the time when this study was initiated, no comprehensive study had yet been undertaken to substantiate unequivocally the involvement of any specific proteinase in the invasion process, using the direct immunocytochemical approach. Abnormally high levels of cathepsin B, as well as unusual, secreted, high molecular weight forms of this enzyme, have, however, been demonstrated in a number of human and animal tumours, using classical biochemical fractionation techniques (Sloane *et al.*, 1987; 1990). The increased amounts of intra- and extracellular cathepsin B (Sloane *et al.*, 1981; 1982; Rozhin *et al.*, 1990), increased number of cathepsin B mRNA transcripts (Murnane *et al.*, 1991), and the association of cathepsin B with the plasma membrane (Sloane *et al.*, 1986; 1987; Erdel *et al.*, 1990) have also been correlated with tumour metastatic potential. In the present investigation, therefore, the aim was primarily to substantiate the validity of extrapolations, from biochemical isolation procedures to invasive potential, by exploring the distribution of cathepsin B in invasive cells, using immunocytochemistry. In principle, either conventional polyclonal or peptide antibodies could be used for this purpose.

Peptide antibodies (Bulinski, 1986) are polyclonal but are directed at a particular peptide sequence, usually consisting of from six to twenty amino acids, selected from the protein primary structure. A peptide, corresponding to the so-called "target" sequence, is synthesised and used to raise peptide antibodies which, ideally, will subsequently recognise and react with the target sequence in the native protein. The fact that the target sequence can be selected, gives peptide antibodies a particular utility, which has been exploited for several different purposes in the present study.

For the immunocytochemical localisation of cathepsin B, peptide antibodies have a potential advantage over conventional antibodies. Most polyclonal antibodies against mature cathepsin B, raised in laboratory animals, only recognise the denatured form of the enzyme (Barrett, 1977; Graf *et al.*, 1981; Wardale *et al.*, 1986). The cryoultramicrotomy approach, selected for this study partly because of its speed, is also particularly mild. This favours the survival of labile antigens and is, consequently, an

advantage in most cases. In the specific case of cathepsin B, however, the enzyme may be insufficiently denatured to be recognised by polyclonal antibodies; this is discussed more fully in Chapter 4. Peptide antibodies, targeting a linear sequence of amino acids, however, may recognise the protein in either its native or denatured forms, depending upon the accessibility of the peptide sequence in the structure of either form, and may, therefore, be more suited to the immunocytochemical localisation of cathepsin B in cryoultrassections.

Peptide antibodies are also potentially useful for labelling studies designed to locate and identify precursor forms of cathepsin B. Various high molecular weight forms of procathepsin B, presumably processing intermediates, have been identified (Mort *et al.*, 1983; Mort and Recklies, 1986; Buttle *et al.*, 1988; Buttle and Abrahamson, 1990). Many of these high molecular weight intermediates are secreted, for example by lactating mouse and rat mammary glands (Recklies *et al.*, 1985), mammary tumours (Recklies *et al.*, 1982a) and malignant human breast tumour explants (Mort *et al.*, 1980) and have been found in the ascitic fluid of patients with neoplasia (Mort *et al.*, 1983). These secreted high molecular weight forms of cathepsin B exist in three different states: fully active, partially active and latent, the active enzyme being secreted to a greater degree by malignant than non-malignant tumours (Mort and Recklies, 1986). These forms are stable at alkaline or neutral (physiological) pH and may be most relevant in tumour invasion, especially as they are resistant to inhibition by the proteinase inhibitor, α_2 -macroglobulin (Mort and Recklies, 1986) and have a decreased tendency to be inhibited by cystatins, their natural inhibitors (Buttle *et al.*, 1988). This is in marked contrast to the mature enzyme which is labile at physiological pH and would, therefore, probably be inactive if secreted, and would also be more subject to inhibition.

The peptide antibody approach also offers a unique way of studying the properties and distribution of intermediately processed forms of cathepsin B. Cathepsin B, like most proteinases, is synthesised in a pre-pro- precursor form. The M_r of procathepsin B, after cleavage of the pre- (signal) peptide, is calculated, from its cDNA, to be 35 900, and measured to be 40 000 in its glycosylated state (Chan *et al.*, 1986). The "latent" and active high molecular weight forms of cathepsin B, found in cell cultures of tumour explants and cancer cells (Mort and Recklies, 1986), were found to have a roughly corresponding molecular weight (M_r 33 000) after deglycosylation.

A similar, high molecular weight, active, form of cathepsin B, stable to alkali pH, has been found in sputum (Buttle and Abrahamson, 1990). Sputum cathepsin B

appears to have a molecular weight intermediate to that of the entire procathepsin B precursor sequence (M_r 35 900 from the cDNA sequence) and the mature form of the enzyme [M_r 27 600 for the human liver enzyme, on the basis of the amino acid sequence (Ritonja *et al.*, 1985)]. For this reason, it has been speculated that this variant may be a procathepsin B intermediate, which has retained only the C-terminal portion of the procathepsin B sequence, to give a "truncated form" of procathepsin B (Buttle *et al.*, 1988; Buttle and Abrahamson, 1990). In order to investigate the relative distribution, and hence the relevance of such cathepsin B variants, in tumours and invasive cells, peptide antibodies which would recognise either the entire procathepsin B or the "truncated" form, were raised. To do this, target peptide sequences were selected from positions "early" and "late" in the pro-peptide sequence.

In addition to the use of anti-cathepsin B antibodies in immunolocation studies, it was decided to take a longer-term view and consider what may be done, by way of therapy, if the immunocytochemical studies were to reveal that cathepsin B was, in fact, involved in tumour invasion. In this regard, it was considered that, by judicious choice of a target peptide, one might be able to elicit peptide antibodies which, upon reaction with the intact enzyme, would block its activity. Such an approach, using immuno-inhibitory peptide antibodies, could be envisaged to lead to immunotherapies of two types; passive immunotherapy, where administration of the antibody itself could be used to block invasion, and active immunotherapy, where the target peptide may be used as a "vaccine", able to induce production of the protective antibodies by the patient's own immune system. Although this approach is conceptually simple, there are many practical problems which would have to be addressed, not least the problem of autoimmunity and that of accessibility of the antibody to the invasion front. Nevertheless, this approach was considered to have sufficient potential to warrant preliminary investigation here. Consequently, in this study, peptide antibodies against mature cathepsin B were selected for their potential in two respects, for immunocytochemistry and for enzyme immunoinhibition, as this was considered the most cost-effective approach.

The ultimate aim of the immunolabelling studies undertaken was to determine the relevance of precursor and mature forms of cathepsin B, as well as other enzymes, in the invasion process of human tumours. Early on it was realised, however, that a great deal of preliminary methods-development work was necessary, as none of the required methods were extant in this laboratory, nor indeed at the University of Natal, at that time. For the purposes of methods-development, a readily accessible model of a human invasive cell was required and for this purpose it was

decided to use human leucocytes, especially neutrophils and monocytes. Also, since it was intended that mainly human tissues were to be studied, peptide antibodies were raised against human sequences. For the limited investigation undertaken, the antibodies that were deemed necessary, were antibodies to cathepsin B (both to the full- and truncated precursors, and to mature cathepsin B); to cathepsin D, a marker enzyme for lysosomes; to the major neutrophil proteinases, elastase and cathepsin G, and to the neutrophil granule marker enzymes, lactoferrin and myeloperoxidase.

3.2 Polyclonal antibodies against whole proteins

Rabbit antibodies against sheep IgG and chicken IgY, and against mature cathepsin D, were raised in this laboratory from PEG precipitates of sheep IgG and chicken IgY (Polson *et al.*, 1985), and from purified antigens from human, porcine and bovine splenic tissue (Jacobs *et al.*, 1989), by Theresa Coetzer and Philip Fortgens, respectively. Sheep antibodies, raised and affinity purified against mature human liver cathepsin B, were provided by Dr D. Buttle, Strangeways Research Laboratory, Cambridge, UK, and rabbit anti-human leucocyte elastase antibody was supplied by Dr J. Travis, Department of Biochemistry, University of Georgia, Athens, GA. The anti-cathepsin G, lactoferrin and myeloperoxidase antibodies were from Serotec Ltd, (Oxford, England), Sigma Chemical Co. (St Louis, Mo.) and Dakopatts (Glosstrup, Denmark), respectively.

3.3 Peptide selection for production of antibodies against pro- and mature cathepsin B

Two peptides in the sequence of human procathepsin B (residues 17-79, numbered from the prepro- amino terminus), were chosen, one from the amino-terminal end of the procathepsin B sequence, residues 22→36 (denoted ppB22-36), and one from the C-terminal end, residues 64→77 (denoted ppB64-77) (Fig. 7). These were selected to represent the complete and the "truncated" forms of the pro-sequence, respectively (Buttle *et al.*, 1988; Buttle and Abrahamson, 1990). The "truncated" sequence would, presumably consist of about 50% fewer pro-sequence residues, the residues being removed from the amino-end of the pro-sequence. Peptides representing these two sequences were synthesised by Dr G. Knight of Strangeways Research Laboratories, Cambridge.

The chosen sequences of the human cathepsin B were compared with the corresponding mouse sequences, to assess the likelihood of cross-species reactivity between the anti-human sequence antibodies produced and mouse cathepsin B (Chan *et al.*, 1986) (Fig. 7). Such cross-reactivity would be useful for envisaged studies on the BL16-BL6 mouse melanoma cell line. In the case of ppB22-36, 11/15 residues are identical, and in ppB64-77, 10/14 are identical, which suggests that cross-reactivity is possible.

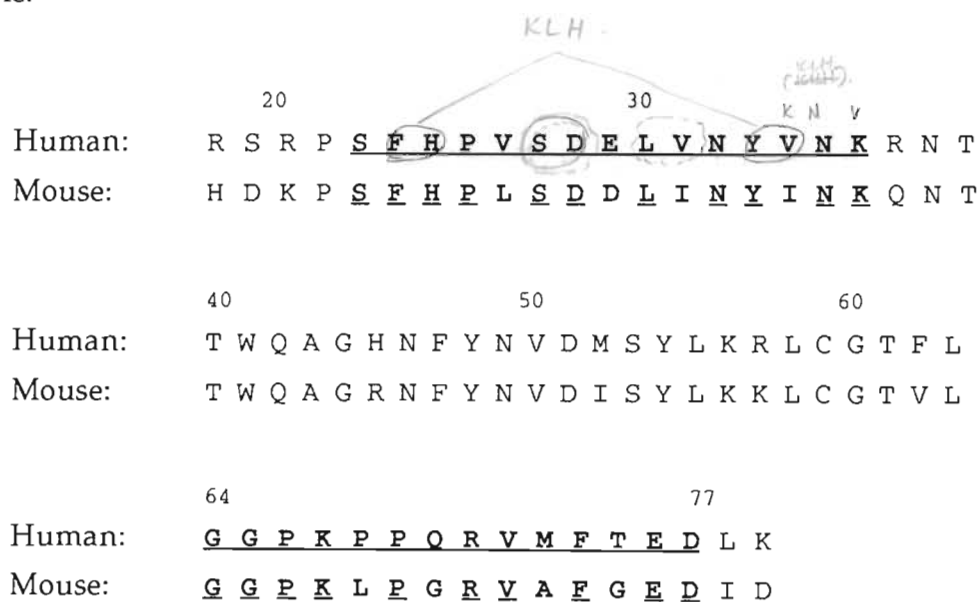


Figure 7. Human and mouse procathepsin B sequences showing selected peptide sequences ppB22-36 and ppB64-77.

Bold, underlined sequences represent human sequences chosen for synthesis. Those underlined with a broken line represent mouse residues homologous to those from the selected human sequence (from Chan *et al.*, 1986).

The procathepsin B sequence is too short to allow much latitude in the selection of target peptide sequences on the basis of the commonly-used criteria of hydrophilicity and flexibility (Figs. 8 and 9). Sequence ppB64-77 is more hydrophilic than sequence ppB22-36 which has both hydrophilic and hydrophobic character (Fig. 8) (Hopp and Woods, 1981; 1983). Both pro-sequence peptides selected were sequences showing peaks of flexibility (Fig. 9).

In the mature enzyme, target sequences were similarly selected by consideration of hydrophilicity and segmental mobility (flexibility) (Figs 8 and 9), and by inspection of the published 3-D structure of the analogous enzyme, papain (since at the time the 3-D structure of cathepsin B had not been published). The 3-dimensional structures of cathepsins B, H and L were deduced, from amino acid sequence information, to be comparable to that of papain (Kamphuis *et al.*, 1985; Dufour, 1988),

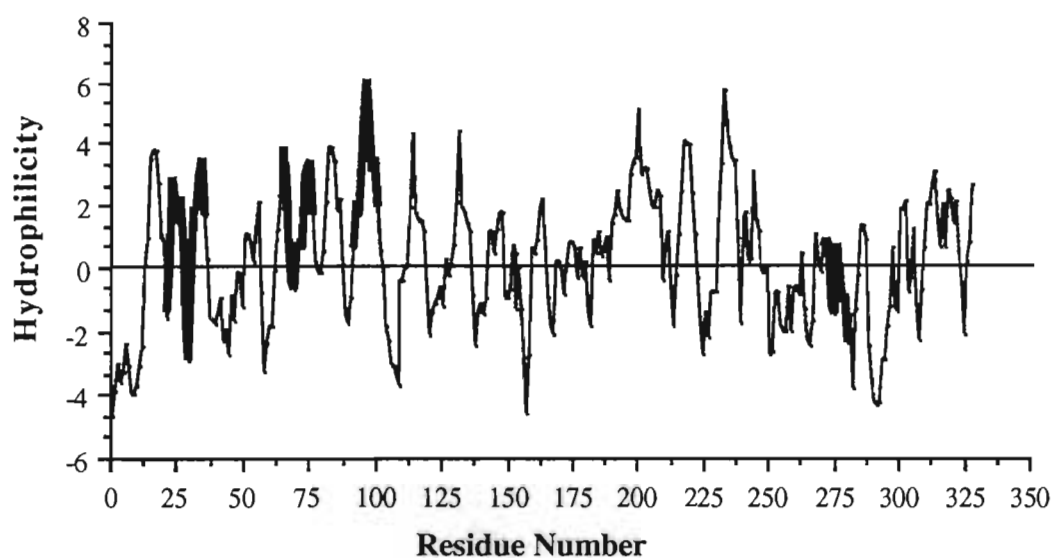


Figure 8. Hydrophilicity plot of the human preprocathepsin B sequence (Hopp and Woods, 1981;1983). The bold lines indicate the four peptides, ppB22-36, ppB64-77, B13-22 and B192-201, selected from the sequence of procathepsin B. The prepro-sequence contains 79 residues, which must be subtracted from the preprocathepsin B residue numbers indicated, to obtain the residue numbers in the mature enzyme.

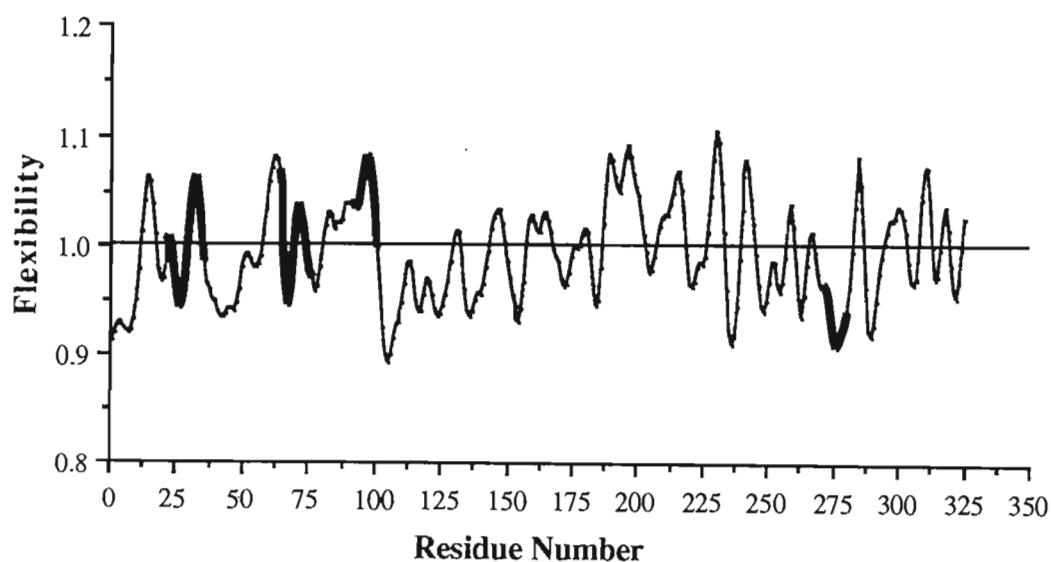


Figure 9. Flexibility plot of the human preprocathepsin B sequence (Westhof *et al.*, 1984). The bold lines indicate the four peptides, ppB22-36, ppB64-77, B13-22 and B192-201, selected from the sequence of preprocathepsin B.

so the 3-D structure of papain, as published by Wolthers *et al.* (1970), was used in the choice of peptide. Consideration of the 3-D structure was necessary in the choice of peptide to raise antibodies having a high probability of being immunoinhibitory. In order to elicit immunoinhibitory antibodies, the peptide sequence should be accessible

to antibody binding and should also involve the substrate-binding cleft, and preferably some active site residues.

The numbering of papain's residues and the alignment of the equivalent residues in cathepsins B, H and L is slightly different in different publications (Wolthers *et al.*, 1970; Kamphuis *et al.*, 1985; Dufour, 1988; Musil *et al.*, 1991; Wiederanders *et al.*, 1991), but for ease of reference the 3-D structure and the numbering of papain according to Wolthers *et al.* (1970), will initially be used (Fig. 10). The selected residues will then be converted to the equivalent numbering of papain and cathepsin B, as given by Musil *et al.* (1991), and aligned with equivalent residues of cathepsins H and L according to Wiederanders *et al.* (1991), using the papain numbering of Musil *et al.* (1991) (Fig. 11).

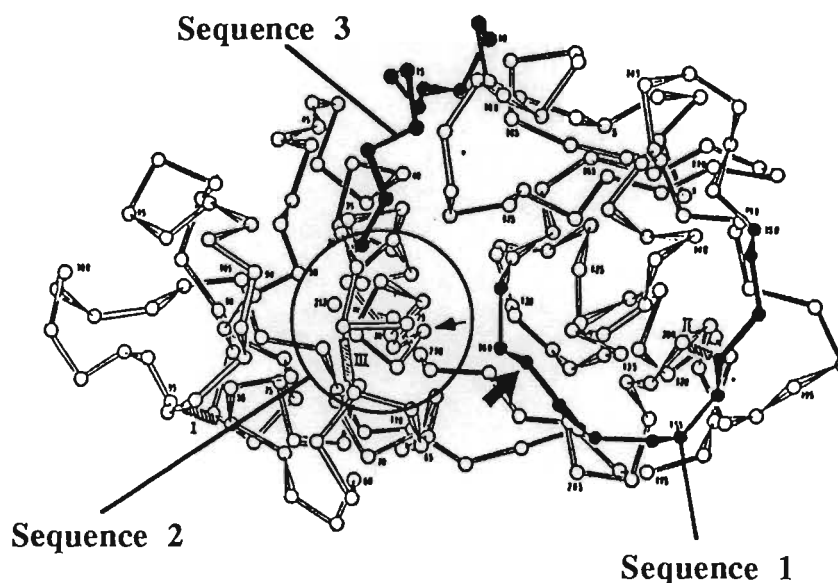


Figure 10. 3-D structure of papain (from Wolthers *et al.*, 1970). The three target sequences for peptide synthesis (sequences 1, 2 and 3) are within the active site cleft, which runs roughly vertically across the centre of the papain structure. Large arrow, active site His-159; small arrow, active site Cys-25.

Inspection of the 3-dimensional structure of papain (Fig. 10), reveals three potential target peptide regions, for raising antibodies with the required properties. The three choices were, either from an accessible loop of amino acids containing the active site histidine (residues 150→161 of the mature papain enzyme, designated "sequence 1"), from a helical sequence around the active site cysteine at position 25 (designated "sequence 2") and a hinge region (residues 9→19, designated "sequence 3", just preceding the active site cysteine sequence) (Fig. 10).

Of these three potential choices, only two peptides were considered suitable. Sequence 1, containing the active site histidine, appeared to be a suitable choice for the production of peptide antibodies against a specific cathepsin, as there is no inter-cathepsin sequence homology in this region (Wiederanders *et al.*, 1991, and Fig. 11).

Human cathepsin B:	Q H V <u>T G E M M G G H A V</u>	(res 192-201)
Human cathepsin H:	<u>S C H K T P D K V N H A V</u>	(res 149-161)
Human cathepsin L:	<u>E P D C S S E D M D H G V</u>	(res 153-165)
Papain	F V G P C G N K V D H A V	

Figure 11. Comparative sequences for cathepsins B, H and L containing the active site histidine (Sequence 1). Alignment has been made according to the histidine residue (bold) using the residue equivalents of Wolthers *et al.*, (1970) but the numbering and sequence of cathepsin B as given by Musil *et al.*, (1991) and of cathepsin H and L as given by Wiederanders *et al.* (1991). [The numbering of res. 150 - 161 of papain, according to Wolthers *et al.*, (1970) is given as res. 149 - 161 by Musil *et al.* (1991)]. Chosen sequences are indicated by underlining.

Residues in sequence 2 (Fig. 10) are, however, highly conserved (Dufour, 1988, Wiederanders *et al.*, 1991), so that antibodies raised against peptides in this region would probably be non-discriminating. This region is also α -helical and the discontinuous strip-of-helix epitope may be more difficult to model than a simple linear epitope. All these factors made sequence 2 an unsuitable choice.

The choice of peptide sequence for the production of immunoinhibitory antibodies to cathepsin B, was, therefore, between sequence 1 and sequence 3. This study was, however, part of a group study (subject to economic constraints) on immunoinhibitory peptide antibodies. Sequence 3 was an unsuitable choice for cathepsin L, due to high sequence homology in this region between cathepsins L and H (Ritonja *et al.*, 1988), but the cathepsin B sequence was different from both the cathepsin L and H sequences in this region. Sequence 3 was, therefore, chosen for the production of anti-cathepsin B antibodies, while sequence 1 was selected for the production of the anti-cathepsin L antibodies. Due to the similarity in structure of the different cathepsins (Dufour, 1988), the decision to choose sequences from different peptide sequences within the 3-D structure, for the production of the anti-cathepsin B and L peptide antibodies, was taken in order to potentially maximise the information gained from the experiments.

Selection of sequence 3 (residues 9→19, Fig. 10) as a target for the production of immunoinhibitory anti-cathepsin B antibodies, was considered a potentially good choice. The sequence is near to the active site cysteine (residue 25), an important region for enzyme activity, as judged by the sequence conservation in this region (residue 23→33) and yet has a reasonably high hydrophilicity (Fig. 8) and segmental mobility profile (Fig. 9), suggesting it would be immunogenic. Antibodies binding to this peptide sequence could also potentially occlude the active site cleft (Fig. 10) and thus immunoinhibit the enzyme. Papain residues 9→19 (Wolthers, 1970) correspond to papain residues 10→18, according to Musil *et al.* (1991), and residues 13→22 in mature human cathepsin B (Chan *et al.*, 1986; Musil *et al.*, 1991). The selected peptide was consequently denoted B13-22.

Human:	W P <u>Q C P T I K E I R D</u> Q G S C
Mouse:	R E <u>Q C P T I</u> G <u>Q I R D</u> Q G S C

Figure 12. Residues 13-22 of human liver cathepsin B chosen for the production of a potentially immunoinhibitory antibody (Sequence 3). The equivalent mouse sequence is given below, with residues common to those chosen from the human sequence underlined (Chan *et al.*, 1986; Musil *et al.*, 1991).

Mouse cathepsin B has 8/10 residues in common with human cathepsin B in the B13-22 peptide, indicating a fairly high probability of useful cross-reactivity (Fig. 12).

As detailed below, in practice the peptide B13-22 proved unsuccessful in eliciting antibodies capable of recognising and inhibiting the activity of mature cathepsin B, whereas antibodies against sequence 1 proved successful in both respects for cathepsin L (Coetzer *et al.*, 1991) and H (Coetzer, 1993), and were subsequently used in labelling studies (see Chapter 5). Consequently, in a second round of peptide synthesis, sequence 1 was selected as a target for raising potentially immunoinhibiting peptide antibodies against human cathepsin B (Fig. 10). The selected peptide, denoted B192-201 (Fig. 13), was compared with that of mouse cathepsin B (Chan *et al.*, 1986). In peptide B192-201, 6/10 residues, in a continuous stretch, are identical, so that cross-reactivity is again at least possible.

Human: Y Q V T G E M M G G **H** A I R L G W G

Mouse: Y K H **E A G D** M G G **H** A I R I L V W G

Figure 13. A peptide sequence of human and mouse cathepsin B (Sequence 1) showing the chosen human sequence B192-201 and the equivalent mouse sequence (Chan *et al.*, 1986). The bold underlined sequence represents the sequence chosen for synthesis, and that underlined with a broken line, represents mouse residues identical to those selected from the human sequence. The active site histidine is indicated.

3.4 Peptide synthesis

The procathepsin B peptides, ppB22-36 and ppB64-77 (Fig. 7), were synthesised by Dr G. Knight, of Strangeways Research Laboratory, Cambridge, UK. An extra glycine and cysteine residue was added to both C-termini to facilitate conjugation via the cysteine residue at the carboxyl end of the peptide, using the heterobifunctional cross-linking reagent, NHS-bromoacetate. The N-terminal serine of ppB22-36 was acetylated in order to "block" the charge on this residue, which is not present in the protein.

The two peptides, B13-22 and B192-201, selected from the mature human liver cathepsin B sequence, were custom synthesised by Multiple Peptide Systems, San Diego, CA. B13-22 was synthesised with an additional cysteine residue added to the carboxyl end of the peptide and an α -amino butyric acid residue was substituted for the existing cysteine. This was done to allow conjugation via the added carboxy-terminal residue, rather than the existing cysteine, using m-maleimidobenzoyl-N-hydroxysuccinimide (MBS). The N-terminal amino acid was acetylated only in the case of B13-22, as it was intended that conjugation of this peptide should be effected via the C-terminus. In the case of B192-201, this was not done as the N-terminal residue was required for coupling, using glutaraldehyde.

The second peptide, B192-201, was also synthesized with alkylation of the two methionine residues and an amide on the carboxyl terminus of the peptide. The modification of the methionine residues was designed to block these reactive sites during synthesis and coupling procedures, while the addition of an amide group blocks the negatively charged carboxyl group and makes the peptide mimic the situation in a protein more closely (Multiple Peptide Systems technical bulletin).

3.5 Peptide/carrier protein conjugation

3.5.1 Procathepsin B peptide-carrier protein conjugation

The procathepsin B peptides (ppB22-36 and ppB64-77) were conjugated to keyhole limpet haemocyanin (KLH), via the amino groups of the KLH and the cysteine residues added to the carboxyl ends of the peptides, using the heterobifunctional cross-linking reagent, NHS-bromoacetate in a modification of the procedures of Davies *et al.* (1988) and Bernatowicz and Matsueda (1986), as reported below. The peptide ppB22-36 was conjugated to KLH in a molar ratio of approximately 1:39 and the antibody raised (using intramuscular injections of 400 µg of peptide, into each of two rabbits) by Dr D. Buttle, Strangeways Research Laboratory, Cambridge, UK. This antibody was supplied as a non-affinity purified, uncharacterised, IgG fraction. A similar conjugation and inoculation procedure was used by the present author for the peptide ppB64-77.

3.5.1.1 Reagents

KLH and N-hydroxysuccinimidyl (NHS-) bromoacetate were obtained from Sigma. Dimethyl formamide (DMF), originally from Holpro, was obtained from the Natal Institute of Immunology, where it had been redistilled. The DMF was tested for free amines (Sanger, 1945) and stored over a molecular sieve.

Reduction buffer A [0.02 M sodium phosphate buffer, 8 M urea, pH 8.0]. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.675 g) and urea (120.1 g) were dissolved in 225 ml of dist. H_2O , titrated to pH 8.0 with NaOH and made up to 250 ml.

Buffer B [0.1 M potassium phosphate buffer, 1 mM EDTA, 0.02% NaN_3 , pH 7.0]. KH_2PO_4 (13.61 g), Na_2EDTA (0.372 g) and NaN_3 (0.2 g), were dissolved in 950 ml dist. H_2O , titrated to pH 7.0 with NaOH, and made up to 1 litre.

Buffer C [0.05 M sodium acetate buffer, pH 4.0]. Glacial acetic acid (1.43 ml, $\delta = 1.05$) was added to 450 ml dist. H_2O , titrated to pH 4.0 with NaOH and made up to 500 ml.

Buffer D [0.02 M sodium phosphate buffer, pH 7.0]. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.675 g) was dissolved in 225 ml of dist. H_2O , titrated to pH 7.0 with NaOH and made up to 250 ml.

3.5.1.2 Procedure

Reduction of the peptide ppB64-77 (8 mg) (4.94×10^{-6} moles, assuming an M_r of 1617), solubilised in reduction buffer A (0.27 ml), was effected by an almost equimolar amount of dithiothreitol (DTT) (7.33×10^{-4} g, 4.76×10^{-6} moles), at pH 8.0 (Cleland, 1964). After overnight reduction at room temperature, the peptide mixture (0.27 ml) was desalted into buffer C, pH 4.0, using a column of Sephadex G-10 (4 x 0.9 cm i.d.). The degree of reduction and concentration of peptide eluted at V_0 was determined using the Ellman's Test (Sedlak and Lindsay, 1968), the number of moles of sulfhydryls, calculated from the extinction coefficient of Ellman's reagent, being equated to the equivalent weight of the peptide in 1 ml of eluate. Removal of urea was checked using the Berthelot reaction (Varley, 1967).

Bromoacetylation of KLH was effected as follows: KLH (30 mg dissolved in 2 ml of buffer B) was extensively dialysed against buffer B, centrifuged to remove any large aggregates (10 000 x g, 10 min, RT) and cooled on ice to 4°C. NHS-bromoacetate (10.6 mg) in amine-free DMF (166 μ l), was added drop-wise, with stirring, the solution was allowed to warm to room temperature, over a period of 30 min, re-cooled on ice and a further amount of NHS-bromoacetate (10.6 mg in 166 μ l DMF) was added and treated as before. The bromoacetylated KLH was desalted in buffer D on a Sephadex G-25 column (26.8 x 1.5 cm i.d.), and the concentration and recovery of bromoacetylated KLH was approximately calculated using the absorbance readings of the recovered protein at 280 nm and an extinction coefficient previously established for this KLH (A_{280} of a 1 mg/ml solution = 1.47). This allowed the calculation of the amount of bromoacetylated KLH to be added to the reduced peptide.

Bromoacetylated KLH (approx. 8 mg) (4×10^{-8} moles) in 1.3 ml buffer D was added to the reduced cysteine residues in the peptide (2 mg) (1.23×10^{-6} moles) in 300 μ l of buffer C, in a molar ratio of *ca.* 1:30 of KLH to peptide. The peptide was added to the bromoacetylated KLH, stirred for 2 h at room temperature, and the KLH-peptide conjugate was re-applied to the Sephadex G-25 column (26.8 x 1.5 cm. i.d.) and eluted in buffer D.

3.5.1.3 Results

The degree of reduction and concentration of peptide, eluted at V_0 after reduction with DTT, was 99% and 6.4 mg/ml, respectively.

The concentration of bromoacetylated KLH recovered in the pooled fractions of the eluted peak was roughly assessed to be 6 mg/ml. A total amount of approx. 8 mg of bromoacetylated KLH was added to 2 mg of the peptide; a mass ratio of 4:1 or a molar ratio of 1:30 of KLH to peptide. From the calculated amount of bromoacetylated KLH re-applied to the column and the absorption, at 280 nm, of the single eluted peak, the recovery of KLH was estimated to be 94% (7.5 mg from 8 mg applied) and the eluted conjugate concentration, 2.7 mg/ml KLH. This gives a concentration of 0.54 mg/ml of conjugated peptide, assuming 100% coupling efficiency, or 0.12 mg/ml of peptide at a 22% coupling efficiency previously reported by Bernatowicz and Matsueda (1986).

As the desired inoculation dosage for each of two rabbits was 100 μ g peptide, 195 μ l of conjugate (equivalent to 102 μ g peptide, at 100% conjugation, or 20 μ g, at a 22% coupling efficiency) was used per rabbit.

3.5.2 Mature cathepsin B peptide-carrier protein conjugation

Peptides B13-22 and B192-201 were conjugated using two different conjugation procedures (the MBS and glutaraldehyde methods) and two different carrier proteins (KLH and ovalbumin). Using MBS, B13-22 was conjugated, to free amino groups in KLH, via the added cysteine residue, using a modification of the method of Robertson and Lui (1988). B192-201 was conjugated to ovalbumin, using glutaraldehyde (Briand *et al.*, 1985). These different approaches reflect an evolution of opinion in this laboratory, an evolution which was partly influenced by the results of the present study.

3.5.2.1 Reagents

M-maleimidobenzoyl-N-hydroxysuccinimide (MBS) was from Sigma and glutaraldehyde [25% (m/v), E.M. grade] was from Merck.

Buffer A [0.02 M sodium phosphate buffer, pH 8.0]. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.276 g) was dissolved in 950 ml of dist. H_2O , titrated to pH 8.0 with NaOH, and made up to 1 litre.

Buffer B [0.05 M sodium acetate buffer, pH 4.0]. Glacial acetic acid (2.86 ml) was added to 950 ml of dist. H_2O , titrated to pH 4.0 with NaOH, and made up to 1 litre.

Buffer C [0.1 M sodium phosphate buffer, pH 7.0]. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (13.8 g) was dissolved in 950 ml of dist. H_2O , titrated to pH 7.0 with NaOH, and made up to 1 litre.

Buffer D [0.1 M sodium phosphate buffer, pH 6.5]. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (1.38 g) was dissolved in 95 ml of dist. H_2O , titrated to pH 6.5 with NaOH and made up to 100 ml.

Buffer E [0.1 M sodium phosphate buffered saline, pH 7.2]. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ((1.15 g), NaCl (8 g), KCl (0.2 g) and KH_2PO_4 (0.2 g) were dissolved in 950 ml dist. H_2O , titrated to pH 7.2 with NaOH, and made up to 1 litre.

3.5.2.2 Procedure

The MBS coupling procedure requires reduction of any oxidised cysteine residues before the peptide conjugation. Peptide B13-22 (8.1 mg) (6.92×10^{-6} moles, assuming molecular weight 1169) was dissolved in buffer A (0.3 ml), and reduced (1 h, RT) by the addition of an approx. 10% molar excess of DTT (1.17 mg) (7.59×10^{-6} moles) (Cleland, 1964). The peptide mixture (0.3 ml) was desalted into buffer B, using Sephadex G-10 (4 x 0.9 cm i.d.). The number of moles of reduced cysteine residues (and hence number of moles of peptide) available for conjugation in the peptide eluate peak (0.5 ml) was determined using the Ellman's Test (Sedlak and Lindsay, 1968).

The maleimide group was incorporated into KLH, following the method of Robertson and Lui (1988). KLH was extensively dialysed against buffer C and centrifuged (10 000 x g, 10 min, RT) to remove large aggregates. MBS (3.7 mg) (1.18×10^{-5} moles in 0.58 ml of amine-free DMF) was added to KLH (30 mg in 1.5 ml buffer C) and allowed to react, with stirring, for 1 h. The resulting mixture (2.0 ml) was applied to a Sephadex G-25 column (4.7 x 1.6 cm i.d.) and eluted with buffer D.

The total number of maleimide groups introduced was calculated by taking an aliquot of the KLH-MBS conjugate (30 μ l), adding a known amount of mercaptoethanol (2 μ l, 9 nmoles) and relating the number of free sulfhydryls consumed (determined using the Ellman's test) to the number of MBS groups introduced, and relating this to the total volume of the KLH-MBS eluted in the Sephadex G-25 peak (2.4 ml) (Kitagawa and Aikawa, 1976).

The peptide (0.5 ml, containing 4.375×10^{-6} moles reduced cysteine residues, equivalent to 5.02 mg of peptide) was added to the KLH-MBS conjugate (2.4 ml, containing 7.08×10^{-6} moles of MBS residues). The mixture (2.9 ml) was allowed to react for 3 h at room temperature, while stirring, and 2.6 ml was subsequently applied to Sephadex G-100 (9.6 x 1.5 cm i.d.), equilibrated with buffer C. The elution profile was monitored at 280 nm and two peaks were identified. The major peak contained the KLH-MBS-peptide (5.8 ml), and the second peak (9.3 ml) contained free peptide.

The second peptide, B192-201, was conjugated in this laboratory, in parallel with other peptides, by Theresa Coetzer, using the method of Briand *et al.* (1985). Ovalbumin (25 mg) (0.55×10^{-8} moles) in 1.1 ml buffer E was extensively dialysed against buffer E and centrifuged (10 000 x g, 10 min, RT). Peptide (5 mg) (0.5×10^{-7} moles of peptide, assuming an M_r of 1002) was dissolved in the supernatant, giving a molar ratio of 9.09:1, peptide:ovalbumin. Glutaraldehyde [568 μ l of a 25% solution (Merck, E.M. grade, $d = 1.06$)] was added dropwise over 5 min, with constant stirring. After reaction for 1 h, at room temperature, sodium borohydride (1 mg/ml) was added to terminate the reaction, and the conjugate was dialysed extensively against buffer E. The final volume of conjugate was 7.7 ml, so the concentration of peptide was estimated at approximately 324 μ g/ml, assuming a 50% coupling efficiency.

3.5.2.3 Results

The amount of reduced B13-22 peptide added to the MBS-derivatised KLH and the number of MBS groups introduced into the KLH, were estimated, using the Ellman's test, as being 4.375×10^{-6} moles reduced cysteine residues (equivalent to 5.02 mg of reduced peptide), and 7.08×10^{-6} moles of MBS residues, respectively. By relating the number of MBS groups to the original amount of KLH derivatised, it was estimated that the MBS groups would be contained in 18.9 mg of KLH (9.45×10^{-8} moles). The coupling molar ratio would, therefore, have

been 1:44, KLH to peptide. The ratio of MBS to peptide groups allowed to react was 1:1.5. The low coupling efficiency estimated (59%), however, indicated that final coupling was possibly of the order of 23:1 peptide to KLH. This coupling efficiency result was confirmed using the micro-biuret assay (Itzhaki and Gill, 1964). Of the two peaks identified after the final conjugation step, the second peak (9.3 ml) was estimated to contain 2.0 mg of free peptide, indicating 60% conjugation (i.e. the conjugate peak contained 3.02 mg peptide in 5.8 ml = 0.52 mg/ml). It was, therefore, calculated that 400 μ l of conjugate (equivalent to 200 μ g of peptide) should be used per rabbit for the production of antibodies.

The efficiency of coupling of the B192-201 peptide to ovalbumin, using the glutaraldehyde procedure was not estimated, but was assumed to be about 50% (a ratio of approximately 4.50:1 peptide to ovalbumin, giving a concentration of 324 μ g/ml of peptide). Inoculation of 330 μ l of conjugate (equivalent to approx. 100 μ g of peptide) was estimated to be required per rabbit.

3.6 Inoculation protocols for antibody production

Antibodies were raised in rabbits, two rabbits being inoculated with each immunogen. The inoculation protocol used for all peptide conjugates is summarised in Table 6. The indicated dose of conjugate was triturated 1:1 with Freund's Complete or Incomplete adjuvant (Difco), to form a stable emulsion, and injected intradermally in at least two sites, at the intervals indicated in Table 6.

Free, unconjugated, peptides were also used as immunogens in two cases. In the first case, the protocol of Richardson *et al.* (1985) was used. In the case of the first mature cathepsin B peptide (B13-22), an inoculation regime as given above (Table 6) was adopted, except that the final inoculations at 13, 28 and 30 weeks, were with free peptide. In the case of peptide B192-201, 0.5 mg of free peptide in 1 ml PBS, was emulsified with an equal volume of either complete or incomplete Freund's adjuvant (used alternately, the first inoculation being with complete adjuvant) and 1 ml of the preparation (250 μ g of peptide) was injected subcutaneously into two sites on the back of the neck of each rabbit. A greater amount of free peptide was inoculated to compensate for the anticipated multiple conformations that the peptide could possibly adopt, due to the lack of constraint on conformation otherwise imposed by conjugation procedures (Todd *et al.*, 1982).

Table 6. The inoculation protocol used to raise anti-cathepsin B peptide antibodies.

Weeks	Freund's Adjuvant	Site	Dose
0	Complete	i.d. ^a	200 µg ^b 100 µg ^c 200 µg ^d 100 µg ^e
2	Incomplete	i.d.	As previous
3			Bleed
6	Incomplete	i.d.	As previous
8			Bleed
10	Incomplete	i.d.	As previous
	none	i.v. ^f	1 mg free peptide
12			Bleed

^a intradermal injection into skin at the back of the neck of rabbit

^b procathepsin B peptide (ppB22-36)

^c procathepsin B peptide (ppB64-77)

^d first mature cathepsin B (B13-22)

^e second mature cathepsin B (B192-201)

^f intravenous injection into marginal ear vein (Richardson *et al.*, 1985)

used only with the first mature cathepsin B peptide (B13-22). Inoculation of free peptide again at 13, 28 and 30 weeks, with bleeds two weeks after.

3.7 Assessment of immune response, and antibody purification

Antibody response and recognition of the peptide sequence was generally monitored using whole antisera and the ELISA procedure (Coetzer *et al.*, 1991).

3.7.1 ELISA procedure for assessment of antibody recognition of peptide and enzyme

ELISA assays against the peptide and the mature enzyme, were conducted in a similar fashion, using whole sera, before the removal of antibodies directed at KLH carrier molecules, as detailed below.

3.7.1.1 Reagents

Phosphate buffered saline pH 7.2 (PBS). $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (1.15 g), NaCl (8 g), KCl (0.2 g), KH_2PO_4 (0.2 g), were dissolved in 950 ml dist. H_2O , titrated to pH 7.2 with NaOH, and made up to 1 litre.

PBS-Tween. Tween 20 (1 ml) was made up to 1 litre with PBS.

Blocking solution [0.5% (w/v) bovine serum albumin in PBS] (PBS-BSA). BSA (0.5 g) was dissolved in 100 ml PBS.

150 mM citrate-phosphate buffer, pH 5.0. Citric acid solution (21.0 g/l) was titrated to pH 5.0, with a solution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (35.6 g/l).

ABTS substrate [0.5% 2,2'-azino-di(3-ethyl)benzthiozoline in 150 mM citrate buffer, pH 5.0, containing 0.0015% H_2O_2]. ABTS (7.5 mg) and H_2O_2 (7.5 μl , added immediately before use) were dissolved in 150 mM citrate-phosphate buffer, pH 5.0 (15 ml). This provides sufficient reagent for one ELISA plate.

50 mM carbonate buffer. NaHCO_3 (0.21 g) was dissolved in 45 ml of dist. H_2O , titrated to pH 6.0 with HCl, and made up to 50 ml.

Stopping buffer [150 mM citrate-phosphate buffer containing 0.1% NaN_3]. NaN_3 (0.1 g) was dissolved and made up to 100 ml in 150 mM citrate-phosphate buffer.

3.7.1.2 Procedure

Nunc High Bond Immunoplate wells were coated with antigen (150 μl of peptide in PBS, pH 7.2, or enzyme in 50 mM carbonate buffer, pH 6.0) overnight at room temperature, diluted to give a final optimal coating (ppB22-36, ppB64-77, and B192-201, 2 μg ; B13-22, 5 μg and whole cathepsin B, 1 μg). Wells were blocked with BSA (200 μl of PBS-BSA, 1 h, 37°C), and washed 3X with PBS-Tween. Dilutions of primary antiserum (100 μl , in PBS-BSA) were added and incubated for 2 h at 37°C.

After washing 3X in PBS-Tween, a sheep anti-rabbit-IgG horseradish peroxidase conjugate (120 μ l in PBS-BSA) was added and incubated (30 min, 37°C). The plate was again washed 3X in PBS and ABTS substrate (150 μ l) was added, incubated for 15 min, and the A₄₀₅ measured using a Bio-Tek EL307 plate reader.

3.7.1.3 Results

Some differences were observed in the responses to inoculation protocols using conjugated peptide followed by free peptide, as used to raise the B13-22 antisera (Fig. 14, graphs a & b), or inoculation with conjugated or free peptides alone (each used throughout the inoculation protocol), as used with peptide B192-201 (Fig. 14, graphs e & f, and c & d, respectively). All inoculation regimes elicited responses which differed in duplicate experimental animals but all produced antisera which recognised their respective free peptides coated to ELISA plates (Fig. 14). In the case of the B13-22 peptide, where conjugated peptide was followed by free peptide, an initial decrease in level of response was seen in the rabbit that responded best to inoculation with conjugated peptide (Fig. 14, rabbit 1b) while the rabbit that responded the least to the conjugated peptide showed an increasing response to inoculation with free peptide (Fig. 14, rabbit 1a).

Assays for the recognition of mature cathepsin B, coated to ELISA plates at pH 6.0, were only performed for peptide B13-22 antiserum, but no recognition of the native protein was observed in this case (results not shown).

3.7.2 Isolation of IgG fraction

The IgG fraction was separated from rabbit serum using the method of Polson *et al.* (1964).

3.7.2.1 Reagents

10 mM borate buffer, pH 8.6. Boric acid (2.16 g), NaCl (2.19 g), NaOH (0.2 g) and HCl (0.62 ml of 37% solution) were dissolved in 950 ml dist.H₂O. The pH was checked and adjusted to pH 8.6, if necessary, and the solution made up to 1 litre.

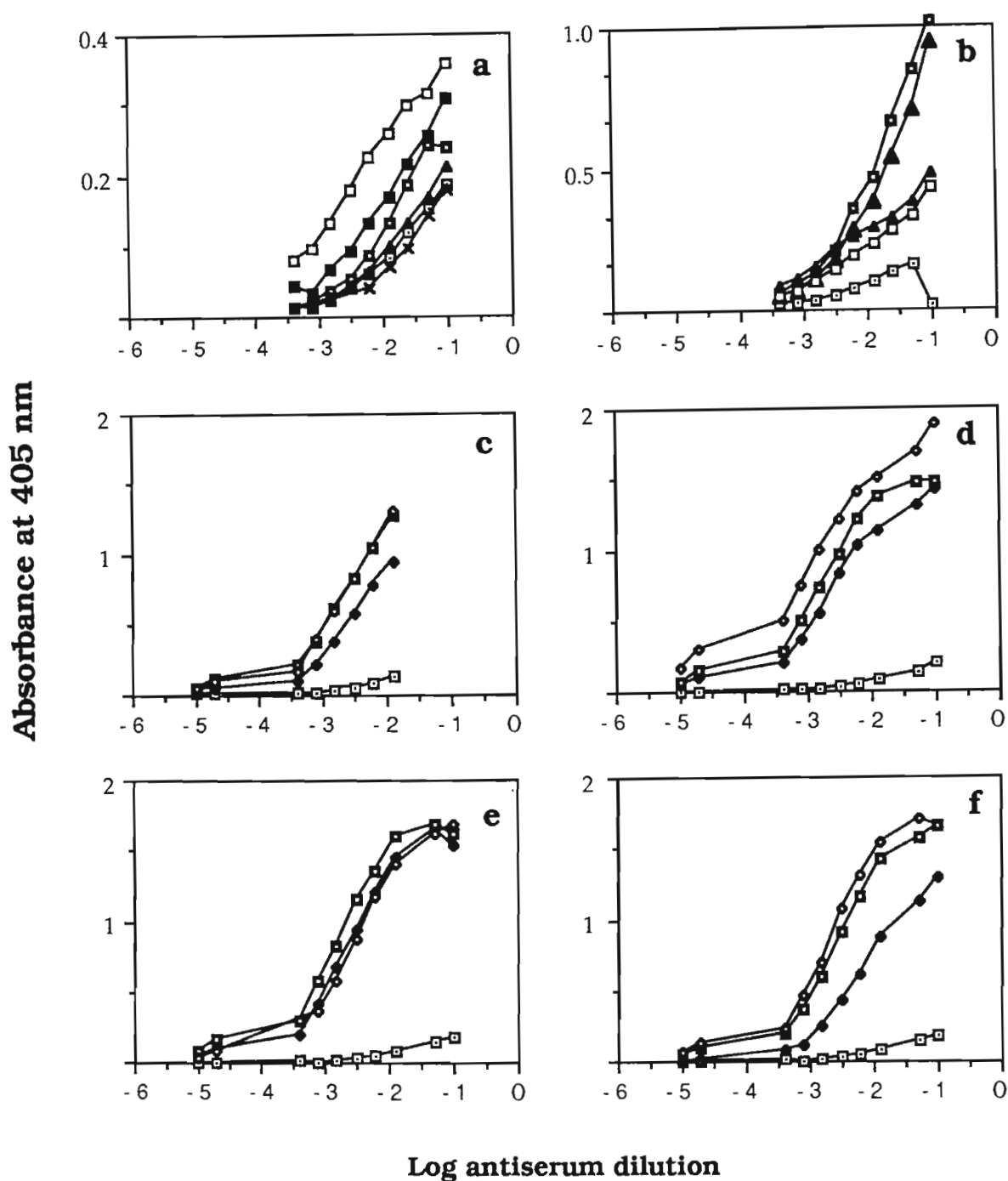


Figure 14.

Response curves showing recognition of coated free peptides by antibodies.

Response curves of:- a and b, two rabbits (rabbits 1a and 1b) inoculated intradermally with 200 μ g KLH-conjugated B13-22 peptide, followed by one inoculum of 1 mg of free peptide into the marginal ear vein (Note that the Y-axis in a is expanded 5X and in b is expanded 2X); c and d, two rabbits (2 and 3) inoculated with 100 μ g of ovalbumin-conjugated B192-201 peptide; e and f, two rabbits (3 and 4) inoculated with 250 μ g of free B192-201 peptide.

□ = preimmune; X = 3-week; ◆ = 5-week; ■ = 8-week; ◇ = 12 week; ▲ = 14-week; ■ = 17-week; ▲ = 30-week; □ = 32-week

0.1 M sodium phosphate buffer pH 7.6. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (1.4 g) and NaN_3 (0.01 g) were dissolved in 95 ml of $\text{dist.H}_2\text{O}$, titrated to pH 7.6 with NaOH and made up to 100 ml.

3.7.2.2 Procedure

One volume of serum was diluted with two volumes of borate buffer and polyethylene glycol (PEG) (M_r 6 000) was added slowly, while stirring, to a final concentration of 14% (w/v). The mixture was centrifuged (12 000 x g, 10 min, RT) and the pellet re-dissolved in the original volume of 0.1 M sodium phosphate buffer, pH 7.6. PEG was again added slowly to a final concentration of 14% (w/v), allowed to dissolve, and the solution was again centrifuged (12 000 x g, 10 min, RT). The pellet, containing the rabbit IgG, was dissolved in half the original serum volume of 0.1 M sodium phosphate buffer, pH 7.6, and stored at 4°C.

3.7.3 Removal of KLH antibodies

Contaminating antibodies, generated against the KLH carrier molecules of the peptide-KLH conjugate, are known to cause cross-reactivity to a large number of epitopes. The anti-B13-22, ppB22-36 and ppB64-77 IgG fractions were consequently affinity purified, the unwanted anti-KLH antibodies being removed by adsorption to a KLH-derivatised Sepharose-4B affinity column, prepared by the method of March *et al.* (1974).

3.7.3.1 Reagents

2 M sodium carbonate. Na_2CO_3 (21.19 g) was dissolved in $\text{dist.H}_2\text{O}$ and made up to 100 ml.

Wash A [200 mM NaHCO_3 , pH 9.6]. $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ (12.4 g) and NaHCO_3 (8.4 g) were each dissolved in $\text{dist.H}_2\text{O}$ and made up to 500 ml. The Na_2CO_3 solution was titrated against the NaHCO_3 solution (100 ml) to pH 9.6.

Wash B [100 mM sodium bicarbonate buffer, pH 9.2]. $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ (0.25 g) and NaHCO_3 (0.84 g) were each dissolved in $\text{dist.H}_2\text{O}$ and made up to 200 ml. The Na_2CO_3 was titrated against the NaHCO_3 (100 ml) to pH 9.2.

Coupling buffer [100 mM sodium bicarbonate, 500 mM NaCl, pH 8.3]. Na₂CO₃·H₂O (5.3 g) and NaHCO₃ (4.2 g) were each dissolved with NaCl (14.61) in dist.H₂O and made up to 500 ml. The Na₂CO₃ solution was titrated against the NaHCO₃ solution (250 ml) to pH 8.3.

Keyhole limpet haemocyanin (KLH) (5 mg/ml). KLH (50 mg) was dissolved in coupling buffer (10 ml), centrifuged (10 000 × g, 15 min, RT) (to remove aggregates) and the absorbance read for later use in calculating coupling efficiency ($E_{280\text{ nm}}^{1\text{ mg/ml}} = 2.0$ for KLH).

Blocking agent [1 M ethanolamine-HCl, pH 8.0]. Ethanolamine (6.06 ml) was diluted in 80 ml dist.H₂O, titrated to pH 8.0 with HCl and made up to 100 ml.

Wash C [100 mM sodium acetate, 500 mM NaCl, pH 4.0]. Acetic acid (5.72 ml) and NaCl (14.6 g) were dissolved in 450 ml dist.H₂O, titrated to pH 4.0 with NaOH and made up to 500 ml.

Chromatography buffer [20 mM sodium phosphate, 500 mM NaCl, pH 7.4]. NaH₂PO₄ (1.38 g), NaCl (14.61 g) and NaN₃ (0.1 g) were dissolved in 450 ml dist.H₂O, titrated to pH 7.4 with NaOH and made up to 500 ml.

Elution "buffer" [3.5 M potassium isothiocyanate]. KSCN (34.0g) was dissolved in 100 ml dist.H₂O.

3.7.3.2 Procedure

Sepharose-4B (10 ml of packed gel) was washed in a Büchner funnel, on a Whatman No 1 filter paper disc, with 150 ml dist.H₂O, transferred to a small beaker, allowed to settle and the supernatant removed. Dist.H₂O (10 ml) and 2 M Na₂CO₃ (20 ml) were added to the gel, and the mixture was placed on ice, in a fume hood, and mixed gently until the temperature decreased to 4°C. The rate of stirring was increased and 1 M CNBr solution in acetone (2 ml) was added as rapidly as possible.

The gel mixture was stirred rapidly for not longer than 90 min, transferred to a Büchner funnel and washed with dist.H₂O (100 ml), wash A (100 ml) and wash B (100 ml). During washing, the gel must be stirred gently with a glass rod

to prevent it from "caking" during filtration, especially during the initial washing step, as the gel tends to become sticky in the final stages of activation, unless the activating agent is quickly and uniformly removed. The activated gel was washed with excess coupling buffer, transferred to a glass bottle and allowed to settle. Excess coupling buffer was removed, and the KLH in coupling buffer (5 mg/ml, 10 ml) was added to the suspension and mixed, end-over-end, for 14 h at 4°C.

The gel was allowed to settle and the excess coupling buffer, containing any unbound KLH, was removed and kept. The absorbance at 280 nm of the supernatant, determined against a coupling buffer blank, was compared with the absorbance of the original KLH solution, and the coupling efficiency calculated by difference.

Blocking agent (10 ml) was added to the remaining gel and the slurry was mixed, end-over-end, for 2 h at room temperature, to block the remaining reactive groups. The gel was washed on a Büchner funnel, alternately with coupling buffer and low pH buffer, wash C, to ensure that no free ligands remain ionically or non-covalently linked to the gel or ligand. The gel was finally washed and stored in chromatography buffer (4°C).

The Sepharose-4B affinity resin was packed into a column (5.3 x 0.5 mm i.d.) and washed with chromatography buffer (3 column vols), elution "buffer" (1 column vol, to elute any loosely bound KLH) and again with chromatography buffer (3 column vols). IgG (3-5 mg, in 0.5-0.6 ml of chromatography buffer) was applied to the column and eluted with chromatography buffer. The break-through peak, being the desired IgG fraction, was collected, while the contaminating KLH antibodies were eluted with elution "buffer" (1 column vol.). The resin was subsequently washed and stored in chromatography buffer.

3.7.3.3 Results

Judged using the ELISA technique, levels of contaminating anti-KLH antibodies in the break-through peak appeared to be very high, even after successive purification steps, and with very little IgG loaded. When assayed using a dot blot system, however, the KLH-binding activity apparently disappeared after two passages of the IgG fraction through the column.

3.8 Antibody characterisation

3.8.1 Western blotting of SDS-PAGE gels

Western blotting was done as described in Chapter 2.

3.8.1.1 Reagents

Secondary antibodies, antigens and locating agents. Sheep IgG was isolated (section 3.7.2) and conjugated to HRPO according to the method of Hudson and Hay (1980). Biotinylated goat-anti-rabbit IgG and donkey anti-sheep IgG were from Sigma. Human liver cathepsin B, affinity purified anti-human liver cathepsin B antibodies and crude preparations of mucoid and purulent sputa were provided by Dr D. Buttle, Strangeways Research Laboratory, Cambridge, UK. Human leucocyte elastase was from Dr J. Travis, Department of Biochemistry, University of Georgia, Athens, GA. Recombinant human procathepsin B, recombinant human cathepsin B, isolated human liver cathepsin B and a second affinity-purified anti-human liver cathepsin B antibody were from Dr L. Mach, Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna, Austria. Streptavidin-biotin-peroxidase complex was from Amersham and the Protein A gold and immunogold probes were prepared as described in Chapter 2.

3.8.1.2 Procedure

For the evaluation of anti-procathepsin B peptide antibodies, and anti- (mature) human liver cathepsin B, three (purported) sources of procathepsin B were used. Firstly, a neutrophil fraction, containing monocyte/macrophages, and hence also cathepsin B (Burnett *et al.*, 1983; Crocker *et al.*, 1984) and presumably its pro-forms, was used. Secondly, mucoid and purulent sputa, reported to contain the whole (M_r 40 000) and truncated (M_r 37 000) forms of procathepsin B, respectively (Buttle *et al.*, 1988), were used. Finally, and arguably most definitively, the antibodies were evaluated by probing electroblots of recombinant procathepsin B and mature cathepsin B molecules, expressed in yeast.

3.8.1.3 Results

The characterisation of the ppB22-36 and ppB64-77 peptide antibodies proved to be problematic, due mainly to the difficulty in finding a suitable source of procathepsin B. Crude leucocyte fractions did not show any bands of reactivity with anti-mature liver cathepsin B antisera, used at a level of 20 $\mu\text{g}/\text{ml}$, even when the leucocyte fractions were loaded in excess of 15 μg per lane, and a sensitive immunogold or protein A gold method, with silver amplification, was used (Fig. 15, blot B, lanes a and b, and blot D lanes a and b, respectively). Such overloading of protein caused non-specific targeting of some bands on these blots, with similar non-specific bands being seen in the IgG controls used at the same concentration as the anti-cathepsin B antibody (Fig. 15, blots A and C, lanes a and b).

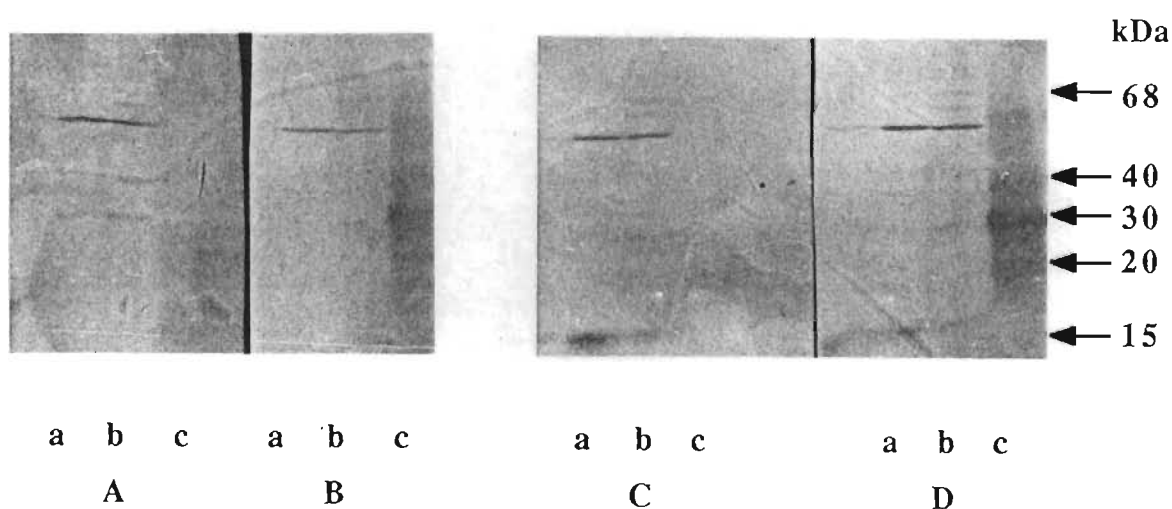


Figure 15. Western blots of leucocyte fractions and human liver cathepsin B. Western blot of a 12.5% reducing SDS-PAGE separation. Approx. 15 μg of crude lymphocyte (lane a), and neutrophil fraction (lane b), and human liver cathepsin B (1 μg) (lane c), were loaded. A and C were treated with a pre-immune IgG preparation (20 $\mu\text{g}/\text{ml}$), while B and D were treated with a sheep anti-human liver cathepsin B IgG preparation (20 $\mu\text{g}/\text{ml}$), and probed with a rabbit anti-sheep gold probe (blots A and B) or a protein A-gold probe (after the use of a rabbit anti-sheep linking antibody) (blots C and D), and silver amplified. The anti-cathepsin B antibody located bands at M_r 57 000, 40 000, 27 000, 25 000 and 20 000.

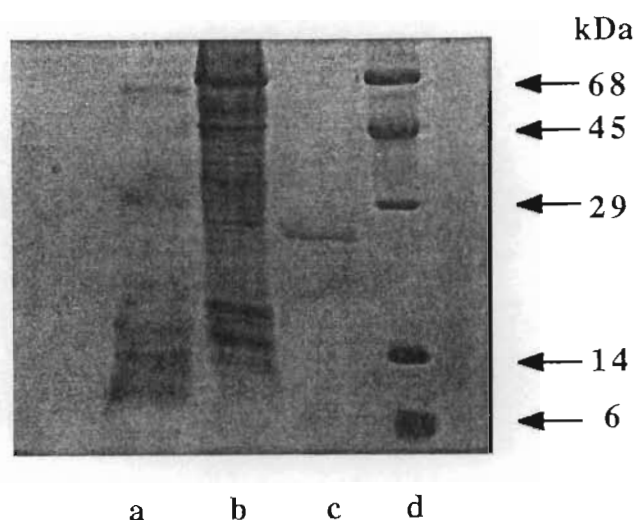


Figure 16. Reducing SDS-PAGE reference gel (12.5%) for blots of lymphocyte and neutrophil fractions and human liver cathepsin B (Fig. 17). Gel shows electrophoretic pattern for the lymphocyte fraction (lane a) and the neutrophil fraction (lane b) (15 μ g), human liver cathepsin B (1 μ g) (lane c) and molecular weight markers BSA, M_r 68 000, ovalbumin, M_r 45 000, carbonic anhydrase, M_r 29 000, lysozyme, M_r 14 000 and insulin, M_r 6 000. (lane d).

Although only a single band (M_r 27 000) of human liver cathepsin B was seen on SDS-PAGE gels loaded with 1 μ g of human liver cathepsin B (Fig. 16), blots probed with the anti-human liver cathepsin B, followed by an immunogold or protein A-gold probe, with silver amplification, indicated the presence of trace amounts of approx. M_r 57 000, 40 000, 25 000 and 20 000 forms of cathepsin B, in addition to the M_r 27 000 mature form (Fig. 15, blots B and D, lane c). The M_r 57 000 form was possibly a dimer and the M_r 25 000 and 20 000 forms, minor products of autocatalytic processing. Different gold probes were used to check whether any of the bands may be due to intrinsic non-specificity in either of the gold probe systems. Subsequently, a peroxidase system was used, and the western blot results for the lymphocyte, neutrophil and human cathepsin B fractions, were verified (Fig. 17).

The M_r 40 000 form was considered to possibly be the pro-form of cathepsin B, and hence the human liver preparation potentially provided a source of precursor for blots using the anti-ppB22-36 and ppB64-77 antisera. However, when the amount of human cathepsin B and leucocyte fractions loaded into each well for SDS-PAGE was increased to 15 μ g or more, to increase the amount of putative procathepsin B blotted, bands were targeted in a manner which could not be due to the expected targeting of the anti-ppB22-36 (Fig. 17, blot A) and which was, therefore, most likely due to non-specific interactions. [The ppB64-77 antiserum gave no labelling of the leucocyte and human liver cathepsin B fractions (results not shown)]. Similarly, mucoid and purulent sputa, which have been reported to contain whole and

truncated forms of procathepsin B (Buttle *et al.*, 1988), also gave a pattern of inexplicable, non-specific binding effects (e.g. Fig. 18).

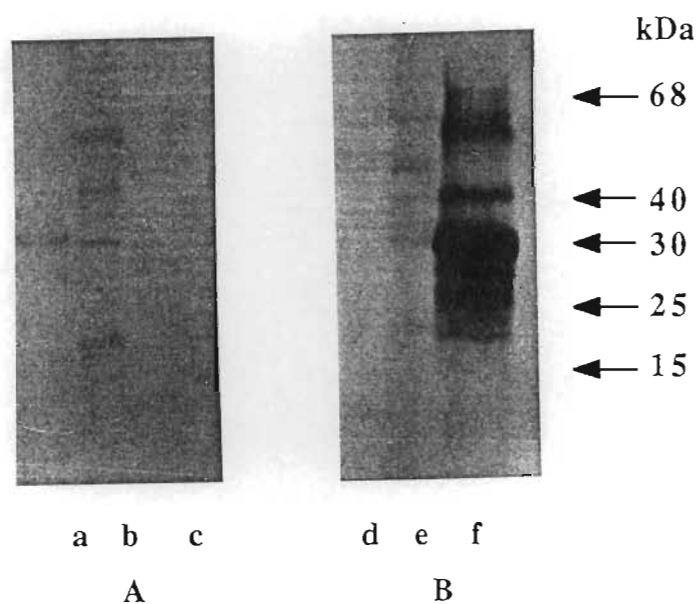


Figure 17. Western blot of lymphocyte and neutrophil fractions and human liver cathepsin B. a and d, lymphocyte fraction; b and e, neutrophil fraction (loaded in excess of 15 μ g protein); c and f, human liver cathepsin B (15 μ g of protein/well). Blot A was probed with anti-ppB22-36 IgG (100 μ g/ml) and blot B with anti-human liver cathepsin B (10 μ g/ml), and labelling detected with sheep anti-rabbit HRPO.

In an attempt to find a more defined and suitable source of procathepsin B, a collaboration was established with Dr J. Glössl, and his (then) student, Lukas Mach, of the Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna, Austria. These workers had available, mature cathepsin B, isolated from human liver, as well as mature and procathepsin B, expressed in yeast, and, in exchange for samples of the anti-ppB22-36 and anti-B64-77 peptide antibodies, Lukas Mach agreed to do western blots of their cathepsin B and procathepsin B samples, using these antibodies.

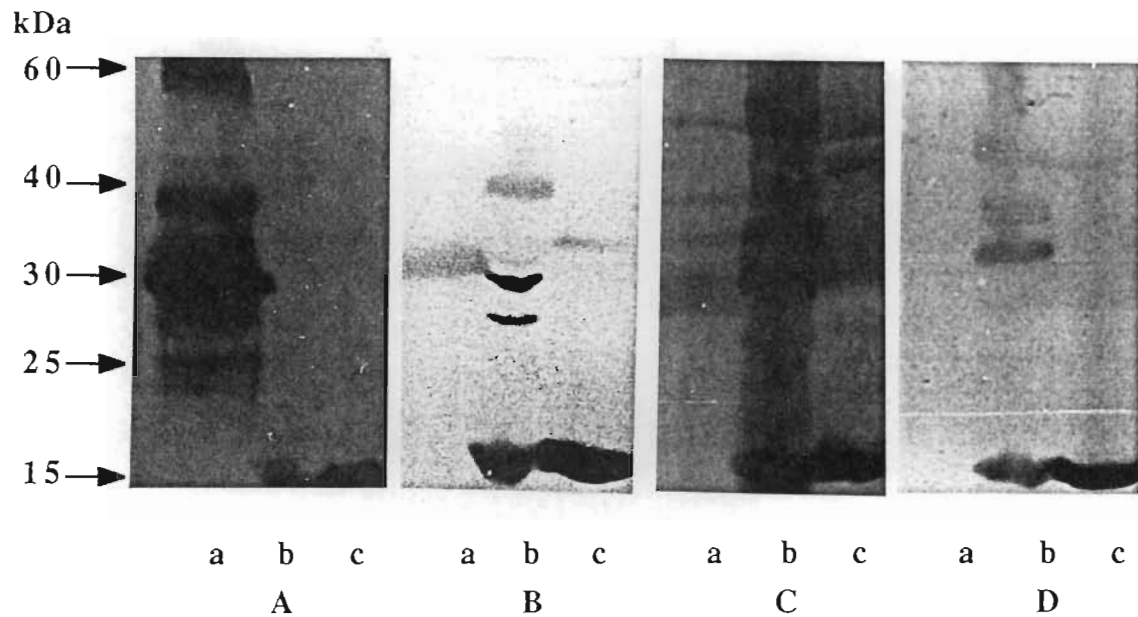


Figure 18. Western blot of reducing 12.5% SDS-PAGE of mucoid and purulent sputa, and human liver cathepsin B. Lane a, human cathepsin B (15 mg/ml); lane b, a crude fraction of purulent sputum; lane c, a crude fraction of mucoid sputum. Probed with; A, anti-human liver cathepsin B (10 $\mu\text{g/ml}$); B, anti-ppB64-77 (200 $\mu\text{g/ml}$); C, anti-ppB22-36 (200 $\mu\text{g/ml}$); D, pre-immune IgG (200 $\mu\text{g/ml}$). Targeting of antibodies was located using the streptavidin-biotin-peroxidase system.

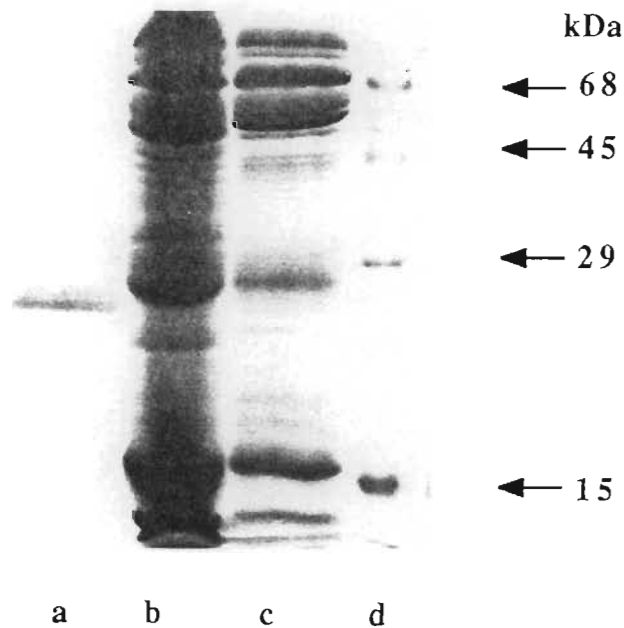


Figure 19. Reducing SDS-PAGE reference gel (12.5%) for blot shown in Fig. 18. Lane a, Human liver cathepsin B (15 $\mu\text{g/ml}$); lane b, purulent sputum; lane c, mucoid sputum; lane d, molecular weight markers, BSA M_r 68 000, ovalbumin M_r 45 000, carbonic anhydrase M_r 29 000, lysozyme M_r 14 000.

The anti-ppB22-36 antibody reacted strongly with recombinant procathepsin B (Fig. 20, blot A, lane a), but not with the mature recombinant or mature isolated human liver cathepsin B (Fig. 20, blot A, lane b and c, respectively). The anti-ppB64-77 antibody did not recognise isolated mature human liver cathepsin B (Fig. 20, blot B, lane c), reacted extremely weakly with the recombinant human pro-enzyme (Fig. 20, blot B, lane a) and weakly with the recombinant mature enzyme (Fig. 20, blot B, lane b). All three forms of cathepsin B were recognised by the anti-human liver cathepsin B antisera (Fig. 20, blot C, lanes a, b and c).

The reaction of the anti-ppB22-36 peptide antibody was as anticipated. Similarly, the reactivity of the anti-ppB64-77 peptide antibody with the recombinant procathepsin B (entire sequence), was as anticipated. The weak reactivity with the recombinant mature cathepsin B and lack of reactivity with the mature liver cathepsin B may be explained by the fact that recombinant mature cathepsin B contains an N-terminal extension of, probably, six residues, after autocatalytic cleavage of procathepsin B, by cathepsin B itself (Hasnain *et al.*, 1992a). In the yeast this sequence remains, whereas, in mammalian cells, this extension seems to be removed by sequential exopeptidolytic trimming, possibly by cathepsin C (Hasnain *et al.*, 1992a). The epitope recognised by the anti-ppB64-77 antibody is, therefore, possibly comprised of only 5 amino acids, residues 73-77 (the difference between the C-terminal extension and the target peptide).

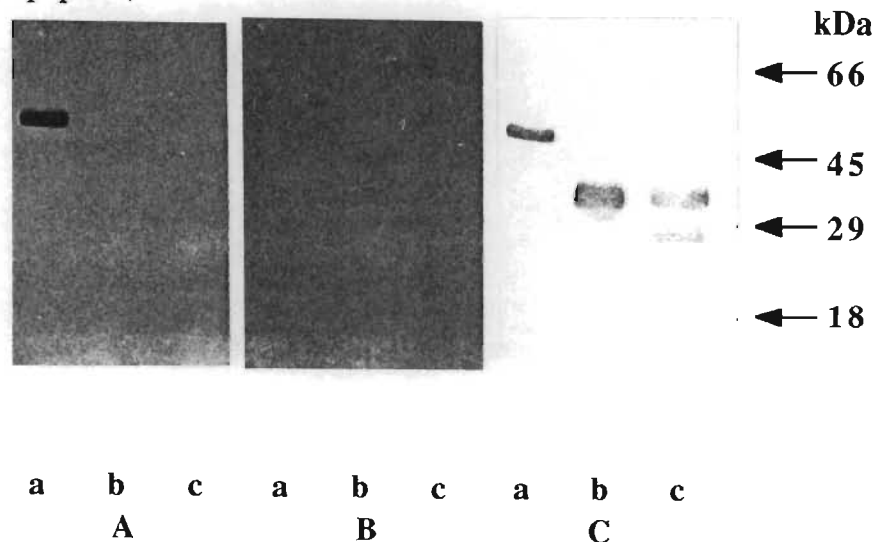


Figure 20. Western blots of recombinant procathepsin B, recombinant mature human cathepsin B and mature isolated human cathepsin B. **a**, recombinant yeast procathepsin B (0.5 μ g); **b**, recombinant mature human cathepsin B, from yeast (0.5 μ g), **c**, isolated mature human liver cathepsin B (0.5 μ g). Blots were probed with; **A**, anti-ppB22-36; **B**, anti-ppB64-77; **C**, anti-human liver cathepsin B (all at 2.5 μ g/ml), and visualised using the streptavidin-biotin-peroxidase system.

As the reactivity of the anti-ppB64-77 antibody was extremely weak, this, unfortunately, precluded studies into the presence of the stable, truncated, form of procathepsin B in cancer and normal cells. The success of the ppB22-36 antibody, however, meant that this could be applied to the detection of procathepsin B (see Chapters 4 and 5).

With regard to peptide antibodies to mature cathepsin B, it was found that IgG fractions from different rabbits reacted differently with respect to their recognition of human liver cathepsin B in reducing SDS-PAGE blots (Table 7).

Table 7. Recognition of whole mature cathepsin B by anti-peptide antibodies in reducing SDS-PAGE western blots.

Rabbit Number	Inoculate	Recognition of Cath B in SDS-PAGE blots by corresponding antibody
1A	KLH-conjugated B9-19	–
1B	KLH-conjugated B9-19	–
2	Free B192-201	–
3	Free B192-201	+
4	Ovalbumin-conjugated B192-201	+
5	Ovalbumin-conjugated B192-201	++

The IgG fractions that reacted positively, varied in their response to different bands in the human cathepsin B (Fig. 21). The IgG fractions from rabbits 3 and 4 targeted the heavy chain of mature cathepsin B, at *ca.* M_r 26 000 and a dimer form at *ca.* M_r 57 000, while that from rabbit 5 targeted all of the components of mature cathepsin B (Fig. 21). The reactivity of these peptide antibodies was as expected and was consequently considered acceptable.

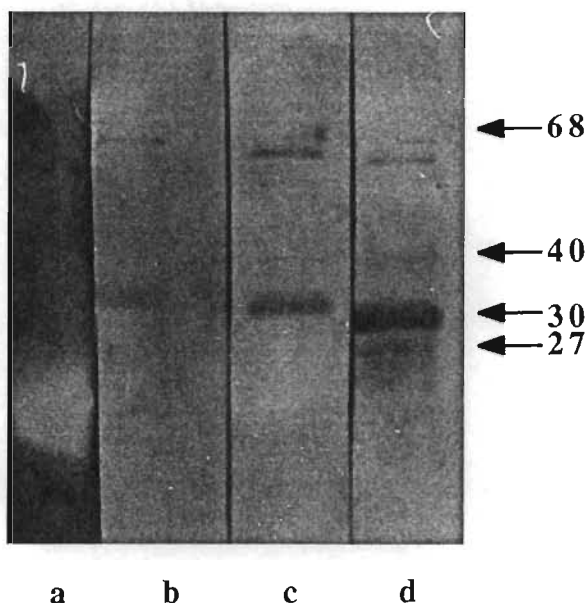


Figure 21. Western blot of 12.5% SDS-PAGE gel of human liver cathepsin B, probed with the antisera from different rabbits inoculated with the B192-201 peptide. Human liver cathepsin B (2 $\mu\text{g}/\text{ml}$) was loaded and probed with IgG (100 $\mu\text{g}/\text{ml}$): blots a and b, IgG from rabbits 2 and 3, respectively, inoculated with free B192-201 peptide; blots c and d, IgG from rabbits 4 and 5, respectively, inoculated with B192-201 conjugated to ovalbumin.

The anti-human leucocyte elastase IgG fraction, targeted the M_r 28 000-30 000 doublet in purified fractions of human leucocyte elastase, (Fig. 22), but failed to recognise elastase in crude homogenates of density gradient-separated leucocyte fractions (result not shown). Since neutrophils contain large amounts of elastase, this failure could be ascribed to non-optimal Ab/Ag ratios in the western blot. However, at all ratios tested, the homogenates gave either no labelling or, at higher Ab levels, generally non-specific labelling. This seems to reflect a peculiar property of the leucocyte homogenate as the anti-elastase antibody subsequently gave specific immunolabelling of cell sections (Chapter 4).

The rabbit anti-human cathepsin D sera recognised the M_r 48 000, single-chain form in crude fractions of human leucocyte homogenates, and the M_r 48 000 single-chain and M_r 30 000 two-chained form of human cathepsin D, in purified fractions of human cathepsin D (Fig. 23, blots A and B), whereas no equivalent targeting was seen with pre-immune IgG (result not shown), used at the same concentration. In crude leucocyte homogenates, an additional doublet at approx. M_r 15 000 was seen; this could represent the light chain of the two-chained form of human cathepsin D.

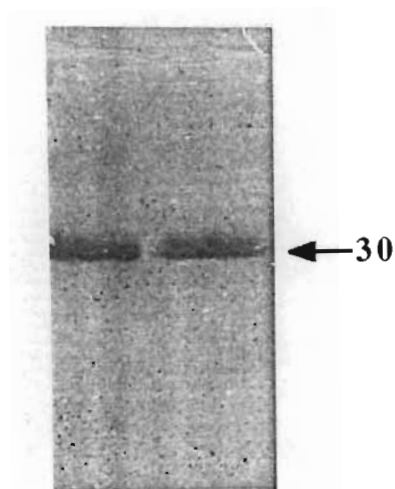


Figure 22. Western blot of 12.5% reducing SDS-PAGE gel of human leucocyte elastase. Human leucocyte elastase (7.5 $\mu\text{g}/\text{ml}$) was separated by SDS-PAGE, electroblotted and probed with rabbit anti-human elastase and pre-immune IgG (86 $\mu\text{g}/\text{ml}$). Labelling was visualised with a sheep anti-rabbit HRPO conjugate. Pre-immune IgG gave no reaction (result not shown).

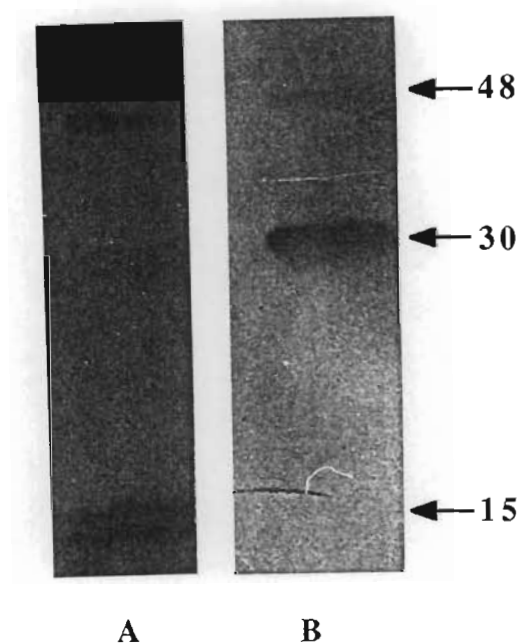


Figure 23. Western blots of 12.5% reducing SDS-PAGE gel for the characterisation of rabbit anti-human cathepsin D antibodies. **A**, a crude homogenate of human neutrophils (5 μg per well); **B**, a purified fraction of human spleen cathepsin D (5 μg per well). Blots were probed with a rabbit anti-human cathepsin D antiserum (1 in 40 dilution) and visualised with a sheep anti-rabbit HRPO conjugate.

The chicken anti-porcine cathepsin D showed a similar pattern of labelling, strongly targeting the M_r 48 000, single-chain form and the M_r 30 000 heavy chain form in the purified fraction from porcine spleen and the corresponding bands, though only weakly, in crude extracts (Fig. 24).

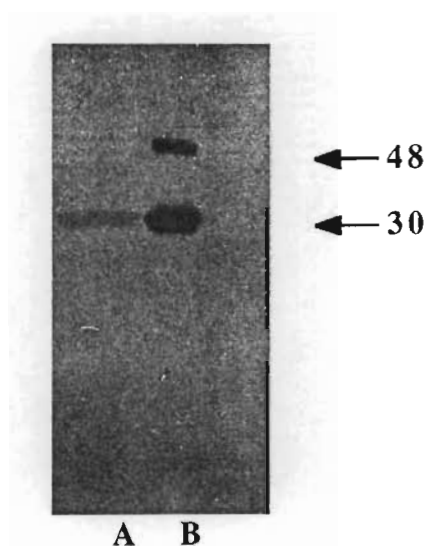


Figure 24. Western blots for the characterisation of chicken anti-porcine cathepsin D antibodies. A, a crude homogenate of porcine spleen (5 μ g per well); B, a purified fraction of porcine cathepsin D (5 μ g per well). Blots were probed with a chicken anti-porcine cathepsin D antiserum (188 μ g/ml) and visualised using a rabbit anti-chicken antiserum (1 in 240 dilution), followed by a sheep anti-rabbit HRPO conjugate (1 in 400 dilution).

3.8.2 Immunoinhibition assays

The anti-mature cathepsin B antibodies, anti-B13-22 and anti-B192-201, were tested for their recognition of the active enzyme, especially for immunolabelling studies in the "cryo" system, where antigens tend not to be denatured, and as an indication of the possible use of such antibodies for anti-proteinase immunotherapy of cancers.

3.8.2.1 Reagents

Assay buffer [352 mM KH_2PO_4 , 48 mM Na_2HPO_4 , 4 mM Na_2EDTA , 0.02% (m/v) NaN_3 , 8 mM Cysteine.HCl, pH 6.0]. KH_2PO_4 (23.95 g), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (4.27 g), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (0.75 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist. H_2O , adjusted to pH 6.0 with NaOH and made up to 500 ml. Prior to the assay, cysteine.HCl (0.007 g) was added to 5 ml of buffer.

1 mM substrate stock solutions. Z-Arg-Arg-NHMec (1.1 mg) was dissolved in DMSO (1.5 ml), aliquotted and stored at 4°C.

20 µM substrate solutions. Substrate stock solution (0.1 ml) was diluted to 5 ml with dist.H₂O.

Brij 35 diluent [0.1% (m/v) Brij 35]. Brij 35 (0.1 g) was made up to 100 ml with dist. H₂O.

Antibody diluent. Tween 20 was added to assay buffer (containing no cysteine.HCl) to 0.1% (v/v).

3.8.2.2 Procedure

Assays for the immunoinhibition of cathepsin B were carried out using the synthetic substrate Z-Arg-Arg-NHMec, as described by Barrett (1980). Immunoinhibition of cathepsin B by antibody preparations was assayed by continuous monitoring of the cleavage of Z-Arg-Arg-NHMec. Human cathepsin B (150 ng) was diluted to 500 µl in 0.1% Brij 35, and the relevant IgG preparation was added at 2 mg-, 1 mg-, or 0.5 mg/ml, in antibody diluent (500 µl). The mixture was incubated at 40°C for 15 min, and an aliquot (500 µl) was removed and activated with assay buffer, containing 8 mM cysteine (250 µl), for 1 min. Z-Arg-Arg-NHMec substrate (250 µl of 20 µM) was added, and the increase in fluorescence was monitored for 5 min at 40°C in a temperature controlled cell in a Hitachi Model F-2000 spectro-fluorimeter, with excitation at 370 nm and emission at 460 nm. The slope of the linear increase in activity, in the presence of the different IgG preparations, was calculated, and the percentage inhibition was expressed in comparison to the slope in the presence of non-immune IgG at the same concentration. The inhibition of enzyme activity by the antibodies was expressed as a percentage of the activity in the presence of non-immune rabbit IgG. Continuous monitoring of substrate hydrolysis established, using minimal amounts of enzyme, whether a particular antibody preparation was immunoinhibitory and whether optimal conditions prevailed for the duration of the assay, such as the amount of substrate available.

3.8.2.3 Results

The peptide antibody IgG fractions against peptide B13-22 showed no effect on enzyme activity (results not shown). By contrast, all IgG preparations from rabbits inoculated with ovalbumin-conjugated, or free peptide B192-201, showed slight activation (approx. 10%) of human liver cathepsin B, rather than an immunoinhibitory effect (results not shown). None of the anti-cathepsin B peptide antibodies were, therefore, suitable for studies on the immunoinhibition of invasion.

3.9 Discussion

Proteins or peptides may be “immunogens”, i.e. structures which elicit an immune response, leading to the production of antibodies, and/or “antigens”, i.e. structures which react with the antibodies produced. In the case of whole proteins, the protein molecule is usually both immunogenic and antigenic, and the distinction between these terms becomes of academic interest only. The same may not hold for peptides, and peptide antibodies intended to react with whole proteins.

So-called “peptide antibodies” are raised by choosing a peptide sequence of *ca.* 10 amino acids from the primary amino acid sequence of the protein of interest, synthesising the peptide and using this to inoculate an animal, either in the form of a conjugate with a carrier molecule or as the free peptide. The resulting peptide antibodies invariably react with the peptide itself, i.e. they are invariably “peptide-reactive”, but they may or may not react with the protein of interest, i.e. they may or may not be “protein-reactive”. The protein-reactivity, moreover, may be specific for the native protein or for more or less denatured forms of the protein.

A major challenge in peptide antibody technology is to be able to predict, in advance, whether a given peptide will elicit protein-reactive antibodies, especially against the native protein, since these antibodies would be the most useful. The terminology in this field is not well developed and so, for economy of expression in this thesis, the term “protein antigenic” will be used to describe a peptide which elicits protein-reactive peptide antibodies. The term “peptide antigenic” will be used for peptides which elicit peptide antibodies able to recognise the peptide, but not necessarily the protein from which the peptide was selected. Predictive methods are, therefore, largely aimed at the identification of “protein antigenic” peptides.

During the raising and characterisation of specific antisera, three aspects of the immune response should be borne in mind. Firstly, protein molecules or peptide sequences are only antigenic if matched by a complementary recognition paratope (antigen-binding site) of an antibody that arises from the pool of immunoglobulin-producing cells, in the inoculated animal (Van Regenmortel, 1986; Larsson, 1988a). Secondly, injection of a foreign substance into an animal, induces the proliferation of - and antibody production by - a particular subset clone of lymphocytes, producing antibodies with a "best fit" paratope (Larsson, 1988a). Thirdly, since the subset clone selection is on the basis of the "best fit possible," the precision of "fit", or complementarity, between the paratope and epitope can vary considerably and hence binding of similar-but-different epitopes may occur, though usually with different avidities (Van Regenmortel, 1986). This fact can give rise to considerable differences in apparent specificity or non-specificity of antigen recognition, given that many epitopes are similar, and may explain the recognition or non-recognition of different epitopes by antibodies diluted to different extents.

Westhof *et al.* (1984) and Tainer *et al.* (1984) found that antibodies preferentially bind to protein regions with a high degree of mobility or flexibility. The mobility of the antigenic region may facilitate the binding of an antigen to an antibody which does not possess an ideal complementary paratope. This may occur as highly mobile regions of antigens stand a better chance of selecting a clone producing antibodies, by adopting a conformation which fits the paratope (Westhof *et al.*, 1984). For recognition, therefore, not only are the residues comprising the epitope (the peptide sequence) important, but so too is the conformation or potential conformations that the epitope may adopt.

Generally, approaches which have endeavoured to identify "protein antigenic peptides" on the basis of primary structure have been found to be less successful than those based on 3-D information and X-ray data (Stern, 1991). This is because most anti-native protein antibodies recognise assembled topological determinants and these are difficult to predict using only primary sequence data (Margalit *et al.*, 1987). The conformation dependence of epitope recognition explains how easy it is to alter, create or destroy the epitope by treatments that alter the conformation of the target antigen. Where the conventional preparation of anti-protein antibodies involves the injection of a conformationally restricted form of the protein molecule, anti-peptide antibodies are produced using a conformationally less restricted synthetic peptide. It is estimated that possibly as few as one in 10^4 or 10^5 peptides retain the conformation recognisable to an antibody raised against the native protein (Todd *et al.*, 1982). Possibly for this

reason, many peptide antibodies do not recognise, or react only weakly with, their target sequences in whole proteins (Palfreyman *et al.*, 1984; Tanaka *et al.*, 1985).

Many methods, based on assumptions on the nature of the immune response and antigenicity, and based on primary sequence data, have been proposed for the predictive identification of protein antigenic peptide sequences (Hopp and Woods, 1981; 1983; Atassi, 1984; Palfreyman *et al.*, 1984; Tainer *et al.*, 1984; Westhof *et al.* 1984; Parker *et al.*, 1986; Van Regenmortel, 1989; Stern 1991). The aim of all of these methods is to facilitate the selection of linear antigenic peptide sequences, usually on the basis of a physical property, that has been found to predispose parts of a protein sequence to constitute a protein antigenic epitope, when the whole protein molecule is injected into an animal.

At the commencement of this study, 3-D structural information, on cathepsin B itself, was not available, and potentially protein antigenic peptide sequences were selected by extrapolation from the 3-D structure of papain, and on the basis of the hydrophilicity, mobility and accessibility of the target peptide (Hopp, 1989; Van Regenmortel, 1989). Hydrophilicity provides an indication that the peptide sequence chosen occurs on the outer aspect of a protein, where it should be accessible to antibody binding (Hopp and Woods, 1981). Mobility parameters (Tainer *et al.*, 1984; Westhof *et al.*, 1984; Van Regenmortel, 1989) have been found to correlate well with the successful choice of peptides for the production of peptide antibodies (Stern, 1991), the more mobile residues proving the most protein antigenic (Tainer *et al.*, 1984; Westhof, *et al.*, 1984). Hydrophilicity (Hopp and Woods, 1981; 1983; Fraga, 1982; Kyte and Doolittle, 1982) and accessibility programmes (Chothia, 1976; Janin, 1979; Novotny *et al.*, 1986) have, however, been found to correlate poorly (Stern, 1991).

Most hydrophilicity (Hopp and Woods, 1981;1983; Kyte and Doolittle, 1982) and mobility predictive programmes (Karplus and Schultz, 1985), are based on protein primary sequence and are designed to make predictions for proteins for which only the primary sequence is known. The Hopp and Woods (1981) programme is reported (Van Regenmortel and de Marcillac, 1986) to correlate less favourably with protein-antigenicity, than hydrophilicity predictions using HPLC methods (Parker *et al.*, 1986), but was used in this study because the programme was readily available. Mobility measurements using X-ray crystallographic temperature information (Tainer *et al.*, 1984), and the less favoured Karplus and Schultz (1985) programme, could similarly have given superior protein-antigenic reactivity predictions to that given by the

programme of Westhof *et al.* (1984) (Van Regenmortel, 1986; Stern, 1991) but these programmes were also not available at the time of selection of peptide sequences.

Subsequent to the selection of the four peptides used in this study, various additional predictive programmes were obtained, for the analysis of primary structure sequence data for hydrophilicity (Kyte and Doolittle, 1982; Parker *et al.*, 1986), flexibility (Karplus and Schultz, 1985), antigenicity (Welling *et al.*, 1985) and accessibility (Chothia, 1976; Janin, 1979; Novotny *et al.*, 1986). This provided the opportunity for a retrospective analysis of the sequence of procathepsin B and an assessment of the accuracy of the programmes' predictions. Only the antigenicity prediction of Welling *et al.* (1985) (Fig. 25) and the accessibility plot of Chothia (1976) (Fig. 26) appeared to correlate with the results obtained for the "protein antigenicity" of the four peptides chosen for the present study.

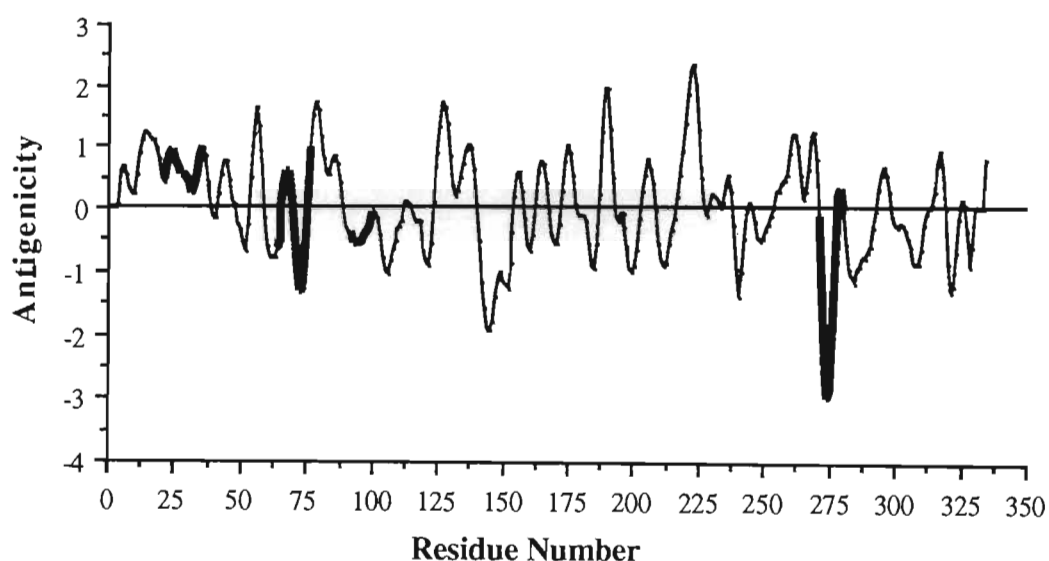


Figure 25. Antigenicity plot of the human preprocathepsin B sequence (Welling *et al.*, 1985). The bold lines indicate the four peptides, ppB22-36, ppB64-77, B13-22 and B192-201, selected from the sequence of procathepsin B. The prepro-sequence contains 79 residues, which must be subtracted from the preprocathepsin B residue numbers indicated, to obtain the residue numbers in the mature enzyme.

The Welling predictive programme uses the calculated relative occurrence of each amino acid in the sequences of known antigenic regions of 20 proteins and compares this with the primary sequence of the unknown protein to predict antigenic epitopes. If peaks occurring above the zero base line are taken as "protein antigenic" regions, and recognition of proteins in western blots is taken as "protein reactivity", then the Welling antigenicity plot correctly predicts the success of the three "protein antigenic" peptides (ppB22-36, ppB64-77 and B192-201) and the lack of protein

antigenicity of peptide B13-22 (though, it must be admitted, that B192-201 appears to barely meet the criterion).

The overall trend of these results is also reflected by the accessibility plot of Chothia (1976), in which only peptides producing peaks greater than 0.5 on the accessibility scale produced protein reactive antibodies. This may indicate that the lack of protein recognition, of the B13-22 antibody, was on account of the lack of accessibility of the target peptide in the whole molecule (as seems to be the case from the 3-D structure of cathepsin B, as discussed below).

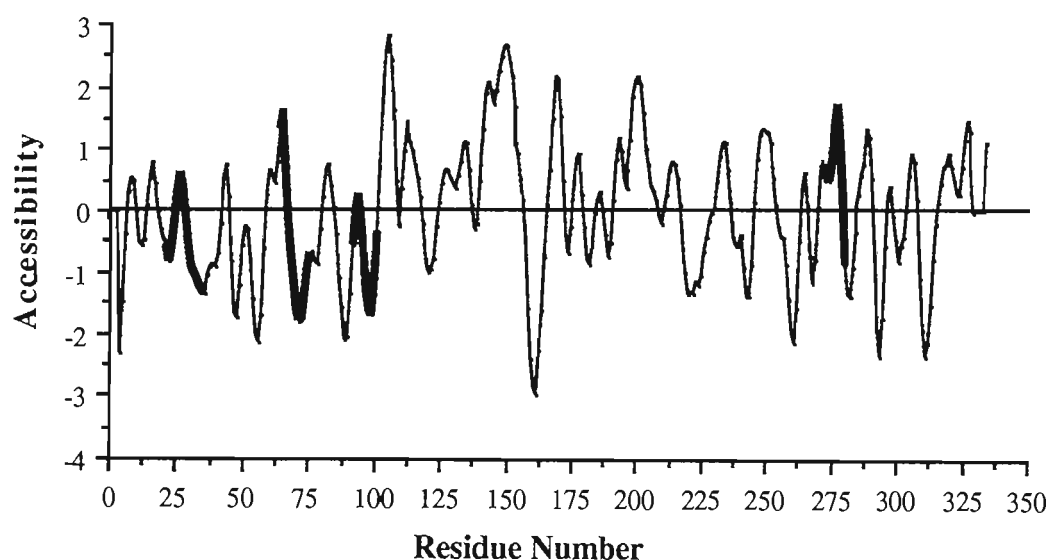


Figure 26. Accessibility plot of the human preprocathepsin B sequence (Chothia, 1976). The bold lines indicate the four peptides, ppB22-36, ppB64-77, B13-22 and B192-201, selected from the sequence of procathepsin B. The prepro-sequence contains 79 residues, which must be subtracted from the preprocathepsin B residue numbers indicated, to obtain the residue numbers in the mature enzyme.

In this study, the hydrophilicity plots of Hopp and Woods (1981) (Fig. 8) and of Parker *et al.*, (1986) (not shown) and the segmental mobility plots of Westhof *et al.* (1984) (Fig. 9) and of Karplus and Schultz (1985) (Fig. 27), seem to indicate that neither hydrophilicity or segmental mobility *per se* are reliable indicators of protein-antigenicity. One common feature of the protein-antigenic peptides (ppB22-36, ppB64-77 and B192-201), however, is that they all contain at least some hydrophobic residues; the peptide ppB64-77 that produced the more weakly reacting anti-peptide antibodies, containing the fewest hydrophobic residues (Fig. 8).

Although, as discussed above, flexibility favours immunogenicity, in this study the flexibility plot of Karplus and Schultz (1985) seems to indicate that some immobility of amino acid residues in the peptide favours protein antigenicity (Fig. 27).

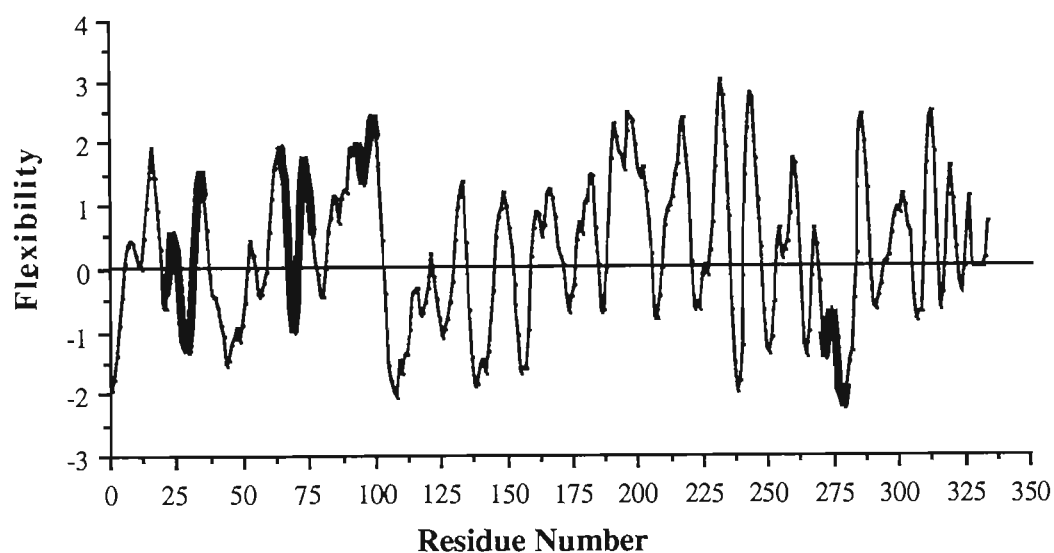


Figure 27. Segmental mobility plot of the human preprocathepsin B sequence (Karplus and Schultz, 1985). The bold lines indicate the four peptides, ppB22-36, ppB64-77, B13-22 and B192-201, selected from the sequence of procathepsin B. The prepro-sequence contains 79 residues, which must be subtracted from the preprocathepsin B residue numbers indicated, to obtain the residue numbers in the mature enzyme.

The presence of some hydrophobic residues and limited peptide flexibility may be important in restraining the peptides in a specific conformation. The conformation adopted by a peptide upon inoculation may be biased by the presence of such hydrophobic residues or by other restraining residues, such as proline (Lerner *et al.*, 1981) or disulfide bonding within the chosen sequence, which would predispose the peptide to adopt a particular conformation. Analysis of the properties of the peptide antibodies raised during this study, seems to indicate that the successful raising of protein reactive peptide antibodies may correlate with the peptides' propensity to adopt specific conformations, due to restraining hydrophobic domains. The possibility that the peptides were adopting helper T-cell binding site conformations (Cornette *et al.*, 1989) was, therefore, investigated.

T-cell presented peptides are reported to be generally amphipathic sequences comprised of hydrophobic and hydrophilic domains (Mouritsen *et al.*, 1991) or α -helices (DeLisi and Berzofsky, 1985). Peptides with amphipathic properties, due to regularly spaced hydrophobic residues, tend to fold into α -helical-like structures on a hydrophobic surface (DeLisi and Berzofsky, 1985), such as the membrane of an antigen-presenting macrophage. The helix-like conformation may be promoted by progressive anchoring of the regularly-spaced hydrophobic side chains into a lipid bilayer, at successive turns of the peptide, assisted by hydrogen bonding along the

peptidyl backbone (Lu *et al.*, 1991). Membrane adsorption may be a step in the transfer of peptides to major histocompatibility complex (MHC) molecules (Berzofsky *et al.*, 1987). The recurring hydrophobic residues would form a longitudinal hydrophobic strip along the helix which could promote its binding to the hydrophobic floor of a scavenging transfer molecule or to an MHC molecule's antigen-binding site. Either as a helical configuration *per se* or within a protective structure, the peptide might be relatively resistant to further proteolytic attack during antigen processing and hence may survive as a presented epitope. The potential amphiphilic helical configuration of T-cell-presented peptides might, thus, reflect selection at a scavenging/transfer step in antigen processing, rather than coiling as a helix within the antigen binding site (Lu *et al.*, 1991). Transfer is perhaps via the surface of an antigen presenting macrophage (together with the Ia receptor) to the T-cell, as free peptide apparently does not cause stimulation in the absence of macrophages (Margalit *et al.*, 1987)

The T-cell receptor sees only a relatively small segment of protein, after the appropriate cleavage, and/or unfolding (Berzofsky, 1985). Thus the experimental modelling approach can be focussed on peptides and short segments of proteins without consideration of protein tertiary structure (Margalit, *et al.*, 1987). X-ray analysis of T-cell sites has revealed that only a limited number of sites are seen by T-cells after immunisation. In order to assess whether any of the chosen peptides could possibly have the propensity to fold into an α -helix and form such an epitope, the "amphipathic helix algorithm" of Margalit *et al.* (1987), was applied. The computer programme (AMPHI), based on this algorithm, predicted 18 out of 23 T-cell antigenic sites correctly (78% correct prediction) in the study by Margalit *et al.* (1987).

This programme identified all of the protein-antigenic peptide sequences, in the present study, as T-cell binding sites. Most of the unsuccessful peptide, B13-22, was identified as an amphipathic region but not as a T-cell binding site. Four amino acids of the peptide ppB22-36 (residues 29→32) and two regions of peptide B192-202 (residues 194→197 and 199→202) were identified as pattern 1 T-cell binding sites (i.e. the pattern, (charged or Gly) - hydrophobic - hydrophobic - (hydrophobic or Pro) -(polar or Gly). Residues 71→75 of the procathepsin B sequence, ppB64-77, were recognised as a pattern 2 T-cell binding site [i.e. (charged or Gly)-hydrophobic-(hydrophobic or Pro)-(polar or Gly)] though the pattern in the peptide is actually, Gly-Pro-Pro-Lys.

There is no doubt that a consideration of the topology of the target peptide sequence in the whole protein is also important. The fact that the peptide antibody against the sequence B13-22 did not recognise the native protein may be due to the

topological position of this sequence within the cathepsin B molecule. On the other hand, the success of the peptide ppB22-36, may be attributed to the fact that it occurs on an accessible loop which occludes the active site, as hypothesised by Mort and Recklies (1986). This peptide occurs on an exposed, flexible region, on the external aspect of the procathepsin B molecule, where it is easily accessible to antibody binding. The overall mobility of the ppB22-36 peptide is lower, however, than the less successful anti-ppB64-77 peptide, showing that mobility alone may not be a reliable criterion for the selection of protein antigenic peptides.

For the choice of a peptide sequence for the production of anti-mature cathepsin B reactive- and, hopefully, immunoinhibitory peptide antibodies, knowledge of the actual 3-D topology of cathepsin B is essential. The structure, published by Musil *et al.* (1991), revealed that the overall folding pattern of cathepsin B, and the arrangement of the active site residues, is similar to that of the related cysteine proteinases papain, actinidin and calotropin D. A few large insertion loops, however, appear on the outer surface of cathepsin B, changing the properties of the molecule and make alignment of equivalent sequences in the different proteinases problematical. Figure 28 shows the structure of cathepsin B (bold), superimposed on the structure of papain (fine lines). The model of the cathepsin B molecule (Fig. 28) is rotated by 45°, around the x-axis, relative to the structure of papain (Fig. 10).

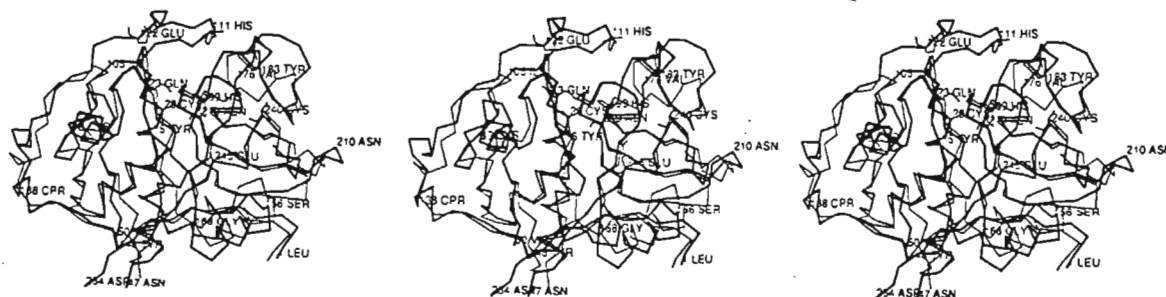


Figure 28. The 3-D structure of cathepsin B, compared to that of papain. (from Musil *et al.* 1991)

A stereo image may be created by viewing the centre and right images through a stereoscope or by viewing the left and centre images with unaided eyes, using proximal convergence (Wood, *et al.*, 1981)

Two of the insertion loops occur in exactly the regions of both of the mature cathepsin B peptides selected for synthesis. Peptide B13-22 occurs on one of the

insertion loops (sequence 16-19, according to the cathepsin B numbering of Musil *et al.*, 1991), and in this region cathepsin B has no residues in common with papain (Table 8).

Table 8. Alignment of cathepsin B and papain¹ (From Musil *et al.*, 1991).

Table I. Alignment of cathepsin B and papain: non-equivalent papain residues are given in lower case letters

Cath B	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Papain	L P A S F D A R E Q W P Q C P T I K E I R D Q G S C G S C W A F G A V E A I S D 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Cath B	41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
Papain	I P E Y V D W R Q k g a v t p V K N Q G S C G S C W A F S A V V T E E G 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72
Cath B	81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
Papain	R I C I H T N A H V S V E V S A E D L L T C C G S M C G D G C N G G Y P A E A W I I K I R T g n L N Q Y S E Q E L L D C D R r s Y G C N G G Y P W S A L 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92
Cath B	121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159
Papain	Q L V A Q Y G I H Y r n T Y P Y E g v q G E G D T P K C S K I C E P G Y S P T Y K Q D K H Y G Y N S Y S V S - N S E K D 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
Cath B	160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198
Papain	R Y C R s r e k g p y a A K T D G V R Q V Q p y N Q G A I M A E I Y K N G P V E G A F S - V Y S D F L L Y K S G V Y Q H V T G E M M G G 121 122 123 134 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158
Cath B	199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235
Papain	L L Y S I A N Q P V S V V L Q a a G K D F Q L Y R G G I F V G p e g n k v d H A I R I L G W G V E N G T P Y W L V A N S W N T D W G D N G F F K I L R - - - 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194
Cath B	236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254
Papain	H A V A A V G Y G p n Y I L I K N S W G T G W G E N G Y I R I K R g i g - G Q D H C G I E S E V V A G I P R T D 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212
	n s y g V C G L Y T S S F Y P v k n

¹Non-equivalent papain residues given in lower case.

The lack of conserved residues, suggests that the sequence B13-22 may be unimportant in the enzymatic activity of cathepsin B, and hence may not be a good choice of peptide for the production of immunoinhibitory antibodies. Such a view is reinforced by an examination of the position of the residues 13→22 in the 3-D structure of cathepsin B (Fig. 28), which reveals that this sequence is occluded by the extra loop in cathepsin B, comprised of residues 108 to 125. Whereas the presumed equivalent sequence (sequence 3) in the papain structure appeared to be accessible to antibody binding (Fig. 10), in the actual 3-D structure of cathepsin B (Fig. 28), residues 13→22 seem to lie buried in the active site cleft, where they are inaccessible to antibody binding. This could explain why peptide antibodies targeting this sequence were not protein reactive.

By contrast, peptide B192-201, in both the speculated position (sequence 1) from the structure of papain (Fig. 10) and in the actual structure of cathepsin B (Fig. 28),

occurs on an exposed loop, part of which (Met-196→His-199) makes up the active site cleft on the right-hand side (Fig. 28). The fact that residues 192→201 are apparently more accessible to antibody binding, than residues 12→22, possibly accounts for the success of this peptide in eliciting antibodies to the native and SDS-denatured protein. Mobility, hydrophilicity, and topological characteristics are all favourable and endorse the success of this peptide.

It was surprising, therefore, that antibodies raised against B192-201 were not immunoinhibitory, as were antibodies to the equivalent sequences in cathepsin L (Dennison and Pike, 1991) and cathepsin H (Coetzer, 1993). This may reflect differences in the catalytic mechanisms of these cathepsins, but it may be noted that the sequence B192-201 is particularly inflexible in cathepsin B (Fig. 9), and this may have some bearing on the lack of immunoinhibitory properties. Because these antibodies were not immunoinhibitory, studies on the immunoinhibition of invasion were temporarily suspended.

Since the target peptides chosen were unsuccessful in eliciting immunoinhibitory antibodies, it might be asked whether, in the light of newer information, another peptide may be selected. Site-directed mutagenesis studies of His-111 (Hasnain *et al.*, 1992b) have shown the importance of this residue in the endopeptidase activity of cathepsin B. The 18-amino acid, internally disulfide-bonded loop that contains His-111 and that partially occludes the binding cleft of cathepsin B, therefore, appears to be a target region that warrants further study in the search for antibodies capable of immunoinhibiting cathepsin B.

Besides the many considerations which bear on the choice of a peptide sequence, the way in which the synthetic peptide is presented to the immune system can also have a significant influence on the outcome. At the outset of this study, "conventional wisdom" was followed and conjugates of the peptides with KLH were used to inoculate test animals. Largely through personal contacts with Dr Atassi, of Texas, and Dr Sylvianne Muller, of Strassburg, however, workers in this laboratory subsequently became aware of the efficacy, and reported advantages, of inoculation with free peptides.

There seems no doubt that free peptides can be as immunogenic as conjugated peptides, judged by the almost equal response seen in ELISA systems (though admittedly a higher dose of peptide was used in the case of the free peptide). The free peptides, however, must elicit a greater number of conformation-specific antibody

types, in an experimental animal. In the present study, the recognition of whole or SDS-denatured, blotted proteins, treated with an equivalent concentration of antibody elicited with the free or conjugated peptide, gave a much weaker reaction in the case of the free peptide, though judged with an ELISA the response is apparently the same. The conjugation procedure possibly helps to restrain the conformations adopted by the peptide, after inoculation (Van Regenmortel and de Marcillac, 1986).

Conjugation to carrier proteins can cause problems of non-specificity (Briand *et al.*, 1985), and in this study removal of anti-KLH antibodies was problematic. Measurement of the initial immune response to inoculation, where KLH was used as a carrier molecule, revealed that anti-KLH antibodies, in control wells, gave far greater absorbance values than those recorded against the coated free peptide. Even subsequent to repeated KLH affinity purification of IgG fractions, the absorbance values still reflected this trend. This is unsatisfactory, especially if the antibodies are to be used for immunolocation studies, as there is always the possibility that anti-KLH antibodies could cross-react with the target peptide, and/or with unrelated peptides. Anti-KLH antibodies are, in fact, known to give non-specific reactions as they cross-react with a large number of different proteins (Dr D. Buttle, pers. comm.). Such observations led to the discontinued use of KLH as a carrier protein, and extra efforts to ensure the specificity of antisera using western blotting.

Many opinions have been voiced on what constitutes a "specific antibody" (Pool *et al.*, 1983; De Mey, 1986; Van Leeuwen, 1986; Larsson, 1988a). In the light of the experience gained in raising peptide and polyclonal antibodies and using these in immunocytochemical studies, however, the author has come to the view that specificity is best judged by a number of different systems and labelling preferably confirmed by verification of the presence of the target antigen by classical biochemical or histological techniques. If this is not possible, preliminary western blot experiments give a good indication of the specificity of the antibody, but most weight should be given to the results of test and control experiments obtained in the system in which the antibody is to be used.

All antibodies used in the present study were checked for labelling specificity, using western blotting against crude and purified fractions of the target antigen. This was found to be the most reliable method for optimising the antibody dilutions to be used in immunolabelling procedures, for checking for the presence of contaminating antibodies, and for checking for cross-reactivity to antigens present in crude extracts of target tissue proteins. It must be noted that for western blots to yield useful

information, the IgG fraction dilution has to be optimal and an optimal protein loading must be used. For testing cross-reactivity, it is normal to load the gels with 10→100-fold the optimal amount of antigen (Larsson, 1988a). To assess non-specific antibody interactions due to the use of too much blotted protein or the use of too-concentrated an antiserum, it was found advantageous to use only IgG fractions and to use a pre-immune IgG control at the same concentration as that used for the test blot. This gave some confidence in results when it was known that antibodies were being used at considerably higher levels than would normally be used. The antibodies mentioned in this chapter, except anti-B13-22, were judged to be biochemically specific, using the western blotting criterion, and were, therefore, considered to be potentially suitable for use in immunolocation studies, described in Chapters 4 and 5.

3.9.1 Summary

In summary, the following points have emerged from the studies reported in this chapter:-

- i) Choice of peptide sequences for the production of peptide antibodies is best made from 3-D structural data, which allows the selection of exposed regions on the protein surface, most likely to be "seen" by the immune system.
- ii) Predictions of potentially "protein-reactive" sequences, from primary sequence data only, are best made using the Chothia accessibility plots (Chothia, 1976), the Welling antigenicity prediction programme (Welling *et al.*, 1985) or the T-cell epitope predicting programme (Margalit *et al.*, 1987) (and possibly best by combining the results of all three programmes).
- iii) For protein-reactive peptide antibodies the flexibility plot of Karplus and Schultz (1985), indicates that some non-mobility in the chosen peptide sequence is desirable. The Hopp and Woods (1981) plot, indicates that a hydrophobic region in the peptide sequence is useful and this is confirmed by the apparent success of the T-cell epitope selection programme.
- iv) Free peptides are immunogenic, but appear to elicit weakly protein-reactive antibodies. Peptides conjugated with ovalbumin gave the best antibodies.
- v) Antibody specificity may be tested primarily in an overloaded western blot system, using the highest concentration of antibody possible, and controlling the results using a pre-immune IgG, at the same level of antibody as the test. (As a secondary, or alternate, test of specificity, the antibody must give labelling of the organelles known, from biochemical analyses, to contain the antigen. A pre-immune IgG preparation (at the same level used for the test antibody) substituted into the labelling regime, must give a negative result).

Of the peptide antibodies raised, only the anti-procathepsin B (anti-ppB22-36) and anti-B192-201 antibodies were potentially applicable to immunocytochemical studies. No immunoinhibitory antibodies were produced.

CHAPTER 4

LEUCOCYTES AS MODEL SYSTEMS OF INVASIVE CELLS

4.1 Introduction4.1.1 Leucocyte and tumour cell invasion

Many parallels exist between the invasive behaviour and physiological processes of tumour cells and those of polymorphonuclear leucocytes (PMNs), monocytes and macrophages of human peripheral blood. In the process of moving into and out of blood vessels - a key step in the successful establishment of metastases - the tumour cell must traverse interstitial stroma and subendothelial basement membrane. Similarly, during extravasation, the blood phagocytes must traverse the same barriers to reach inflammatory tissue sites. In both cases, this requires adhesion, degradation of the extracellular matrix and locomotion (Terranova *et al.*, 1986; Condeelis *et al.*, 1992).

In the case of the tumour cell, the ability to degrade the extracellular matrix correlates strongly with the tumour's metastatic potential (Liotta and Stetler-Stevenson, 1989). Thus, highly metastatic tumours are capable of releasing a variety of degradative enzymes, such as cathepsin B, plasminogen activator, heparin sulphate endoglycosidase, type IV collagenase and elastase (Yusa *et al.*, 1989). A similar subset of proteinases must be involved in the invasive activities of blood phagocytic cells. The two proteinases most likely to be involved in the invasive activity of PMNs are the dominant neutral proteinases, elastase and cathepsin G. These proteinases have been shown by several previous studies to mediate degradation of basement membrane components (Heck *et al.*, 1990). Elastase has, moreover, been associated with mouse mammary tumour cells (Grant *et al.*, 1990) and other human breast carcinoma cell lines (Kao and Stern, 1986). Elastase is also the only neutral proteinase capable of degrading insoluble elastin, a structural component of elastic tissues such as blood vessels, skin, lung and breast tissues (Grant *et al.*, 1990). In monocytes/macrophages the acidic proteinases, cathepsins B (Ward *et al.*, 1990) or D (Bever *et al.*, 1989), may be most relevant in this context.

Many factors that are chemotactic for metastatic tumour cells (Yusa *et al.*, 1989), monocytes (Senior *et al.*, 1982) and PMNs (Blood and Zetter, 1990), are fragments of extracellular matrix molecules produced by proteolytic activity.

Chemotaxis (movement towards a chemotractant through a concentration gradient) is commonly mediated by binding of the chemotractant to a receptor (Zimmermann *et al.*, 1988) and there is evidence that some receptors, such as that for the chemotactic peptide N-formyl-L-methionyl-leucyl-phenylalanine (fMLP) are common to both PMNs and the cells of some tumours, such as Walker 256 carcinosarcoma (Marasco *et al.*, 1985).

Chemotactic stimulation of PMNs results in a change in the shape and polarisation of the granules towards the direction of movement (Zimmermann *et al.*, 1988). Similarly, chemotactic stimulation of tumour cells (for example, by binding of autocrine motility factor to its receptor) initiates the redistribution of some receptors towards the "leading edge" and the extension of pseudopodia (Nabi *et al.*, 1992). In cancer cells, exocytosis of membraned vesicles at the leading edge has been suggested to supply membrane for extension of the leading edge, as well as generating the force for cell locomotion (Bretscher, 1984). Similar principles may apply in the case of PMNs and monocytes. Moreover, if exocytosis of protease-containing vesicles occurs, the proteases may assist the invasive movement of such cells. Elastase receptors have been biochemically demonstrated to relocate to the surface of activated PMNs (Dwenger *et al.*, 1986).

Diapedesis (or movement through a barrier), on the other hand, appears to involve tumour cell receptor-mediated adhesion and signalling, and motility, all of which appear to be closely linked phenomena (Lester and McCarthy, 1992). Some adhesion receptors, such as that to laminin, which may assist in adhesion to basement membranes, have been identified in neoplastic cells, PMNs and monocytes (Huard *et al.*, 1986; Liotta *et al.*, 1986; Mercurio and Shaw, 1988; Terranova *et al.*, 1983; 1986). The 67 kDa laminin receptor, is the dominant laminin-binding protein in PMNs and macrophages (Huard *et al.*, 1986; Mercurio and Shaw, 1988), and is elevated in PMNs by inflammatory agonists such as PMA and fMLP (Yoon *et al.*, 1987). High levels of this laminin receptor are also expressed in oncogenically transformed cells (Hand *et al.*, 1985; Yow *et al.*, 1988), suggesting that increased adhesion to basement membranes is a characteristic of neoplastic transformation, and, in the case of the activated PMN also, is necessary for invasion.

Invasion, after attachment to the basement membrane, involves proteolytic degradation of the extracellular matrix components and diapedesis. The proteases involved in such proteolytic and invasion processes are still unknown. It is also not known whether the proteinases become attached to or associated with the

surface of the invading cell, as indicated by some biochemical studies (Krepela *et al.*, 1987; Sloane *et al.*, 1990), or whether the cells themselves attach to the basement membrane and form a "pocket", into which proteases are discharged, by intimate association between the basement membrane and the invading cell, as occurs in osteoclasts (Eeckhout, 1990).

Attempts to reveal which protease(s) are involved in these processes have been made, using invasion models and employing chemotactic agents and specific proteinase inhibitors (Taylor *et al.*, 1981; Territo *et al.*, 1984; Furie *et al.*, 1987). In these systems, tumour cells and PMNs are stimulated to invade through a membrane analogous to a basement membrane, and the inhibitory effects of incorporation of specific inhibitors observed. Such investigations suffer from a number of shortcomings, arising from two main sources: the penetration of the proteinase inhibitors to the site of action of the proteolytic process (Furie *et al.*, 1987; Milks *et al.*, 1983), and the fact that, once a proteinase has bound to its target substrate, proteinase inhibitors are largely ineffectual in preventing proteolysis (Johnson and Varani, 1981; Campbell *et al.*, 1982). A final complication in studies on PMNs is that some proteinase inhibitors, such as α_1 -proteinase inhibitor, have been found to be inactivated by oxidants generated by neutrophils (Carp and Janoff, 1980; Weiss and Regani, 1984). A better approach might be to observe directly the invading cells, using immunocytochemistry.

4.1.2 The fixation problem

The main focus of the present immunocytochemical study, was to establish whether cathepsin B, one of the main proteinases found in monocytes (Ward *et al.*, 1990) or elastase and cathepsin G, the major proteinases found in PMNs, could become associated with the surface of activated monocytes or neutrophils, respectively, providing a means by which such chemotactically activated cells may be assisted to invade through barrier membranes, as seen *in vivo* and indirectly indicated by some biochemical studies (Dwenger *et al.*, 1986; Sloane *et al.*, 1990).

The first consideration and the major challenge associated with this study was the choice and optimisation of the fixation procedure. For most immunocytochemical studies, a primary requirement is that fixation should immobilise the target antigen, i) where it occurs in the tissue, ii) in a form in which antigenicity is retained, and iii) in a form accessible to the labelling antibody and other immunolabelling reagents (Tokuyasu, 1986). A secondary requirement is that, for

electron microscopy studies, the fixative should preserve adequate tissue ultrastructure to allow the recognition of the subcellular organelles, while producing minimal structural distortions and fixation artefacts. Further tissue processing or embedding procedures should, similarly, not destroy or mask the antigen.

In the present study, the preservation of antigenicity of the two main target antigens for immunolabelling, cathepsin B and elastase, was of primary importance. As the simultaneous identification of granule populations, using labelling of "marker enzymes", was required, a fixation and embedding regime that would allow the labelling of the maximum number of tissue antigens, in a single human tissue or blood sample, was sought. The unpredictable variation in the sensitivity of antigens, to fixation conditions, however, often necessitates the sacrificing of some ultrastructural detail, for the preservation of tissue antigenicity.

Adequate fixation was consequently accepted, and for most of the studies undertaken, paraformaldehyde (PFA), the least denaturing fixative to a wide variety of antigens, was chosen. PFA has low antigen-denaturing properties, but, at physiological pH, it crosslinks tissue residues slowly and produces poor ultrastructure. For electron microscopy, such a weak fixative could only be used in conjunction with the cryoultramicrotomy system of immunolabelling. In this system, tissues or cells are only subjected to mild post-fixation tissue processing procedures. Tissues are frozen prior to sectioning, instead of being embedded in resins, hence obviating the potentially antigen-denaturing and tissue-damaging steps of resin embedding. The "Tokuyasu cryoultramicrotomy technique" was the main tissue processing and sectioning technique adopted for this study.

At physiological pH, PFA was found to be ineffective in fixing elastase in azurophil granules. Extraction and translation of azurophil granule contents has proved a major problem in many studies, using different fixation regimes (Hibbs and Bainton, 1989; Damiano *et al.*, 1986; Cramer *et al.*, 1985; Robinson, 1985). This problem prompted an ongoing investigation, throughout the present study, of ways in which the efficacy of PFA fixation could be improved. Demands on the fixation regime are even more rigorous when dealing with activated PMNs, where granule membranes are considerably more fragile than in unactivated cells and containment of granule contents is consequently more difficult. Investigations of fixation methods were, therefore, conducted using both chemotactically activated and non-activated cells.

Attempts to do double labelling, after cryoultramicrotomy, led fortuitously to the discovery of the benefits of post-labelling fixation, for the improvement of tissue ultrastructure and antigen immobilisation, and to the discovery of the beneficial effect of further, prelabelling, fixation of the sections (lateral fixation). This also provided a novel method for varying the "effective" PFA fixation of a single specimen.

Whereas labellings for most antigens required mild fixation and the use of cryoultramicrotomy, immunolabelling of cathepsin B proved to have unique requirements. Polyclonal antibodies to human liver cathepsin B only recognise cathepsin B in the denatured form. Cryoultramicrotomy and PFA fixation, therefore, proved to be insufficiently denaturing at physiological pH to promote the recognition of cathepsin B by the antibody. It consequently became necessary to explore different fixation and embedding approaches in the hopes of finding a system suitable for cathepsin B, but which would allow the simultaneous labelling of other antigens of interest.

4.1.3 Activation of leucocytes

Activation studies were designed to test the hypothesis that elastase and cathepsin G, and cathepsin B, may become associated with the surface of activated PMNs and monocytes/macrophages, respectively. Difficulties were encountered in these study, however, due to the loss of PMN and monocyte/macrophage fractions by their adherence to the surface of tubes used in the isolation procedure, and because of the reactive nature of the PMNs and the monocytes themselves. For this reason, different density gradient isolation procedures were used and, to obviate the effects of cell isolation procedures, some studies were also performed on leucocytes *in situ* in splenic tissues.

PMNs are easily activated by substances present in the materials used to form density gradients, and appear to have different fragilities in different isolation procedures (Haslett *et al.*, 1985). "Monocytes" are also easily activated during cell fractionation procedures, either by adherence to surfaces or by exposure to contaminating extracellular matrix proteins (Dougherty and McBride, 1989). In the monocyte, this activation triggers morphological, biochemical and functional changes, as the cell becomes the more phagocytically active derivative, a "macrophage".

Unfortunately, the term "monocyte" is loosely used to describe a very heterogeneous group of cells which, upon activation, become even more difficult to classify on morphological grounds (a fact not fully appreciated at the commencement of this study). Classification of these phagocytes is still a topic of some dispute, but is generally carried out by immunolabelling of surface markers (Dougherty and McBride, 1989; Hogg, 1989). In this study, classification was made on morphological grounds, on the basis of descriptions in recent review texts (Van Furth, 1986; Dougherty and McBride, 1989; Kamperdijk *et al.*, 1989; Strachura, 1989) (Section 4.2), and by labelling for marker enzymes.

Finally, to confirm labelling results obtained in the preliminary cryoultramicrotomy studies on monocyte/macrophage cells, some fluorescent microscopy studies were performed on the U937 promonocytic cell line. These cells were differently treated to induce terminal differentiation into PMNs or monocytoïd cells, respectively, in order to confirm cathepsin D and B labelling results observed in isolated PMNs and monocytes. Labelling for these antigens and the M-6-P receptor were also performed to establish reliable "marker(s)" of differentiation to the monocyte (by their presence), or to the PMN phenotype (by their absence), in the U937 cells. Such a "marker", it was thought, could also possibly aid identification of such cells in peripheral blood for future studies.

4.2 Morphological identification of peripheral blood PMNs and monocyte/macrophages

The identification of cells on morphological grounds only, proved quite a challenge. Not only is the classification of the different monocyte-like cell types the subject of some dissent, but ultrastructural detail, required for the identification of the cell type, was often compromised by the fixation protocol used.

Identification of PMNs and their precursors is facilitated by the segmentation and granularity of the nucleus. In cells that have been sectioned, however, information on the segmentation of the nucleus may not always be available, due to the orientation of the cell upon sectioning. Similarly, nuclei which are not segmented may appear to be so, if the "thickness" of the nucleus varies within the plane of sectioning.

A PMN matures from a promyelocyte, with a single round nucleus, to a myelocyte, and then to a metamyelocyte [or band or stab forms (PS), Plate 1, Fig. 2], the nucleus becoming progressively more eccentric and lobulated. Finally the nucleus becomes polylobulated, the more mature PMN having a greater number of nuclear lobes (normally from 3-5), and a greater amount of cytoplasm, containing progressively fewer granules (Plate 1, Fig. 1). Usually only the metamyelocyte and PMN form are present in the peripheral blood, with occasional myelocytes being found (Hayhoe and Flemans, 1980).

Non-activated PMNs are generally round, with an even distribution of granules. Activated PMNs exhibit polarisation of granules towards the direction of chemotaxis and leave behind a trailing "tail" of cytoplasm (Zimmermann *et al.*, 1988) (Plate 1, Fig. 1).

Monocytes arise in the bone marrow as monoblasts and promonocytes. They remain in the bone marrow very briefly before entering the circulation for up to *ca.* 4 days. They subsequently migrate into the tissues, where differentiation and maturation into macrophages occurs as a result of environmental stimulæ. Monocytes display remarkable heterogeneity in ultrastructure, cytochemistry, and peroxidase activity, the latter being used for classification of developmental stage and activation status (Van Furth, 1986; Kamperdijk *et al.*, 1989; Dougherty and McBride, 1989; Strachura, 1989).

One of the main cell types with which monocytes may be confused are the dendritic cells (DCs), a distinct group of antigen presenting cells which play a role, in the presentation of antigens to resting T lymphocytes, as highly efficient stimulators of primary responses (MacPherson, 1989). DCs show irregular outlines with an eccentric, elongated, often lobulated, nucleus and a nucleus/cytoplasm ratio of 1:2 in peripheral blood (Kamperdijk *et al.*, 1989). The plasma membrane is always irregular, some cells possessing moderately smooth surface lamellipodia while others possess blunt pseudopodia of varying length. (Kamperdijk, 1989). Often the nucleus is reniform with many indentations and sometimes it has a nucleolus. The karyoplasm is euchromatic with condensations at the nucleus edge and the cytoplasm contains mitochondria and some strands of rough and smooth endoplasmic reticulum (ER) (see Plate 1, Figs 3 and 4). DCs often contain vacuoles, especially near the cell surface (Plate 1, Figs 3 and 4), but they are non-phagocytic (Kamperdijk, 1989).

Monocytes represent about 5% of the leucocyte population, and show marked endocytic and phagocytic activity, such activity increasing with differentiation from the monoblast to macrophage forms (Strachura, 1989) (see Plate 1, Figs 1 and 2). Monocytes generally have a horseshoe nucleus and faint azurophilic granules which contain myeloperoxidase (Beelen *et al.*, 1989). Their nucleus has heterochromatin (denser chromatin) towards the margins and the cell has a few lamellipods, subsurface vacuoles and a nucleus/cytoplasm ratio of approximately 1:1. They have a lower content of rough endoplasmic reticulum (RER), coated vesicles and lysosomes than macrophages (compare Plate 10, Figs 1 and 2 with Plate 13, especially Fig. 4).

Monocytes stain for esterase-1 and acid phosphatase throughout the cytoplasm, whereas in DCs acid phosphatase is located in a central area near the nucleus. As monocytes mature, the peroxidase content of their granules decreases, and as they differentiate into macrophages, it disappears altogether (Strachura, 1989). A large increase in cytoplasm occurs upon cell activation and transformation into a tissue-specific macrophage, morphology being thereafter determined by external stimulæ (Beelen *et al.*, 1989). Descriptions of the morphology of *in vitro* activated and differentiated monocytes (macrophages) are sketchy, except for a few descriptions that monocytes increase in size, the cytoplasm to nuclear ratio, membrane ruffling, and the number of cytoplasmic granules and lipid inclusions increasing simultaneously (Dougherty and McBride, 1989).

In the case of macrophages, therefore, identification is not simple. In this study, the greatest difficulty was experienced in differentiating metamyelocytes (immature PMNs) (Plate 1, Fig. 2) from monocytes and fully activated macrophages (Plate 1, Fig. 2 and Fig. 1, respectively). These cells contain numerous morphologically similar granules and similar horseshoe-like nuclei and the only differences seem to be in the type and quantity of specific marker enzymes present, in the size of the cell, and the number of mitochondria present (Plate 1 and Plate 13, Fig. 4). If the fixation procedure does not preserve the morphology of mitochondria, then the usefulness of this distinguishing feature is diminished.

In this study, a source of frustration was that serial sectioning was not possible using cryoultramicrotomy, so identifications could not be confirmed by further labellings of marker enzymes on replicate sections. Also, serial sectioning is required to determine the true size of the cell.

4.3 Methods used in the study of peripheral blood leucocytes

Certain methods which were commonly used in the study of PMNs and monocytes are reported in this section. These include gradient fractionation techniques and cell activation protocols.

4.3.1 Gradient separation of PMNs and monocytes

Various density gradient media and many procedural approaches have been advocated for the separation of leucocyte fractions by differences in density (e.g. Boyum, 1968; English and Andersen, 1974; Segal *et al.*, 1980; Jepsen and Skottun, 1982; Haslett *et al.*, 1985; McFaul, 1990). Cell density differences are small, with densities being typically between 1.05 and 1.10 g/l (Miller, 1986). For this reason, no density gradient procedure can ensure absolute separation of the leucocyte populations. Typically, lymphocytes are most easily separated from granulocyte (PMN and monocyte) fractions. Separation of monocytoïd cells and PMNs was found to be impossible, due to the heterogeneity of cell densities and differences in the morphologies of cells classified as monocytes.

Separation protocols generally fall into two groups, those based on Ficoll, a sucrose polymer preparation (Boyum, 1968) and those based on Percoll, a PVP-coated colloidal silica preparation (Dettman and Wilbur, 1979). Most other media are modifications of the Ficoll medium, which include density enhancing additives, such as sodium diatrizoate (known as "Isopaque" or "Hypaque") in the "Ficoll-Hypaque" discontinuous density gradient system of English and Andersen (1974).

Two requirements of density gradient media are that they are non-toxic to cells and have a high molecular weight. A common problem is that gradient forming materials may form significant tonicity gradients also (Miller, 1986). High relative molecular mass preparations often contain large quantities of salt impurities, so cells moving into media of a higher density, also move into regions of higher salt tonicity. Osmotic forces may cause cell shrinkage, thereby increasing cell density and causing cells to move straight through the gradient, to pellet on the bottom of the tube (Miller, 1986). Ficoll gradients are not isotonic at high concentrations of Ficoll, probably due to interactions of Ficoll with water (Williams *et al.*, 1972). Ficoll also tends to enhance cell aggregation, particularly with increasing cell load (Miller, 1986).

Colloidal silica separation media have the principal advantage that high densities can be achieved with very little increase in tonicity or viscosity, and without inducing cell aggregation (Miller, 1986). Colloidal silica, however, is toxic. This may be overcome by the incorporation of a polymer of relatively high molecular mass, such as PVP (Dettman and Wilbur, 1979). Percoll (Pharmacia) consists of silica particles, coated with PVP and, in this study, it was found to be a superior medium for cell separation.

Density gradient cell separation media have been shown to have various effects on cell functions. Increased expression of C3b receptors by PMNs has been observed (Fearon and Collins, 1983) and modulation of multiple PMN functions by trace concentrations of bacterial lipopolysaccharide, have been reported (Haslett *et al.*, 1985). These authors report that cells isolated by the Ficoll-Hypaque method, showed spontaneous shape change, reduced chemotactic responsiveness, and increased production of lysosomal enzymes upon subsequent stimulation with fMLP, in comparison with other methods. Though many different density gradient systems were tried in the present study, the Percoll density gradient method of Haslett *et al.* (1985) followed by the Ficoll-Hypaque gradient separation method of English and Andersen (1974) were the most successful and are, therefore, the only methods described in detail here.

4.3.1.1 Reagents

Dextran (MW 500 000) and Ficoll-Hypaque (marketed as Histopaque 1119 and Histopaque 1077) were from Sigma, BSA (fraction V powder) was from Boehringer and Percoll was from Pharmacia. Sterile saline [NaCl (9 g) in 1 litre dist.H₂O] was from Baxter Laboratories.

Platelet-poor plasma (PPP). PPP was prepared as described in Section 4.3.1.2, below.

6% Dextran. Dextran (MW 500 000) (6 g) was dissolved in sterile saline and made up to 100 ml.

100% Percoll. A stock solution of 100% Percoll was freshly prepared from the commercially supplied solution by diluting the commercial stock 9 parts to 1 part sterile saline.

42% Percoll. Stock Percoll (4.2 ml) was freshly diluted with platelet-poor plasma (5.8 ml).

51% Percoll. Stock Percoll (5.1 ml) was freshly diluted with platelet-poor plasma (4.9 ml).

PBS, pH 7.2. PBS was made up as described in Section 2.3.1.1

2% Bovine serum albumin in PBS, pH 7.2 (PBS-BSA). BSA (2 g) was dissolved in PBS, pH 7.2, and made up to 100 ml.

1% BSA in sterile saline. BSA (1 g) was dissolved in sterile saline and made up to 100 ml.

4.3.1.2 Procedure

PMNs were separated from 10 ml of blood drawn from healthy volunteers into lithium-heparin glass Vacutainers[®], and rapidly transferred to sterile polystyrene tubes (Greiner, Frickenhausen, Germany). Cells were separated using the Percoll gradient method of Haslett *et al.* (1985) or the Ficoll-Hypaque method of English and Andersen (1974).

Following the method of Haslett *et al.* (1985), blood was centrifuged (300 x g, 20 min, 20°C), the platelet-rich plasma supernatant harvested and centrifuged (2 500 x g, 15 min, 20°C) to produce platelet-poor plasma (PPP). 6% Dextran (1 ml) was added to the cell pellet, the volume was made up to 15 ml with sterile saline, mixed, and left for 30 min for erythrocyte sedimentation to occur. The leucocyte-rich supernatant was aspirated and centrifuged (275 x g, 15 min, 20°C). The pellet was resuspended in 2 to 3 ml of PPP and transferred to a 15 ml polystyrene tube, where it was underlayered with 2 ml of freshly prepared 42% Percoll, which, in turn, was underlayered with 51% Percoll and centrifuged (275 x g, 10 min, 20°C).

Lymphocytes, some monocytes and platelets were harvested from the top layer of the gradient (between the plasma layer and the 42% fraction) while PMNs and some monocytes were harvested from the lower wide band between the 42% and 51% Percoll layers. Harvested cells were mostly contaminated with erythrocytes. Attempts to denature these, by osmotic shock, were made by adding

dist.H₂O into the fraction (2 min), after which cells were washed once (400 x g, 10 min, 20°C) with PBS-BSA, before activation and/or fixation.

In the English and Andersen (1974) method, blood was centrifuged (275 x g, 5 min, 20°C), the leucocyte-rich fraction harvested and diluted 50% with sterile saline containing 1% BSA. A discontinuous gradient was created by loading Histopaque 1119 (3 ml) on top of Histopaque 1077 (3 ml), in a 15 ml conical polystyrene centrifuge tube, and the leucocyte-rich fraction (2 ml) was carefully layered on top of the discontinuous gradient. Harvested cells were washed once (400 x g, 10 min, 20°C) with PBS-BSA, before activation and/or fixation.

4.3.1.3 Results

PMNs, especially, showed many more osmotic effects and spontaneous shape changes, when separated on a Ficoll-Hypaque density gradient (Plate 1, Fig. 1), compared to Percoll (Plate 1, Fig. 2). They were also more fragile to subsequent processing procedures; some showed evidence of activation, and fractions were generally more contaminated with lymphocytes and platelets (Plate 1, Fig. 1) than fractions separated on Percoll gradients. Ficoll-Hypaque density gradient separated fractions also appeared to contain a greater number of activated monocytes (macrophages) and dendritic-like cells (Plate 1, Fig. 1).

Percoll fractions appeared to show the greatest enrichment of PMNs, relative to other leucocyte fractions. The actual concentration of leucocytes finally isolated, however, was always relatively low, as PMN/monocyte fractions were always diluted by the large number of erythrocytes that survived the osmotic shock treatment. At best, a large number of erythrocyte "ghosts" or membrane fragments are evident in Percoll cell fractions (Plate 1, Fig. 2). Platelets contaminated all cell fractions (Plate 1, Figs 1 and 2).

4.3.2 Leucocyte activation

Formylated peptides, characteristic of prokaryote proteins, are extremely potent chemotactic agents for PMNs (Schiffman *et al.*, 1975) and monocytes (Estreicher *et al.*, 1990). In both cell types, chemotactic activity is stimulated by a cascade of messenger molecules, triggered by binding of the formylated peptide to the specific receptor on the surface of the cell. Receptor occupancy triggers the rapid exchange of GDP by GTP on a pertussis toxin - sensitive G (G α) protein, resulting in activation of

the G protein. The G protein activates phospholipase - C, leading to the formation of inositol-1,4,5 triphosphate and diacyl glycerol (DAG), which triggers the release of calcium (Verghese and Snyderman, 1989). Further cell signalling events result in increased actin polymerisation, changes in cellular morphology and chemotactic movement, though the mechanism for these effects is still not known (Niggli and Keller, 1993). Phorbol esters, such as PMA, however, activate protein kinase-C and mimic the effect of endogenously generated DAG (Verghese and Snyderman, 1989).

4.3.2.1 Reagents

PMA, fMLP and trypan blue were from Sigma.

2% BSA in phosphate-buffered saline (PBS-BSA). PBS-BSA was made up as described in Section 4.3.1.1.

PMA stock (0.5 mg/ml or 1.23 mM). PMA (1 mg) was dissolved in DMSO (100 μ l) and made up to 2.0 ml with dist.H₂O. The solution was stored at -20°C.

fMLP stock (0.625 mg/ml or 1.43 mM). fMLP (1 mg) was dissolved in DMSO (100 μ l) and 1.5 ml of dist.H₂O added. The solution was stored at -20°C.

Dilute fMLP (5 μ g/ml). Stock fMLP solution (10 μ l) was made up with 1.5 ml with PBS-BSA.

4.3.2.2 Procedure

Cells (2.4×10^6 /ml) were activated by the introduction of fMLP (30 μ M) or PMA (25 or 40 μ M) into the medium (30-45 min, 37°C), before being washed twice (400 x g, 5 min) in PBS-BSA. Trypan blue exclusion tests indicated a cell viability of 90-95%.

4.4 Fixation for studies on PMNs

Extraction and translocation of azurophil granule contents is reported to be a common problem in immunolabelling of PMNs (Hibbs and Bainton, 1989; Damiano *et al.*, 1986; Cramer *et al.*, 1985; Ganz *et al.*, 1985). It is important, however, for the correct classification of granules, on the basis of their complement of antigens, that all of the antigens remain in place, during immunolabelling. This is also necessary for studies on activated PMNs, aimed at identification of surface-bound proteases, and at defining their origin.

Some antigens occur at very low levels, in some granule populations, so it is also important that fixation procedures preserve antigenicity at the highest level possible. For this reason, PFA, the least denaturing fixative to a wide variety of antigens (Larsson, 1988b; Seligman *et al.*, 1950), was chosen for the present study and strategies to improve PFA fixation were employed.

The pH (Berod *et al.*, 1981; Eldred *et al.*, 1983; Bourgnon and Charlton, 1987; Larsson, 1988b), and the concentration of the PFA fixative (Larsson, 1988b), has been shown to affect the fixation of various tissue antigens differently. In this study, the main problem was to fix the contents of PMN azurophil granules and, to do this, it is necessary for the fixative to be able to penetrate the walls of these large granules. At a low concentration, PFA exists in a less polymerised form which will be more penetrating. Having penetrated, however, a subsequent increase in concentration would cause PFA to polymerise to a form more effective in crosslinking (Walker, 1964). Moreover, PFA mainly reacts with uncharged amino groups (Kallen and Jencks, 1966) and these become more numerous as the pH rises. These considerations were the basis of the "low-high" step approaches used in this study, as variations in pH and concentration were employed in PFA fixation regimes.

If the initial fixation is too effective, however, it might over-fix the granule walls, preventing further penetration of PFA into the granule interior - a "nutshell" effect. A possible way of overcoming this, which was developed in the course of this study, was to float the newly-cut section onto fixative, prior to labelling, so that the fixative could gain access to the granule contents, laterally, through the cut surface, without having to cross the granule wall.

Alternatively, an azurophil granule-stabilising technique, previously described by Hibbs and Bainton (1989) was employed, in some cases. The present study was

conducted mainly on non-activated PMNs. Results of such fixation studies will be described in this section and the results of activation studies will be described mainly in Section 4.5.

4.4.1 Reagents

Rabbit anti-human cathepsin D was prepared in this laboratory by Philip Fortgens and anti-human leucocyte elastase (HLE) IgG was from Athens Research and Technology, Athens, Ga. USA (see Section 3.8). Rabbit anti-human lactoferrin IgG, globulin-free bovine serum albumin and diaminobenzidine were from Sigma Chemical Co., St. Louis, Mo. USA. Rabbit anti-human myeloperoxidase IgG was from Dakopatts, Glostrup, Denmark. Foetal calf serum was from Gibco, Paisley, Scotland. Protein A gold labels were produced according to the tannic acid method of Slot and Geuze (1985), as described in Section 2.3.6, unless otherwise stated. Antibodies were diluted in 1% BSA and gold labels were diluted in 0.1% globulin-free bovine serum albumin (Section 2.3.7.1) or 5% foetal calf serum in PBS (Section 2.3.3.1), and other tissue "blocking" reagents were made as described in Section 2.3.3.1.

0.1M Tris buffered saline (TBS), pH 7.6. Tris base (12.1g) and sodium chloride (9 g) were dissolved in approx. 900 ml dist.H₂O, titrated to pH 8.0 with HCl and made up to 1 litre.

Diaminobenzidine (DAB) solution. DAB (0.025 mg) was dissolved in 0.1M Tris buffer, pH 7.6, and 30% H₂O₂ (1 µl) was added, immediately before use.

4.4.2 Procedure

PMNs were separated from blood drawn from healthy volunteers and fractionated by density gradient centrifugation, as described in Section 4.3.1.2. Cell fractions were activated (30 min, 37°C) as described in Section 4.3.2.2 and fixed with either a "single" or "double" procedure. In the "single" fixation protocol, cell pellets were fixed for 15 min, at room temperature, in either 4% or 8% PFA in 200 mM HEPES buffer, pH 7.2 or pH 8.0 (Section 2.3.2.1). In the "double" fixation protocol, cells were fixed for 5 min in 4% PFA and 10 min in 8% PFA in 200 mM HEPES buffer, at pH 7.2 or pH 8.0.

In some preparations, after fixation with 4% PFA followed by 8% PFA (both in sodium cacodylate buffer, pH 7.2), the generation of a myeloperoxidase

reaction product precipitate (oxidised diaminobenzidine) in azurophil granules, was used to stabilise granule contents. In this case, the cells were incubated in DAB solution, for 20 min at room temperature (Graham and Karnovsky, 1966), washed twice in PBS, cryoprotected, and sectioned as below.

Alternatively, after initial fixation, the pelleted cells were simply cryoprotected, frozen and sectioned as described in Section 2.4.3.2. Sections were retrieved on a drop of 2.3 M sucrose, placed on a 100 mesh hexagonal, formvar- and carbon-coated grid and floated out onto chilled PBS, or fixative, for 30 min.

Sections were first blocked by incubation in 0.02 M glycine (10 min), followed by 1% BSA or 5% foetal calf serum in PBS (15-30 min). Incubation (1 h) in either rabbit anti-HLE (0.88 μg in 10 μl), rabbit anti-human lactoferrin (0.36 μg in 10 μl), rabbit anti-human myeloperoxidase (0.5 μg in 10 μl) or rabbit anti-cathepsin D antiserum (1/40) was followed by washing in several changes of PBS (20 min in total). After incubation on 5 or 10 nm protein A-gold probe (10 μl , A_{520} 0.1 – 0.5), grids were again washed in several changes of PBS (20 min in total). All labellings were performed either without or with post-labelling fixation, with 1% glutaraldehyde in PBS (5 min) (Tokuyasu, 1986). Double labelling was performed, using the method of Slot *et al.* (1991), in which the sections are treated with 1% glutaraldehyde in PBS (5 min) and blocked with 0.02 M glycine (15 min) and 1% BSA (5 min), between the two labelling steps. Consistency of labelling with both sizes of gold probe was tested by performing labelling for the different antigens in different orders, using both sizes of gold probe (always using the smaller gold probe first). Finally, all grids were washed in distilled water, contrasted in acidic uranyl acetate/methylcellulose as described in Section 2.4.3 and viewed in a Jeol 100CX transmission electron microscope at 80 kV.

Controls included substituting pre-immune IgG or serum for antibodies used in the labelling scheme, omitting the primary antibody, and, where relevant, omitting the anti-primary antibody.

4.4.3 Results

In assessing different fixation regimes, their success in meeting the following criteria must be considered: i) the preservation of antigenicity, ii) the preservation of ultrastructure, and, iii) the immobilisation of the antigen.

Effect of paraformaldehyde concentration on antigenicity and ultrastructure. PFA was used at two concentrations, 4% and 8%, and at two pH values, pH 7.2 and 8.0, in various combinations. For clarity, the two values will be referred to as "low" and "high", respectively, in each case. For example, "low PFA" means 4% PFA.

Most of the PFA fixation protocols used yielded high density immunolabelling, for all antigens tested. Exceptions occurred where extensive antigen extraction occurred (Plate 2, Fig. 1), or in double-labelling where glutaraldehyde was used between labelling steps, lowering the labelling density of the second antigen (compare labelling of lactoferrin, Plate 3, Fig. C insert, with that of Fig. B, same plate). No obvious differences in labelling densities were evident after flotation of sections on fixative, prior to labelling (compare Plate 2, Fig. 5, and Plate 3 Fig. C).

Double fixation, at low pH, with low PFA, followed by high PFA, (Plate 2, Figs 2, 3a and 3 b), gave slightly better antigen immobilization and improved ultrastructure, compared to that given by the individual fixatives (e.g. Plate 2, Fig. 1), particularly when sections were floated on high PFA, at low pH, after sectioning, and also fixed after labelling (Plate 2, Fig. 3 a and b). Elastase was not totally immobilised by fixation at pH 7.2, however, even when this was assisted by both pre- and post-labelling fixation, and some elastase was still translocated across the cell and surrounding support film (Plate 2, Fig. 3 b).

Effect of granule and membrane stabilisation techniques. Granule stabilisation, with peroxidase reaction products (Hibbs and Bainton, 1989), did not result in the complete immobilisation of the elastase antigen, even if fixation was extended for as long as long as 1 h (Plate 2, Fig. 4), though the azurophil granule appears to be largely stabilised.

Flotation of sections on fixative prior to immunolabelling. Improved results were obtained with sections floated on high PFA, prior to immunolabelling. Cells (fixed, at low pH, with low PFA, followed by high PFA) collected on high PFA (at low pH), and fixed post immunolabelling (Plate 2, Fig. 3a and b), had better ultrastructure, granule preservation and antigen immobilisation than those only post-fixed after immunolabelling (Plate 2, Fig. 2). However, the complete immobilisation of elastase was not achieved by any PFA fixation regime at pH 7.2 only. Cells [fixed with low PFA, at high pH, followed by high PFA, at low pH] collected on high PFA, at

high pH, showed better immobilisation of elastase, but possibly some distortion of the azurophil granules (Plate 3, Fig. C, arrowheads).

The effect of paraformaldehyde concentration and pH. Brief fixation with low PFA, at high pH, followed by high PFA, at low pH (Plate 3, Figs A, B and C), gave the best combination of azurophil granule preservation and antigen immobilisation. With collection of sections on high PFA, at high pH, prior to labelling, the elastase appeared largely contained within granules (Plate 3, Fig. C), though some of the larger azurophil granules appear to be distorted and swollen. It was initially thought that vacuolation of the cytoplasm, especially of more mature PMNs, may have been exaggerated by this flotation regime (e.g. Plate 3, Fig. C). Immobilisation of elastase was better than with low PFA followed by high PFA, at low pH (Plate 2, Fig. 3 b), and azurophil granule structure preservation appeared better than with high PFA, alone, at high pH (Plate 2, Fig. 5).

At first it seemed that fixation with high PFA at high pH only, appeared to rupture and extract azurophil granules to some extent, especially in older cells, resulting in "organelles" which label for azurophil antigens, but which are not electron dense (Plate 2, Fig. 5). (Though Plate 2, Fig. 5, illustrates a PMA-activated PMN, similar results were obtained in some "unactivated cells"). Subsequently, it seemed these "organelles" may rather be due to inadvertent activation of the PMNs, as these electron-translucent bodies seem to be characteristic of activated PMNs (see Section 4.5).

Less translation of the elastase antigen was seen in cells fixed with low PFA, high pH, followed by high PFA at low pH and floated on high pH PFA, high concentration fixative prior to labelling, than with fixation with high PFA at high pH only (compare Plate 3, Fig. C and Plate 2, Fig. 5).

4.4.4 Discussion

Elastase proved to be the most problematic antigen in PMNs, because it occurs in high concentration and is difficult to contain within granules. Since it was the most difficult antigen in this regard, elastase immobilisation became the primary yardstick whereby different fixation methods were assessed.

The effect of PFA concentration on fixation. The fixation achieved on PMNs, indicates that the concentration of the fixative does influence the quality of fixation. Better stability of fixation was evident when high PFA was used. This effect can best be ascribed to the different species of fixative present at different concentrations. Solid PFA consists largely of polymeric species, partially soluble in alkaline solution, which dissolve by slowly depolymerising to smaller, more soluble polymers in alkaline solutions (G. Griffiths, pers. comm.; Walker, 1964; Larsson, 1988b). Stored unfrozen, depolymerisation of the solute soon reaches equilibrium (Walker, 1964; Larsson, 1988b). Above 1→2% (w/v) PFA, the degree of polymerisation and concentration of polymers at equilibrium increases progressively with increasing PFA concentration (Walker, 1964).

At low concentrations (1-2%), PFA reacts mainly, and most rapidly, with deprotonated primary or secondary amines to form hydroxy-methylene bridges (Kallen and Jencks, 1966). At higher concentrations, it reacts with both protonated and unprotonated amines (Kallen and Jencks, 1966), producing a more extensive cross-linking, at physiological pH.

In the present study, more extensive crosslinking may have been facilitated by the increased length of PFA polymers, at high PFA, resulting in crosslinking of reactive residues some distance apart. Coupled with greater reactivity of PFA at high concentration (with both protonated and deprotonated amino groups), this should lead to better preservation of cellular ultrastructure. Fixation with high concentrations of PFA alone, especially at physiological pH, however, appears not to be adequate to prevent the translocation of azurophil antigens, such as elastase.

High PFA may cross-link efficiently, but may not penetrate as well as lower PFA. In the present study, it was found that fixation with low PFA, to ensure rapid penetration of fixative, followed by high PFA, to ensure adequate tissue cross-linkage, gave better containment of elastase and better ultrastructural preservation than any single concentration of fixative. Fixation with any PFA regime, at pH 7.2, however, failed to completely immobilise elastase, or to stabilise azurophil granules, possibly indicating that fixation is too slow and perhaps reversible at this pH.

Effect of pH on fixation. PFA reacts primarily with lysine amino groups and, according to Kallen and Jencks (1966), its affinity for protonated amines is two to three orders of magnitude less than for the non-protonated amine. At pH 7.2, only a small proportion of amino groups would be in the reactive, deprotonated form, so

fixation would be less effective at this pH. Both proteins, and lipids with primary amino groups, should, therefore, be more rapidly and adequately fixed at higher pH. In the present study, the rate and extent of fixation was found to be markedly affected by pH; high pH giving the best antigen immobilisation and, in some cases, the best lipid fixation (see macrophage Plate 13).

The pH not only affects the rate of reaction of PFA with primary amines, but the size of the PFA polymer is also pH dependent. An increase in pH, above pH 4.3, results in a progressive depolymerisation of PFA (Kallen and Jencks, 1966), favouring the formation of smaller, more penetrating, species of PFA. Variations in pH may thus also produce polymers of different sizes which may be more or less suited to bridge tissue reactive groups distributed at a fixed distance apart. Differences in the composition and spatial distribution of such reactive groups may explain the different pH optima, for fixation, reported for different tissues (Berod *et al.*, 1981; Bourgnon and Charlton, 1987; Larsson, 1988b).

Inadequate penetration of fixative to internal structures in a cell, has been reported to be a problem when an attempt is made to improve fixation by increasing the pH (Berod *et al.*, 1981; Eldred *et al.* 1983); the rapid, efficient fixation of outer membranes may impede subsequent fixative penetration. In such cases, the "pH shift" method of Berod *et al.* (1981) and Eldred *et al.* (1983) may be advantageous. In this method, fixation is effected with 4% PFA, to allow rapid penetration, at a relatively low pH (pH 6.5 or pH 7.2), followed by fixation with 4% PFA at pH 11.0 or pH 10.4, the high pH facilitating rapid crosslinkage and immobilisation of the antigen. In the present study, a "reverse pH-shift" strategy, combined with an increase in PFA concentration, was most successful in immobilising azurophil granule antigens. Possibly the low penetration, caused by the increased efficiency of fixation of the azurophil granule membranes, at the higher pH, and hence their impermeability to the fixative, was partially offset by the low concentration of PFA, and the decreased size of the polymers present at the higher pH used for initial fixation.

Pre- and post-labelling fixation. The present study has suggested that floating the newly-cut section onto fixative is an effective way of fixing PMN granule contents. Also, post-labelling fixation with glutaraldehyde (Tokuyasu, 1986) has proved generally beneficial in stabilising sections through subsequent steps. Glutaraldehyde fixation not only strengthens the section, by crosslinking tissue components, but may also fix the antibodies and gold labels in position, preventing them from subsequently dissociating and translocating.

In summary, for the immobilisation of the azurophil granule antigen, elastase (and possibly other azurophil granule antigens), in PFA-fixed, unembedded PMNs, the following points have emerged from this study:-

- a) *ca.* 4% PFA, which would contain a low proportion of polymeric forms of PFA, is advantageous for initial infiltration of the cell pellet.
- b) For initial infiltration, short fixation at pH 8.0 is perhaps optimal as the rate of fixation is increased at this pH, while the smaller polymers prevalent at this pH favour rapid tissue penetration. The short fixation time may limit over-fixation of azurophil granule membranes which, otherwise, may prevent further penetration of the fixative.
- c) For secondary infiltration, *ca.* 8% PFA, at pH 7.2, may be used to advantage. The higher concentration and lower pH promotes the formation of larger polymers, and consequently may also promote more effective bridging of distant groups, while the lower pH attenuates the rate of fixation, thereby ensuring that penetration of the fixative into the granules is not blocked.
- d) After sectioning, and before labelling, the section may, with advantage, be collected onto *ca.* 8% PFA at pH 8.0. After sectioning, the fixative has easy lateral access to all parts of the section and so the most effective fixative, which does not destroy antigenicity, may be used without consideration of penetration limitations.
- e) After immunolabelling it is no longer necessary to preserve antigenicity and so 1% glutaraldehyde, at pH 7.2, may be used to stabilise antibody/antigen/immunolabel complexes against the acid uranyl acetate/methyl cellulose contrasting-and-sealing process, and fortify the tissue against the osmotic shock of distilled water washes.

In this study, the approach of immunolabelling a section, lightly fixed with PFA, and subsequently re-fixing the section with strongly-crosslinking glutaraldehyde, facilitated the simultaneous labelling of two PMN azurophil granule marker antigens, myeloperoxidase and elastase, as well as cathepsin D and lactoferrin - in unembedded tissues. Cathepsin D has not been previously immunolocated in PMNs, at the electron microscopy level, though it has been demonstrated biochemically in PMN fractions of density gradient-separated human leucocytes (Ishikawa and Cimasoni, 1977).

4.5 Immunolabelling of activated PMNs and spleen cells

The fixation procedures used were subject to continuous development throughout the investigations on PMNs and splenic tissues. The fixation used for most of the studies on activated PMNs, for which the results are reported here, i.e. 8% PFA in HEPES buffer, pH 8.0, was considered to be optimal, at the time when these studies were done. In the light of subsequent studies on fixation, reported in Section 4.4, it emerges that this may not, in fact, have been an optimal protocol. Nevertheless, the studies reported in this section complement those on fixation, as insight was obtained into the effect of different activating agents on granule morphology.

For example, in the fixation study reported in Section 4.4, it was considered that perhaps the use of a high PFA, high pH, fixation regime, was responsible for the unusual morphology of some azurophil granules. This may equally have been the result of inadvertent partial activation of the PMNs used in the fixation study. In these "unactivated" cells, besides the known primary and secondary ("specific") granules, a third type of elastase-containing compartment was evident. This is a swollen, electron-translucent compartment, which, as will become evident, is especially prevalent in PMA and fMLP-activated cells, and may have a secretory function.

The view that this compartment may be a consequence of activation, rather than an artefact of fixation, is supported by the observed morphology of unactivated splenic PMNs in tissues fixed at an even more alkaline pH (8% PFA, pH 9.0), where there was no evidence of such compartments (Section 4.5.2). Also, the similarity of the compartment in "unactivated" PMNs and in PMNs activated with two different agents, seems to support such reasoning.

An alkaline phosphatase-positive compartment, distinct from the azurophil and specific granules, which forms large irregular tubular structures, which increase in size upon cell activation, has been described previously (Kobayashi and Robinson, 1991). From the results of the studies reported here, it seems that similar structures, manifest as electron-translucent compartments, may be formed by elastase-containing azurophil granules, upon activation of PMNs.

In the second part of this section, the results of studies of spleen tissue/phagocyte interactions are presented. PMN azurophil granules contain large amounts of elastase (1.59×10^{-15} g/granule) (Damiano *et al.*, 1986), which, if it should be released

upon activation of the cells, would be potentially damaging to host tissues. Immunolabelling results on splenic tissues provided insights into a mechanism by which endothelial cell damage, by elastase released from activated PMNs, may be minimised. On the basis of these results, it is proposed that a possible secondary function of splenic endothelial stave cells is the removal of tissue-damaging proteinases from the splenic chord spaces.

4.5.1 Elastase and cathepsin G in activated peripheral blood PMNs

Although alkaline phosphatase has been found to associate with the surface of fMLP- and PMA-activated PMNs (Kobayashi and Robinson, 1991), elastase and cathepsin G have not previously been demonstrated on the surface of activated PMNs. The association of these potent proteinases with the "leading edge" of the cell, could, however, be relevant in assisting invasive cell movement. Differences in cell shape and in the surface distribution of elastase and cathepsin G were, therefore, examined using both fluorescent labelling, and immunogold labelling of cryoultramicrotomy sections, of PMNs activated *in vitro* with PMA and fMLP.

4.5.1.1 Reagents

Sheep-anti-cathepsin G was from Serotec and rabbit-anti-elastase was from Athens Research and Technology, Athens, Ga. USA (see Section 3.8). Goat anti-rabbit-FITC conjugate, the rabbit anti-sheep-FITC conjugate, and poly-L-lysine were from Sigma Chemicals, St Louis, Mo. USA. Polyclonal rabbit anti-sheep IgG was prepared in this laboratory by Dr Theresa Coetzer. A 3 nm protein A-gold probe was prepared as described in Section 2.3.6. 5% Foetal calf serum (FCS), 1% bovine serum albumin and 0.02M glycine in PBS were made up as described in Section 2.3.3.1.

0.05% Poly-L-lysine. Poly-L-lysine (0.025 g) was dissolved in PBS (50 ml), stored frozen and thawed for use.

Glycerol-phosphate buffered saline mounting medium. Glycerol-PBS mounting medium was freshly prepared by diluting 9 parts of glycerol (AR) with 1 part of PBS, pH 7.2.

4.5.1.2 Procedure

Human PMNs were isolated from heparinised peripheral blood, using Percoll or Ficoll-Hypaque density gradients, and prepared as described in Section 4.3.1.2.

Part of the cell fraction was spread on slides coated with 0.05% polylysine, fixed with 8% PFA, pH 8.0, (5 min) followed by 8% PFA, pH 7.3, (15 min) in HEPES buffer, and labelled with fluorescent conjugate (as described below). The other part of the fraction was similarly fixed and processed for cryoultramicrotomy (Section 2.4.3) and immunolabelled (as described in Section 4.4.2) for elastase (0.88 μg in 10 μl), and cathepsin G (antiserum diluted 1 in 80).

For immunofluorescence studies on proteases associated with the external surface of cells, non-permeabilised cells were used. Residual aldehyde reactive groups on slide preparations were quenched with 0.02 M glycine (15 min) and 5% FCS (15 min), and each slide was incubated in rabbit anti-elastase (22 μg in 250 μl , 1 h) or sheep anti-cathepsin G serum (diluted 1 in 40, 250 μl), washed in PBS (20 min) and the goat anti-rabbit-FITC conjugate (diluted 1 to 15), or rabbit anti-sheep-FITC conjugate (diluted 1 to 20) was applied (250 μl , 1 h). Slides were washed in PBS (20 min) and the labelled preparation was covered with a coverslip, mounted with glycerol mounting medium and viewed using an Olympus-BHT microscope fitted with an epifluorescence attachment and a 490 nm exciter and a 520 nm barrier filter.

For control labellings the primary antibody was omitted or a pre-immune IgG preparation substituted.

4.5.1.3 Results

Since immunolabelling for elastase and cathepsin G gave similar results, for purposes of illustration, only the results of one or the other will be shown.

Unstimulated cells are rounded, and showed little or no elastase or cathepsin G association with the cell surface (results not shown, but see Plate 3, Fig. C). In fMLP- and PMA-activated PMNs, however, a surface distribution of elastase and cathepsin G is evident in both immunofluorescence and I.E.M. labelling; a polarised distribution occurring in fMLP-activated PMNs (Plate 4, Figs 1 and 2 and Plate 5).

Electron microscopy immunolabelling, of fMLP-activated PMNs, for elastase and cathepsin G, reveals a polarisation of elastase- and cathepsin G-containing azurophil granules towards the "leading edge", and concomitant association of elastase and cathepsin G with the diffuse cell surface (Plate 5, Fig. 1, large arrows indicate leading edge and elastase association with cell surface). Some elastase and cathepsin G is also associated with the equally diffuse, ruffled surface (Plate 5, Figs 1 and 2, arrowheads), but not with the "trailing edge" of the cell (Plate 5, Fig. 2, arrows). Some degranulation is evident (Plate 5, Fig. 1, small arrows), but no pinocytosis or phagocytosis. A few electron-translucent, elastase and cathepsin G-containing vesicles are evident.

PMA stimulation has been reported to result in surface ruffling, increased pinocytosis and decreased locomotion (Zimmermann *et al.*, 1988). The present study, however, suggests that these effects are dependent on the degree of activation. Low dose stimulation (25 μ M) results in the formation of a trailing edge "tail" on one side of the cell (Plate 6, large fat arrows), and some polarisation of granule distribution towards the "leading edge" (Plate 6, thin arrows), giving rise to a cell shape which resembles that of fMLP-activated cells. Marked pinocytosis is, however, evident (Plate 6, P = pinocytotic vesicles). A small amount of elastase and cathepsin G is associated with the "leading edge", which is smoother than that of fMLP-stimulated cells (Plate 6 and Plate 5, respectively). Fewer elastase and cathepsin G-containing electron-translucent granules appear near the "leading edge" surface of the PMN activated with the low level of PMA, than are present in fMLP-activated cells. More, and larger, electron-translucent, elastase and cathepsin G-containing compartments appear within the body of PMA-activated PMNs, than occur in fMLP-activated cells.

Immunofluorescent labelling indicates that activation with 40 μ M PMA gives rise to a uniform distribution of both elastase and cathepsin G over the external surface of non-permeabilised neutrophils (Plate 4, Fig. 2). Electron microscopy examination reveals that these cells are rounded, with few or no surface projections (Plate 7, Fig. 2). Degranulation appears to occur by two mechanisms, one by which the entire granule is lost, or its contents are expelled (Plate 7, Fig. 1, large arrow), and one by an exocytosis mechanism, in which the granule membrane appears to integrate into the external surface of the cell, giving a "cup-shaped" structure to which elastase and cathepsin G adhere (Plate 7, Fig. 4, short arrows). This exocytosis occurs on one side of the cell (Plate 7, Fig. 2, arrows). On the opposite surface the

membrane becomes diffuse and both enzymes are associated with the diffuse surface (Plate 7, Fig. 3, arrows).

4.5.1.4 Discussion

Chemotactic peptides activate PMNs via a receptor-mediated, protein kinase C-independent mechanism, while phorbol esters (PMA) or diacylglycerols (DAGs) activate protein kinase C. In either case, however, both elastase and cathepsin G appear to become associated with the surface of activated cells to a greater or lesser extent.

The fMLP-induced changes in cell morphology and apparent function, observed in this study, are similar to those reported for other chemotactic peptides (Zimmermann *et al.*, 1988). Differences in the responses to various levels of PMA were, however, observed here. These responses appear to resemble those described for activation with DAGs (Zimmermann *et al.*, 1988). Locomotion appears to be induced by activation with low levels of PMA, though this is a low level response compared to that seen with chemotactic peptide activation. Compared to activation with fMLP, motility, as evidenced by the shape of the cell, seems to be replaced by endocytotic activity. Similarly, association of proteinases with the cell surface occurs to a lesser extent.

Judging by the shape of the cell, low level (25 μ M) PMA activation, seems to induce phagocytosis and the formation of large, electron-translucent, compartments in the body of the cell. These organelles may possibly represent tubular structures, like the recently-described alkaline phosphatase-containing organelles (Kobayashi and Robinson, 1991). They could only be discerned in thick sections of PMNs, however, so sections viewed here may be too thin to reveal the precise 3-D nature of these organelles. Upon PMN activation (with 50 μ M PMA), the alkaline phosphatase-containing compartment is reported to become distended, forming tubules of increasing length, and to empty its enzyme content out onto the surface of the cell. At a lower concentration of PMA (25 μ M) perhaps only swollen organelles similar to those seen in the present study would be seen. These swollen organelles may form as a result of granule fusion. Double labelling studies, additionally labelling for other marker enzymes for other granule compartments (for which the labelling has been optimised, as described in Section 4.4 above), will be useful for future definition of the origin of these organelles.

Activation with high levels of PMA (40 μM) resulted in the association of large amounts of both cathepsin G and elastase with the cell surface, the induction of the swollen organelles and increased exocytosis, as compared to other activation protocols. A similar response was reported during activation of PMNs with 50 μM PMA, in investigations into alkaline phosphatase-containing organelles (Kobayashi and Robinson, 1991). The surface-bound proteinase response and exocytosis of proteinases is the response seen during overwhelming (suicidal) activation of PMNs in close proximity to an infection site.

Low level fMLP activation, however, appears to give rise to cell movement, which is possibly accompanied by "leading-edge" exocytosis and the association of both enzymes with the "leading-edge" of the chemotactically stimulated cells. This apparent phenotype could be most relevant in PMNs activated by a bacterial peptide, in moving them towards the site of infection. The results suggest that both elastase and cathepsin G could be relevant in assisting this "invasive" type movement of activated PMNs. Results, more like a true chemotactic response, may be obtained if activation was induced by a gradient of chemotractant and not by the introduction of the chemotractant directly into the medium. Such experiments remain an option for the future.

Perhaps the most significant results emanating from the present study of activation, may be the description of the apparently novel, electron-translucent, intracellular compartment with elastase and cathepsin G secretory properties, that appears to be formed in activated PMNs, and the apparent association of elastase and cathepsin G with the surface of activated PMNs.

4.5.2 Elastase and cathepsin D in splenic PMNs and stave cells

Human leucocyte elastase (HLE) has been implicated in a number of inflammatory pathologies, of which pulmonary emphysema has been recognised as possibly the most important (Reilly and Travis, 1980; Barrett, 1981). HLE has a wide variety of substrates with elastin, the integral component of the connective tissue framework of the lung, being one of the most actively degraded (Barrett, 1981). HLE degrades elastin at a rate 78% faster than does cathepsin G, a second elastinolytic enzyme found in the same azurophil granules (Reilly and Travis, 1980).

The source of HLE is the activated PMN, which has been identified as the main source of all the elastolytic enzymes contributing to pathological lung damage (Reilly and Travis, 1980). The lung pathologies of adult respiratory distress syndrome, cystic fibrosis and emphysema are all related to the accumulation of PMNs in the lung tissues and the release of HLE from these PMNs (Reilly and Travis, 1980; Jonas *et al.*, 1991).

Barrett (1981) reports that HLE is not found in any other type of human cell besides the PMN, but the amounts of this enzyme found in the spleen are surprisingly high, in view of the relative scarcity of PMNs in splenic tissues. The fact that the amount of HLE was high was considered an advantage, in the initial studies of PMN fixation and optimisation of PMN immunolabelling protocols, in which splenic tissue was used to obviate the effect of the density gradient separation protocols on PMN physiology. In turn, these early studies led to a study of possible interactions between activated PMNs and splenic endothelial cells and of the distribution of HLE in other splenic tissues and cells. The results, reported below, embody several novel aspects, including observations on the structure and possible function of endothelial cells, on the interaction of activated PMNs and endothelial cells, and on the colocalisation of HLE and cathepsin D in activated PMN azurophil granules. Moreover, in demonstrating the *in vivo* association of HLE with the surface of activated PMNs, the study substantiates the results of the *in vitro* activation study reported in Section 4.5.1.3.

4.5.2.1 Reagents

Most of the reagents used were as previously described in Sections 2.4.3 and 4.4.1. A membrane-stabilising staining procedure, using neutral uranyl acetate, was additionally incorporated into the procedure (Tokuyasu, 1986). The staining reagents are described below.

Stock 4% uranyl acetate (UA). Uranyl acetate (4 g) was dissolved in dist.H₂O, made up to 100 ml and stored in the dark at 10°C, for up to 2 months.

0.3 M potassium oxalate. Potassium oxalate (5.53 g) was dissolved in 75 ml of dist. H₂O, adjusted to between pH 7 and pH 7.4, with 10% NH₄OH, and made up to 100 ml.

2% Neutral uranyl acetate. A 2% solution of neutral uranyl acetate was freshly prepared by mixing equal volumes of 4% uranyl acetate and 0.3 M potassium oxalate, immediately before use.

4.5.2.2 Procedure

Samples of human spleen tissue (2 x 2 mm), from a 10 year old female, subjected to a therapeutic splenectomy for portal hypertension, were fixed in 8% PFA in 200 mM borate buffer, pH 9.0, (20 min, RT), cryoprotected with 2.1 M sucrose, mounted on a copper stub and frozen in liquid nitrogen as previously described (Section 2.4.3). Sections were cut, using a cryoultramicrotome, and double immunolabelled for elastase and cathepsin D, using 3 nm and 10 nm protein A gold probes as described in Section 4.4.2. After labelling, sections were contrasted by the "positive-negative" contrasting procedure in which sections are rinsed briefly in dist.H₂O, stained in neutral uranyl acetate (10 min), and further contrast stained as described in Section 2.4.3.

4.5.2.3 Results

The fixation regime chosen for the fixation of splenic tissues seemed adequate, for the preservation of both tissue ultrastructure and tissue antigenicity. Labelling for elastase and cathepsin D was facilitated by this fixation protocol.

Sections, cut across the axis of a splenic sinus of red pulp (Plate 8, Figs 1 and 2), show the primary function of the splenic endothelial stave cells in separating the splenic sinus (S) from the cordal spaces (C) of the spleen (see especially Plate 8, Fig. 2) (Chen and Weiss, 1972). The red pulp cords are supported by a delicate reticulin skeleton (Plate 8, Fig. 1, arrows lower left-hand-side of plate) which supports a population of highly phagocytic macrophages (M), some PMNs (P), and fibroblasts responsible for reticulin formation. The phagocytic cells of the cords are speculated to effect the final destruction of aged or damaged blood cells (Chen and Weiss, 1972).

The venous sinuses (S) are lined by unusual, highly elongated endothelial cells which lie parallel to the axis of the sinus (Plate 8, Fig. 1, E = endothelial stave cell). These sinuses have been likened to tall wooden barrels, the endothelial cells representing the wooden staves, hence their name. A cross-section of a sinus (Plate 8, Fig. 2), shows how the endothelial stave cells line a sinus, with

their nuclei bulging into the sinus lumen at intervals. The "staves" of the "barrel" are seen to be held together with a band of basement membrane on their basal surface (Plate 8, Fig. 1 arrows right-hand-side and middle of plate, and Fig. 2, arrows). The basement membrane is usually discontinuous over the basal surface of the stave cells, allowing blood cells, particularly erythrocytes, to squeeze through, to and from the pulp cords and venous sinuses, hence imposing a sieving barrier to the passage of cells. Effete or less resilient cells are retained or destroyed by the stress of passage.

Endothelial stave cells also appear to have some phagocytic and pinocytotic capabilities. A phagocytosed platelet is seen in the central endothelial stave cell (Plate 8, Fig. 1 Pl = platelet) and the luminal surface of the endothelial stave cells may be seen to contain numerous pinocytotic vesicles (Plates 8, Fig. 1, small vesicles on the surface opposite to the basement membrane indicated with arrows, center and right-hand-side of plate, and Plate 9, small arrows). Evidence of pinocytotic removal of the HLE (3 nm gold particle) and cathepsin D (10 nm gold particle) from the splenic sinus occupied by the activated PMN, is given by Plate 9 (small arrows). Only in the vicinity of activated PMNs could the endothelial stave cells be seen to contain HLE, such as seen in Plate 9.

Large vesicles in the endothelial stave cell, labelled with cathepsin D only (Plate 9, large arrow), are possibly lysosomes. Notice that labelling for cathepsin D largely appears membrane associated (e.g. Plate 9, possible lysosomal organelle indicated with large arrow).

In some PMN granules labelling for cathepsin D and elastase (an azurophil granule marker enzyme) is colocalised in the same granule, whereas some granules were labelled only for cathepsin D. This supports the hypothesis that subpopulations of azurophil granules exist (Damiano *et al.*, 1988). The association of both cathepsin D and elastase with the surface of activated PMNs also appears to occur *in vivo* (Plate 9, middle-sized arrows and arrowheads, respectively), verifying *in vitro* studies which indicated the association of other azurophil granule antigens with the surface of activated PMNs.

In other sections of blocks fixed with PFA at pH 9.0, in this study, numerous artefactual vesicles were seen to form on the surface of cells. Vesiculation is reportedly caused by staining with acidic uranyl acetate, and stabilisation of membranes by pretreatment with neutral uranyl acetate, has been recommended in such cases (Tokuyasu, 1986). Staining with neutral uranyl acetate, prior to the usual

acid uranyl acetate staining, and post-labelling fixation, does not seem to have been successful in preventing the formation of artificial vesicles on the surface of the endothelial stave cell (Plate 9, two large vesicles mid-centre to right of plate).

4.5.2.4 Discussion

Splenic tissue samples fixed for a study similar to that described for PMNs (Section 4.4), were to be used in this study. The pH of fixation of one block of tissue, however, had been increased in an exploratory manner, in order to facilitate the denaturation of cathepsin B and favour its labelling. The fixation achieved with this high pH fixation regime and 8% PFA was considered more acceptable than other blocks fixed in the pH ranges described for the fixation study on PMNs (Section 4.4). Fixation at high pH was subsequently found to be one of the regimes that favoured cathepsin B immunolabelling (Section 4.6).

The precise mechanisms by which the spleen performs its functions of removing effete cells and particulate matter from the blood circulation, are still largely unknown. The reticular meshwork of the cord cells, and the phagocytic macrophages in the sinuses of the splenic red pulp, are thought to be responsible for the removal of aged erythrocytes (RBCs) from the blood (Chen and Weiss, 1972). As an erythrocyte ages, it becomes progressively more fragile and less able to survive this chordal pathway. Low oxygen tension, cholesterol content and glucose concentrations in the chordal spaces, has also been suggested make RBC membranes more rigid by their effects on haemoglobin, the plasma membrane and energy-producing capacity. Such induced rigidity may prolong retention of the RBCs in the spleen, and physiological stresses may predispose marginally viable RBCs to phagocytosis.

Phagocytosis, and removal of aging RBCs may also be favoured by prolonged exposure to the high concentrations of hydrolytic enzymes in extracellular fluids in the sinuses, secreted by macrophages (Weiss and Greep, 1977), and possibly also by activated PMNs. The presence of such hydrolytic enzyme may lead to the observed loss of sialic acid residues, and exposure of galactose sugars on the outer membrane of aging cells may facilitate recognition and removal of damaged RBCs by phagocytic cells (Weiss and Greep, 1977). In the present study, immunolabelling of splenic tissue for elastase and cathepsin D has revealed that the regulation of the levels of extracellular hydrolytic enzymes, in the splenic sinuses, may be achieved by pinocytotic uptake of these enzymes by endothelial stave cells.

Endothelial stave cells are also known to stimulate the release of significant amounts of elastase and other enzymes by PMNs (Jonas *et al.*, 1989). A dual mechanism for the control of proteinase levels, by stave cells, can, therefore, be envisaged; stimulation of the release of proteinases from phagocytic cells, on the one hand, and the uptake of proteinases, by pinocytosis, on the other. Further it seems that stave cells may participate in the phagocytic activities of the spleen, in the removal of effete cells, as evidenced by phagocytosis of a platelet (Plate 8, Fig. 1). Pinocytic activity, but not phagocytic activity, has previously been reported in endothelial stave cells (Weiss and Greep, 1977).

Examination of many neutrophils in splenic tissues, using immunolabelling for elastase as a measure of PMN exocytic activity, reveal that not all PMNs are stimulated to release enzymes in the presence of endothelial stave cells. Such stimulation of phagocytic cells may be the result of specific, controlled activation, resulting from the release of specific PMN- or macrophage-stimulating signal substances.

This study also shows, for the first time, colocalisation of cathepsin D and elastase enzymes in some granules, whereas other granules seem to contain only cathepsin D. Quantitation of the labelling, by direct enumeration of the number of gold particles present, indicates that the amount of cathepsin D in PMNs appears to be ten-fold lower than the level of elastase. This estimate concurs with biochemical analyses, which indicate that the amount of elastase in PMNs is ten fold higher than cathepsin D. This makes the content of cathepsin D in neutrophils of the order of 0.16 pgs per PMN [calculated from the amount of elastase per cell given by Damiano *et al.* (1988)], assuming that the labelling efficiency is the same for both enzymes. The content of cathepsin D in macrophages is reported to be much higher, of the order of 1.17 pg per macrophage (Bever *et al.*, 1989). This result was verified by fluorescent microscopy labelling (Section 4.7).

Studies reported in Section 4.5.1 show that elastase and cathepsin G become associated with the surface of PMNs, activated *in vitro*. As shown here, cathepsin D and elastase also appear to become associated with the surface of PMNs activated *in vivo*. All three enzymes may, therefore, be involved in the invasive activities of PMNs.

4.6 Immunolabelling of peripheral blood monocytes and macrophages, and splenic plasma cells

A considerable number of studies have been performed to identify and describe the distribution of PMN proteinases, and the mechanism of their differential release during degranulation (Schiffman, *et al.*, 1975; Bainton, 1977; Brederoo, *et al.*, 1983; Perez *et al.*, 1987; Damiano *et al.*, 1988; Sommerhoff *et al.*, 1990). The two major PMN serine proteinases, elastase (HLE) and cathepsin G, are now known to be proteinases of the azurophil granules and their activities have been well-characterised (Kargi *et al.*, 1990). Much less, however, is known about the serine proteinases of human mononuclear phagocytes.

Originally it was thought that only PMNs contained HLE and cathepsin G (Barrett, 1981), but recent studies have proved that this is not true (Campbell *et al.*, 1989; Kargi *et al.*, 1990). Several research groups have described serine proteinase or elastase-like or elastolytic activity in human peripheral blood monocytes (Werb and Gordon, 1975; Lavie *et al.*, 1980; Campbell *et al.*, 1989) and in macrophages (White *et al.*, 1977; Chapman and Stone, 1984). Definitive work on the precise nature of the enzyme activity, its subcellular distribution and its relationship to the degree of differentiation of the mononuclear phagocyte, has not, however, been reported in all cases (Kargi *et al.*, 1990). Investigations on peripheral blood monocytes are hampered by the fact that it is more difficult to obtain pure fractions of these cells, in high yield, than is the case with PMNs, as less than 10% of leucocyte populations are of the monocyte/macrophage type (Kargi *et al.*, 1990).

In the present study, elastase labelling in human peripheral blood monocytes and macrophages is demonstrated, and, for the first time, elastase-like immunolabelling of splenic plasma cells is shown. Immunolabelling of precursor and mature cathepsin B in unactivated and activated monocytes, is also demonstrated for the first time. Granule populations of fMLP-stimulated monocytes (macrophages) show high concentrations of precursor cathepsin B, but activated cells (macrophages) show no evidence of any surface-association of precursor, or mature, cathepsin B, or of elastase. A secondary objective of this study was to optimise fixation procedures for future application to tumour cells, so comment is also made on the fixation and other technical procedures found to be optimal for antigen- and tissue ultrastructural preservation.

4.6.1 Elastase in peripheral blood monocytes and macrophages and elastase and cathepsin D in splenic plasma cells

A membrane-bound elastase-like proteinase has been demonstrated on the surface of mononuclear leucocytes, including monocytes (Lavie *et al.*, 1980; Zucker-Franklin *et al.*, 1981; Estreicher *et al.*, 1990). This was shown to either be HLE, or to be very similar to it, as it is inhibitable with both diisofluorophosphate (DIFP) and the elastase inhibitor, Ac-Ala-Ala-Pro-Val-CH₂ (Zucker-Franklin *et al.*, 1981). Surface elastase may be relevant for the "invasive movement" of mononuclear leucocytes through barrier membranes, to sites of inflammation or tumour activity (Gauci and Alexander, 1975), as in the case of PMNs. Consequently, in the first part of this study peripheral blood monocytes and macrophages, were examined for surface elastase, using the cryoultramicrotomy labelling technique.

In studies on human spleen, the discovery of enzymes immunologically resembling elastase and cathepsin D, in plasma cells, was a novel and unexpected finding. No immunolabelling on the surface of these cells was seen.

4.6.1.1 Procedure

Peripheral blood monocytes/macrophages were isolated using the Ficoll-Hypaque method as previously described (Section 4.3.1). Cells were fixed immediately, or first activated with fMLP as described in Section 4.3.2. Fixation was effected using 4% PFA (5 min) followed by 8% PFA (10 min) both in HEPES buffer, pH 7.2, and sections were floated on 8% PFA, pH 7.2 (Section 4.4), until used for labelling. Sections were labelled for elastase and post-fixed with 1% glutaraldehyde, as described in Section 4.4.

Human splenic tissue, fixed and processed as described in Section 4.5.2.2, was simultaneously labelled for elastase and cathepsin D as described in Section 4.4.

4.6.1.2 Results

Immunolabelling for elastase was observed in monocytes (Plate 10, Figs 1 and 2) as well as macrophages (Plate 10, Figs 3 and 4) isolated from human peripheral blood. Immunolabelling was observed in both small electron-dense granules (presumed to be primary lysosomes) and in more electron translucent

vacuoles (Plate 10, Figs 1 and 2, arrowheads). Endocytic compartments appear to empty their contents into a few larger electron-translucent compartments, and lysosome-like granules, containing elastase, seem to have fused with or become expelled into the larger electron-translucent compartment, forming a secondary lysosome-like compartment (Plate 10, Figs 1 and 2, arrowheads). The elastase present, therefore, could easily have arisen from endocytosis. Immunolabelling for cell surface-associated elastase is, however, not evident in monocytes (Plate 10, Figs 1 and 2).

The activated equivalent of the monocyte, the macrophage, is seen to have a larger cytoplasm to nucleus ratio and shows phagocytic activity, rather than the less noticeable endocytic activity of the monocyte (Plate 10, Figs 3 and 4, and Figs 1 and 2). Generally fewer electron-dense granules labelled for HLE are present, possibly because many of the granules could have been discharged into the phagosome (Plate 10, Figs 3 and 4, arrowheads). Large aggregates of phagocytosed material are visible in some phagosomes but, once again, there appears to be no significant association of HLE with the surface of the macrophage (Plate 10, Figs 3 and 4).

Splenic macrophages showed similar low labelling densities for elastase as the human peripheral blood macrophage (results not shown). Some of the few plasma cells (B-cells) seen in the splenic red pulp samples, however, manifest a high labelling density for HLE on their endoplasmic reticulum (ER) (Plate 11, Fig. 2). No association of elastase with their outer surface was, however, seen. These cells also exhibited sparse labelling for cathepsin D (Plate 11, Fig. 1, arrowheads).

4.6.1.3 Discussion

There is an on-going debate concerning the protein complement of typical monocyte/macrophages, and the identity of elastase-like proteinases reportedly attached to the surface of monocytes and present in lymphocytes (Zucker-Franklin *et al.*, 1981; Pierce and Senior, 1981; Campbell *et al.*, 1989; Kargi *et al.*, 1990). This debate is fuelled by the heterogeneity among different subtypes of monocytes (Campbell *et al.*, 1989; Kargi *et al.*, 1990) and by an apparent difference in the type of elastase expressed by monocytes and macrophages from different animal species (Campbell *et al.*, 1989).

Studies on human peripheral blood monocytes and murine macrophages indicate that human monocytes contain elastase that is

immunologically and catalytically indistinguishable from HLE, whereas the murine cell lines contain and secrete a metalloproteinase (Senior *et al.*, 1982). A widely-held opinion, however, is that the elastase of monocytes may be, in fact, a metalloproteinase (J. Travis, pers. comm.). Studies on monocytes separated from peripheral blood using Ficoll-hypaque density gradient separation, followed by elutriation centrifugation and a sensitive competitive-binding ELISA assay, however, have shown that monocytes contain approximately 6.3% and 5.2% of the mean content of HLE and cathepsin G, respectively, of PMNs (Campbell *et al.*, 1989). The determination of endogenous proteinase content is, however, complicated by the fact that mononuclear phagocytes are able to take up elastase, and other enzymes, secreted from PMNs during density gradient separation (Campbell and Wald, 1983).

The origin of the elastase in monocytes cannot be determined by looking for its mRNA, as no elastase mRNA can be demonstrated in mature monocytes. It appears that in both monocytes and PMNs, elastase is formed in the bone marrow precursor and the capacity to synthesise this enzyme is lost by the time the mature cells reach the bloodstream (Campbell *et al.*, 1989). Alveolar macrophages (Campbell and Wald, 1983) and monocytes (Kargi *et al.*, 1990) have receptors for HLE, however, and are capable of uptake and storage of elastase for as long as 5 days or more (Campbell and Wald, 1983). Cells that are this active in endocytosis and phagocytosis, may thus become HLE positive by uptake of HLE, perhaps from activated, degranulating, PMNs, to which they might be exposed during the isolation procedures. This complicates determination of the origin of elastase in monocytes/macrophages.

Studies on the proteinase content of the human promonocytic cell line, U937, have been useful in resolving this question, especially when the mRNA for the different enzymes is also analysed (Senior *et al.*, 1982; Ward *et al.*, 1990; Lindmark *et al.*, 1990). HLE and cathepsin G, the major proteinases found in PMNs, have been found in U937 cells which have not been exposed to exogenous sources of PMN enzymes. Under the influence of differentiation agents such as PMA, however, the monocyte phenotype of the U937 cell line is expressed and there is an associated increase in the monocyte proteinase, cathepsin B, and its mRNA, but a decrease in levels of PMN elastase and elastase mRNA (Ward *et al.*, 1990). Such studies confirmed previous findings on density gradient separated cells and have established cathepsin B as a marker for monocyte differentiation and elastase as a quantitative marker for the PMN phenotype.

The bulk of the protein-degrading activity of intact human lymphocytes and monocytes, is reported to be due to a surface-bound elastase (Lavie *et al.*, 1980). No plasma membrane-bound HLE was seen in labelling experiments, in the present study, however. This may be because surface expression of HLE may only be a feature of chemotactically activated cells. Repetition of these studies, with fMLP-activated monocyte/macrophages, proved difficult, however, due to the low yield of monocytes and the heterogeneity of monocyte populations. According to Campbell *et al.* (1989), only about 20% of monocytes are replete with HLE and other serine proteinases. Finding such cells is, therefore, difficult.

Stimulated monocytes are known to release enzymes, such as elastase taken up from activated PMNs, to a much greater extent than the PMNs themselves, in proportion to their total cellular content (Campbell *et al.*, 1989). Monocytes are hence thought to play a major role in pulmonary emphysema, despite their smaller proportion of enzymes such as elastase (Campbell and Wald, 1983). If elastase is, therefore, involved in the invasive activity of monocytes/macrophages, the mechanism might be by release of elastase rather than by an association of elastase with the external membrane.

Enzymes which occur to a much greater extent, in monocytes (and macrophages), are cathepsin B and cathepsin D (Moreland and Pedersen, 1979; Lesser *et al.*, 1989). Cathepsin D is present in larger amounts than cathepsin B, but, surprisingly, little importance seems to have been attached to the possible role of cathepsin D, in lung pathologies, cathepsin B being the major focus of attention to date (Moreland and Pedersen, 1979; Lesser *et al.*, 1989).

In tumour tissues too, cathepsin B has been the focus of many studies (Sloane *et al.*, 1990). Isolation procedures on tumour homogenates have often indicated the presence of high levels of cathepsin B relative to normal tissue, a phenomenon which has been attributed to the tumour itself (Sloane *et al.*, 1990). Stimulated macrophages, however, greatly increase in cathepsin B content (Lesser *et al.*, 1989), and the prognosis of post-surgical remission in cancer patients, has been linked, inversely, to the macrophage content of tumours (Gauci and Alexander, 1975). It is clearly important, therefore, in investigating the proteinases purportedly involved in tumour invasion, not to overlook the contribution which may be made by enzymes from cells of the immune system, such as macrophages.

An interesting result in the present study was the extensive labelling for HLE seen in splenic B cells (plasma cells). Immunolabelling for HLE in such cells has not previously been reported, and it is difficult to imagine what the potential role of elastase might be in an antibody-producing cell. Though this is a curious and unexpected result, it is not completely without precedent. Use of a radiolabelled inhibitor, $^3\text{HAc-Ala-Ala-Pro-Val-CH}_2\text{Cl}$, specific for HLE, has demonstrated the presence of an elastase-like enzyme on the surface of intact lymphocytes (Lavie *et al.*, 1980), though the type of lymphocyte was not identified. Similarly, using a radiolabelled serine proteinase inhibitor, DIFP, 5-8% of lymphocytes are reported to show surface labelling for a serine proteinase, which may be elastase (Zucker-Franklin *et al.*, 1981). Such labelling, may, however, relate to T cells only; HLE being found in both a membrane-bound and soluble form in CD4^+ and CD8T^+ T cells (Bristow *et al.*, 1991).

The B cell-like lymphocyte shown in this study to label with anti-elastase antibodies, fits the description of an OKT3 negative, B lymphocyte [classed as a "lymphoplasmacytoid cell" by Matutes and Catovsky (1982)], rather than a T cell. Immunoreactivity with the anti-HLE antiserum was also shown in eosinophils (EM results not shown, but see immunofluorescent pictures, Plate 4, Figs 1 and 2).

There is a danger in relying on immunoreactivity alone to indicate the presence of a specific proteinase, in immunolabelling studies on leucocytes, however, in that many proteinases have quite extensive sequence homologies and there is a fair chance that different proteases may cross-react, immunologically. Sequence homologies of 47% and 56% have been described between rat mast cell proteinase II, and a product of activated mouse cytotoxic T cells, and cathepsin G (Salvesen *et al.*, 1987). Similarly, an antibacterial protein, with no proteolytic activity, has been described as having a 33% homology with cathepsin G, a 44% homology with HLE, a 45% homology with PR 3 (an azurophil granule proteinase), and 33% homology with human lymphocyte protease (the human homologue of the murine cytolytic T cell proteinase) (Almeida *et al.*, 1991). The immunological reactivity with anti-HLE antibodies, observed in eosinophils and B cells, therefore, requires biochemical confirmation, before it can be irrefutably concluded that these cells contain elastase.

4.6.2 Cathepsin B in splenic endothelial stave cells, and splenic and peripheral blood macrophages

This part of the present study was aimed at optimising immunolabelling conditions for the labelling of cathepsin B and procathepsin B, and, for this purpose, the distribution of cathepsin B in splenic endothelial stave cells, and in the macrophages of the spleen and blood was studied. The ultimate aim, once immunolabelling conditions had been optimised, was to compare the distribution, and trafficking, of precursor and mature forms of cathepsin B, and other lysosomal enzymes, in tumour and normal tissues.

Most tissue, unfortunately, contains relatively low levels of cathepsin B and even lower levels of procathepsin B. Optimisation of immunolabelling for these two antigens, therefore, proved quite difficult. As previously mentioned, immunolabelling for cathepsin B is in any event difficult because cathepsin B spontaneously denatures at neutral pH and above. For this reason, antisera raised in experimental animals generally recognise only the denatured form of cathepsin B. Immunoreactivity, therefore, requires prior denaturation of the enzyme (Rowan *et al.*, 1992). The rapid fixation with PFA, at physiological pH, and the processing of cells and tissues for cryoultramicrotomy, as used in all other studies, proved to be too mild to promote the denaturation, and consequent immunoreactivity, of cathepsin B.

Efforts to promote the denaturation of cathepsin B, and to improve the fixation of elastase, by exposing the tissue to a high pH, fortuitously led to the solution of both problems. Exposure to the high pH, during short fixation periods, denatured cathepsin B sufficiently to promote immunolabelling and accommodated immunolabelling for procathepsin B also. The only other way in which immunolabelling of cathepsin B could be effected was subsequent to a denaturing, dehydration step, which is a normal part of resin-embedding protocols. For embedding in LR White resin, short fixation periods using PFA proved to be inadequate for adequate ultrastructural preservation of the tissue, however, and it was necessary to use glutaraldehyde, for stronger fixation.

Due to their very low levels in most other tissues, immunolabelling for cathepsin B precursors was initially optimised on monocytes and macrophages, present as contaminants of PMN fractions separated by density gradient centrifugation. Immunolabelling of fMLP-activated cell fractions proved most rewarding. Activated macrophages are known to increase cathepsin B synthesis to

about 8 times the basal level (Padilla *et al.*, 1988) and, in this study, fMLP-activated human peripheral blood monocytes were found to contain unexpectedly large amounts of cathepsin B precursor. No surface expression of precursor or mature cathepsin B was evident in any of these immunolabelling studies, however. Immunolabelling studies of this kind have not been previously reported.

4.6.2.1 Procedure

Splenic tissue, fixed with 8% PFA in PBS, pH 7.2 or 8% PFA in borate buffer, pH 9.0 and processed for cryoultramicrotomy as described in Section 4.5.2.2, was simultaneously labelled for cathepsin B and procathepsin B, using sheep anti-cathepsin B (0.20 µg in 10 µl) and anti-ppB22-36 (2 µg in 10 µl) as described in Section 4.4.

Similarly, splenic tissue samples were fixed with 4% PFA containing 0.2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (Section 2.3.2.1), embedded into LR White resin, and immunolabelled as described in Section 2.5. Double labelling for cathepsin B and procathepsin B was performed, using sheep anti-cathepsin B (0.20 µg in 10 µl) and anti-ppB22-36 (2 µg in 10 µl) and 6 nm and 10 nm protein A gold probes, respectively, and using a 1% glutaraldehyde fixation step between immunolabelling steps, essentially as described in Section 4.4.

Finally, the PMN fraction of peripheral blood, separated using the Percoll gradient centrifugation method (Section 4.3.1.2), was activated using 30 µM fMLP, as described in Section 4.3.2.2, and processed for cryoultramicrotomy, as described in Section 4.5.2.2. Sections were labelled for cathepsin B or procathepsin B, using sheep anti-cathepsin B (0.20 µg in 10 µl) or anti-ppB22-36 (2 µg in 10 µl), as described in Section 4.4.2. Controls additionally included an adsorption control. For the adsorption control, the anti-peptide antibody (ppB22-36, diluted to 200 µg/ml) was preadsorbed with 200 µg/ml of the peptide against which the antibody was raised (30 min, RT), centrifuged (10 000 × g, 15 min, RT), and used in the immunolabelling procedure.

4.6.2.2 Results

For immunolabelling studies on cathepsin B and its precursor, fixation with 8% PFA at pH 8 or 9, for "cryo"-labelling of fMLP-activated monocytes and splenic tissues, proved satisfactory. Fixation with 4% PFA containing 0.2%

glutaraldehyde, at pH7.2, followed by dehydration and LR White embedding, was also satisfactory. Fixation with 8% PFA at pH 7.2 was unsatisfactory for "cryo"-immunolabelling of cathepsin B, as no labelling of this antigen was observed at this pH.

In endothelial stave cells, electron-dense vesicles, possibly pre-lysosomal or lysosomal compartments (Plate 11, Figure 4, medium arrow, Fig. 3, arrows) and Golgi elements (Plate 11, Figure 4, G and small arrows), labelled for both the mature (10 nm) and precursor cathepsin B (3 nm), whereas large electron-translucent vesicles labelled for mature cathepsin B only (Plate 11, Figure 3, arrowheads, lower left). Electron-dense organelles are usually classified as lysosomes, but the presence of large quantities of procathepsin B in the electron-dense compartment, makes this more likely to be a prelysosomal organelle. The presence of mature cathepsin B in the electron-translucent compartment suggests that this is a lysosome.

Sections from LR White-embedded splenic tissue showed much swelling and distortion (Plate 12, Figs 1 and 2). In macrophages, two populations of secretory granules were present; an electron-translucent and a more electron-dense granule population. The more electron-translucent secretory granules of macrophages, however, showed labelling for mature cathepsin B, the larger vesicles giving lower labelling densities (Plate 12 Fig. 1, arrows). The occasional, more electron-dense, larger vesicles, showed labelling for the precursor enzyme, a feature characteristic of immature vesicles in a secretory cell (Plate 12, Fig. 2).

fMLP-activated macrophages (Plate 13), compared to their unstimulated counterparts, showed a greater proportion of cytoplasm and increased numbers of granules and Golgi compartments. A certain population of electron-translucent and semi electron-dense granules showed dense labelling for procathepsin B (Plate 13, Figs 1, 2 and 4). Immunolabelling for procathepsin B was also present in Golgi bodies, as would be anticipated (Plate 13, Fig. 4, arrow indicates procathepsin B labelling). No surface labelling for procathepsin B was seen (Plate 13, Fig. 1). In all activation procedures there are always a few cells that show greater activation than others.

Double immunolabelling of this cell, for the precursor and mature forms of the enzyme would have been useful in both verifying the labelling of the peptide antibody and for the assessment of the relative distribution of precursor

and mature forms of the enzyme in different granule populations. This labelling was obtained in a cryoultramicrotomy system, where serial sectioning is not possible, so immunolabelling of replicate sections of the same cell, as shown in Plate 13, for cathepsin B, could not be performed.

A less satisfactory approach in assessing the relative distribution of precursor and mature forms of cathepsin B, is to compare single labelling results in different cells. The anti-mature cathepsin B antibody will recognise all forms of cathepsin B, including the precursor form, so to obtain a true picture of the distribution of mature enzyme only, it is necessary to assess the **difference** between specific procathepsin B labelling and total cathepsin B labelling. The cell shown in Fig. 3 of Plate 14 (labelled with anti-mature cathepsin B) seems to have slightly more cathepsin B labelling than the cell depicted in Plate 13 (labelled for procathepsin B). This may indicate that the cell depicted in Plate 14 may contain some vesicles that label for mature cathepsin B only, though the majority of vesicles would probably have precursor and mature cathepsin B colocalised.

Cathepsin D labelling was only performed on unactivated monocyte-containing cell fractions, but these monocytes contained moderate numbers of granules that labelled for cathepsin D (Plate 13, Fig. 3).

All control immunolabellings were satisfactory, giving negligible background labelling. The adsorption control performed with the peptide antibody, showed an 80% reduction of immunolabelling .

Though the cells depicted in Plates 13 and 14 were all cut from the same block, the difference in organelle integrity in the different cells, is marked. The section from the macrophage, depicted in Plate 14, Fig. 3, is the thinner section (possibly 80-100 nm), while the sections of cells illustrated in Plates 13 and 14, Figs 1 and 1, respectively, are of approximately the same thickness (possibly 120-150 nm). The cell illustrated in Plate 13, and Plate 14 Fig. 2, however, was found near a grid bar, whereas the cells illustrated on Plate 14, Figs 1 and 3, were mid-way between grid bar meshes. The thickness of the uranyl acetate/methyl cellulose film finally covering the section varies, not only in overall thickness, depending upon how much methyl cellulose is removed prior to the final drying process, but also with distance of the cell from the grid bar mesh.

The preservation of organelle structure, therefore, seems to depend, not only on the fixation regime used, but also on the thickness of the section and the thickness of the final film of methyl cellulose covering the section. Greater membrane definition and cellular integrity seems to be preserved in thicker sections, covered by slightly thicker films of uranyl acetate/methyl cellulose sealing agent. A thicker section may help to prevent granule extraction, but not completely in all cases, as illustrated in Plate 14, Fig 2, where a large granule can be seen to have been extracted from the macrophage depicted (large arrows indicate the hole and the extracted granule).

4.6.2.3 Discussion

Immunolabelling for cathepsin B, with the polyclonal anti-human liver cathepsin B antibody, seems to require that the antigen be denatured by exposure to high pH during fixation for cryoultramicrotomy, or by exposure to dehydration, during resin embedding after fixation at physiological pH. Labelling with the procathepsin B peptide antibody, however, seemed to be favoured by most regimes.

Relatively low levels of mature and precursor cathepsin B were anticipated to be present in most cells. A slight increase in cathepsin B precursor levels, concomitant with increased synthesis of cathepsin B, however, was anticipated in response to cell activation. The observed result, however, indicates a much greater increase.

The increase in cathepsin B activity of stimulated macrophages, from different sources, has been shown to differ to a great extent; pulmonary macrophages showing a greater increase than peritoneal macrophages (Lesser *et al.*, 1983). Cathepsin B activity, in alveolar macrophages, also seems to increase, under the influence of certain stimulæ, where other enzymes, such as cathepsin D, actually decrease (Lesser *et al.*, 1989). No information of how much precursor enzyme is actually present in activated peripheral blood monocytes, however, is available.

In the present study, all immunolabelling controls, including the adsorption control for the peptide antibody, however, appeared to be satisfactory, indicating that immunolabelling results should be reliable. Immunolabelling is always, best verified with biochemical data, however. In this case, PMA-differentiated U937 cells would be a suitable subject for such confirmatory biochemical studies and as

a preliminary to such studies, and to confirm some of the labelling results obtained for cathepsin D and B, the U937 cell line was acquired.

4.7 Immunofluorescence studies on the U937 cell line

The U937 cell line is a promonocytic cell line which grows readily in culture and which, upon treatment with PMA, differentiates to acquire the phenotypic characteristics of normal mature monocytes (Senior *et al.*, 1982; Lindmark *et al.*, 1990; Ward *et al.*, 1990). This cell line, therefore, theoretically provides an ideal model in which monocyte and macrophage proteinases may be studied. Alternatively, differentiation towards the PMN phenotype may be induced by exposure of U937 cells to dimethyl sulphoxide (DMSO) (Lyons and Askam, 1989).

Differentiation to the monocyte phenotype leads to an increase in cathepsin B mRNA and cathepsin B synthesis, and a decrease in elastase mRNA and elastase (Ward *et al.*, 1990), and cathepsin G (Welgus *et al.*, 1986), levels. Other monocyte differentiation and maturation-related antigens recorded include a gelatinase/type IV collagenase, a stromelysin and a matrilysin (Watanabe *et al.*, 1993). Studies on the effect of differentiation on cathepsin D levels, in U937 cells, have not been previously reported, though in density gradient-separated peripheral blood PMNs, cathepsin D has been reported to be between 5 and 10 times lower than the level of elastase i.e. of the order of 0.16-0.8 pgs/PMN (Ishikawa and Cimasoni, 1977; Damiano *et al.*, 1988).

Studies on peripheral blood cells are complicated by poor yields, and by activation, especially of monocytoid cells, by density gradient separation procedures, so it was decided to pursue further investigations, into the proteinases contributing to the invasive capabilities of monocytes and PMNs, using the U937 cell line. Some leukemic cell lines have characteristics different from the corresponding normal cell type (Lyons and Askam, 1989) but the protease profile of the U937 cells, is reported to be the same as that of the normal cell equivalent (Welgus, *et al.*, 1986; Lindmark *et al.*, 1990).

In the studies reported here, the level and distribution of cathepsins D and B, and the M-6-P receptor status of the cells, was assessed by fluorescent immunolabelling of U937 cells, differentiated to the monocytoid phenotype, using PMA, and to the PMN phenotype, using DMSO. This was done mainly to verify the

success of U937 differentiation procedures, preliminary to further studies, and to verify some of the results obtained using isolated peripheral blood leucocytes.

4.7.1 Reagents

The rabbit anti-cathepsin D antibody, was from Dr Hasilik, Physiologisch-Chemisches Institut, Universität Münster, Münster, rabbit anti-bovine 215 kDa mannose-6-phosphate receptor antibody was from Dr Hofflack, European Molecular Biology Laboratory, Heidelberg, and the anti-human liver cathepsin B antibody was from Dr Lukas Mach, Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna, Austria.

The U937 cells were a gift from Dr Josef Glössl, Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna. The RPMI 1640 medium was from Highveld Biologicals, Sandton, and the foetal calf serum from Delta Labs, Sandton, the bovine serum albumin (BSA) Fraction V, was from Boehringer Mannheim, and Saponin and FITC-labelled mouse anti-rabbit monoclonal antibody was from Sigma Chemical Co., St Louis, Mo. Anti-Fade[®], immunofluorescence coverslip mounting medium, was from Molecular Probes Inc. Eugene, Or.

Phosphate Buffered Saline (PBS-F). The PBS used in immunofluorescence labelling studies, unlike that used in the EM immunolabelling studies, contained calcium and magnesium and will, therefore, be referred to as PBS-F. NaCl (7.99 g), KCl (0.199 g), Na₂HPO₄ (1.145 g), KH₂HPO₄ (0.2 g), CaCl₂ (0.147 g) and MgCl₂ (0.102 g) were dissolved in approx. 700 ml Dist.H₂O. The solution was adjusted to pH 7.2 with 1M NaOH, if necessary, and made up to 1 litre.

4% Paraformaldehyde. 4% PFA (16 ml) was made by diluting a 16% stock solution (Section 2.3.2.1) (4 ml) with PBS-F (12 ml).

2% Bovine serum albumin (BSA). 2% BSA was made by dissolving BSA (0.8 g) in PBS-F and making up to 40 ml.

5% Foetal calf serum. Foetal calf serum (0.5 ml) was dissolved in PBS-F and made up to 100 ml.

0.1% Saponin in PBS-F (PBS-saponin). Saponin (0.1 g) was dissolved in PBS-F and made up to 100 ml.

4.7.2 Procedure

U937 cells were cultured in RPMI 1640-supplemented 15% foetal calf serum, at 37°C, in a humidified atmosphere, containing 5% CO₂. A sterile glass coverslip (15 mm) was placed in each well of a 12 well Nunc multiwell plate and each well was seeded with approx. 5×10^4 cells in 1 ml. Differentiation was induced, in duplicate wells, by addition of either PMA (1 µl of a 10 µM solution in DMSO/well) or 1% DMSO (10 µl/well) and incubation for 3 days. A 25 ml flask of similar cell concentration was concurrently incubated to supply a source of unactivated cells.

Cell populations, exposed to either differentiation reagent, generally become adherent to glass upon differentiation. Cells adherent to coverslips, were washed 3 times with PBS-F, and undifferentiated cells in suspension, from the 25 ml flask, were pelleted by gentle centrifugation (100 x g, 5 min, RT), and washed 3 times in PBS with repeated centrifugation (100 x g, 5 min, RT).

The final pellet of undifferentiated cells, resuspended in a suitable quantity of PBS-F, was spread on a sterile coverslip. These cells and the adherent cells were fixed onto the coverslips with 4% PFA (200 µl, 10 min, RT), rinsed 3 times with PBS-F, and non-specific residual fixative binding sites were blocked by incubation in 0.2% BSA (45 min, RT).

The coverslip was incubated in the primary antibody (200 µl, 1 h, RT): the anti-cathepsin B antibody at 40 µg/ml, the anti-human cathepsin D serum diluted 1/40, or the mannose-6-phosphate antibody diluted 1/50 in PBS-saponin. After six rapid washes with PBS-saponin, 5% foetal calf serum secondary block solution (200 µl, 30 min, RT) was applied. After the required incubation period the secondary, FITC-labelled, mouse monoclonal antibody, diluted 1/40 in PBS-F-saponin, was applied (200 µl, 1 h, RT). After six washes with PBS-saponin, cells were refixed with 4% PFA (200 µl, 10 min, RT), washed with PBS-F-saponin, rinsed well in dist.H₂O and allowed to drain dry. The coverslips were mounted, face-down in a minimum amount of Anti-Fade[®], and viewed in an Olympus BH2 microscope, fitted with a BH2-RFC Reflected Light (epifluorescence) Attachment, fitted with a 460 nm interference filter and a 520 nm barrier filter.

4.7.3 Results

U937 cells labelled with the rabbit anti-human liver cathepsin B antibody, showed very weak immunolabelling of undifferentiated cells (Plate 15, Fig. 1a) and strong, polarised, immunolabelling of PMA-treated cells (Plate 15, Fig. 1b). DMSO-treated cells (PMN phenotype) showed no labelling at all (results not shown).

Immunolabelling for cathepsin D showed that cathepsin D is present in the undifferentiated cell line (Plate 15, Fig. 2a) but to a lesser extent than in PMA-treated cells (Plate 15, Fig. 2b). DMSO-treated cells showed only very weak, almost negative, labelling (result not shown).

Finally, immunolabelling for the mannose-6-phosphate receptor, indicated that this is only present in cells differentiated with PMA (Plate 15, Fig. 3a and 3b), indicating that only the monocyte expresses the mannose-6-phosphate receptor.

4.7.4 Discussion.

The combined labelling results indicate the success of differentiation procedures, as cathepsin B (Ward *et al.*, 1990) and D (Bever *et al.*, 1989) are known to be present in monocytes. Mean levels of cathepsin B in undifferentiated U937 cells have been reported as being of the order of 15 ng/10⁶ cells, whereas, upon exposure to PMA, these levels rise to approximately 297 ng/10⁶ cells (Ward *et al.*, 1990). Levels of cathepsin B, in peripheral blood PMNs, have been determined to be of the order of 0.45 ng/10⁶ cells (Assfalg-Machleidt *et al.*, 1991). These results seem to concur with the trends seen in the present immunolabelling study, on the U937 cells. Labelling for cathepsin B in PMNs appears negative, as anticipated, as such levels of cathepsin B are below the detection limit of this immunofluorescence system.

Levels of cathepsin D in U937 cells do not seem to have been previously determined. Cathepsin D levels in human peripheral PMNs, however, have been estimated as being of the order of 0.16 - 0.8 pgs/cell (Ishikawa and Cimasoni, 1977; Damiano *et al.*, 1988). If these estimates are correct, it is not surprising that the immunolabelling for cathepsin D, on DMSO-treated U937 cells, was negative. On the basis of immunolabelling results (Section 4.4.2), it was anticipated that the PMN-like phenotype, induced by DMSO-treatment, would have shown some immunolabelling for cathepsin D. There are two possible explanations for this apparent discrepancy:

i) the PMN labelled for cathepsin D may have been an early form of PMN, and contain more cathepsin D than most mature PMNs, ii) the level of cathepsin D present in PMNs may be below the sensitivity of the present immunofluorescence labelling system.

Immunolabelling for the M-6-P receptor also seems to be a good alternative marker for the monocyte phenotype. Monocytes are known to have two receptor populations that are responsible for lysosomal enzyme clearance and trafficking, the mannose receptor and the M-6-P receptor (Shepherd and Stahl, 1984). Though little seems to be known about the trafficking and processing of enzymes in monocytes and PMNs, it is known that PMNs are devoid of these receptors (Shepherd and Stahl, 1984; Lindmark *et al.*, 1990), a result confirmed in the present study.

The present studies have served to confirm the utility of the U937 cell line in providing a simpler system on which preliminary studies may be conducted before subsequent confirmation on more refractory peripheral blood and bone marrow cells.

4.8 Discussion

4.8.1 Significance of the immunolabelling results

The association of elastase, cathepsin D and cathepsin G with the surface of activated PMNs, demonstrated in the present study, provides the first indication that these enzymes may be involved in the invasive movement of PMNs through basement membranes. Only one other study has previously indicated that elastase may become associated with the surface of activated PMNs. This was a biochemical demonstration that proposed the presence of an elastase receptor on the surface of activated PMNs (Dwenger *et al.*, 1986). Perhaps the main reason why elastase has not previously been demonstrated on the surface of PMNs, is that PMNs are difficult to isolate, they are fragile and easily stimulated to degranulate. PMNs contain a large amount of elastase, which mostly occurs within the granules and, if this is not contained during isolation and immunolabelling, it becomes impossible to determine where else the elastase might naturally occur. Therefore, the development of adequate methods for the fixation of PMNs, although a tiresome necessity, in fact "opened up" PMNs for the studies reported here and has made them accessible for further immunocytochemical studies, in the future.

Cathepsin B and procathepsin B, in activated monocytes, were easier to study, in some respects. These enzymes, however, do not seem to be membrane associated and, therefore, would appear to not play a role in the movement of macrophages through barrier basement membranes. The high content of procathepsin B in fMLP-activated monocytes is interesting. If precursor enzyme was secreted into an acid pocket, such as formed by an osteoclast in intimate contact with its target tissue (Eeckhout, 1990), the precursor enzyme would constitute a readily-available source of mature cathepsin B. In an acid environment, the precursor enzyme is rapidly autocatalytically cleaved to the active enzyme (Mach *et al.*, 1993), and hence could play a role in basement membrane hydrolysis and the "invasive" movement of activated monocytes through barrier tissues.

The interaction of PMNs and monocytes with endothelial cells is also of interest, as, preliminary to leucocyte, or tumour invasion, interactions between the leucocyte or tumour cell and the endothelial cells of the vasculature, take place (Dean *et al.*, 1991; El-Sabban and Pauli, 1991). Macrophages in contact with endothelial cells and fibroblasts, are reported to induce the formation of "cavicolae", or small vesicles, very similar to those seen in the endothelial stave cells in this study. These vesicles, in the macrophage/fibroblast cell environment, are seen to take up products from the macrophage. In the study reported by Dean *et al.* (1991), these were BSA-gold adducts, previously endocytosed by the macrophage, and not ordinarily taken up by fibroblasts, were seen to be transferred from the monocyte to the fibroblast cell, indicating the establishment of possible "communication" channels between cells.

Similarly, the metastatic potential of B16-F10 murine melanoma cells, has been demonstrated to be correlated with the transfer of cytoplasmic dye, from the tumour cell to the vascular endothelium (El-Sabban and Pauli, 1991). In this case tumour-endothelial cell communication is mediated by gap junction channels formed after adhesion to the vascular endothelium. This interaction is cited as playing a possibly crucial role in tumour cell extravasation at a secondary site (El-Sabban and Pauli, 1991) and similar interactions between PMNs and monocytes and the endothelia, may be relevant in leucocyte "invasive" activities. Low molecular weight components, such as the arachidonic acid derivative HETE (Dean *et al.*, 1991), synthesised by the tumour cell or the leucocyte, could be transferred to the endothelium, to stimulate endothelial cell retraction, exposing the basement membrane to proteolysis and thereby assisting tumour cell- or leucocyte invasion. Proteolysis may be effected by proteinases from the invading cell, or else messenger products may stimulate the endothelial cells themselves to secrete proteinases.

4.8.2 The cryoultramicrotomy approach

On the technical side, a novel aspect of this study is the fact that none of the studies reported here have previously been attempted using the cryoultramicrotomy technique. Elastase has been immunolabelled in peripheral blood monocytes (Kargi *et al.*, 1990), but not using cryoultramicrotomy. The cryoultramicrotomy approach allows the use of weak fixation regimes, as tissues do not have to withstand the harsh processes of resin embedding. Because of this, it was possible to develop an optimal fixation protocol, thereby "opening up" PMNs for immunocytochemical study.

At the commencement of this study, the speed with which results may be obtained using cryoultramicrotomy, and the fact that tissue fixation is the only antigen-denaturing step, made the "cryo" approach very attractive for optimisation of fixation, and for antibody optimisation procedures. Processing of a tissue to the final cutting and labelling stages, may be done in one day, under ideal conditions, whereas resin embedding procedures may take up to a week (Griffiths, 1993). With experience, however, some of the draw-backs of the "cryo" technique came to be appreciated

In the "cryo" system, there is no resin matrix holding the elements of the section together, as there is in a resin section. Despite the carbon and formvar support film beneath the specimen, the fully-hydrated specimen is easily damaged by rapid changes in osmotic pressure and by dessication. Osmotic stresses, such as occur when the section is picked up off the microtome knife, with 2.3 M sucrose, placed on the grid and floated off onto buffer, may produce tissue vesiculation. Strategies to overcome this effect have been suggested (Tokuyasu, 1986), e.g. floating sections off onto a protein solution, such as 2% BSA containing 1 M sucrose. In the present study, these were found to offer no significant benefit.

Another effect of capturing the section on sucrose is that it is easy for weakly-held cell components, such as azurophil granules, to be pulled out of the section as the dense sucrose droplet falls away from the grid section, when this is floated on buffer. Floating the sections off onto fixative, thereby effecting lateral fixation, was the only process found to be beneficial, both for osmotic effects and for the retention of the azurophil granules.

The fact that the cryoultramicrotomy section is unsupported, makes crosslinking of the contents of large granules, to hold the granules in place, much more critical. Lateral fixation, by flotation of the section on fixative, subsequent to cutting and before immunolabelling, assisted in retaining the azurophil granules. It was only appreciated later, however, that with increasing thickness of the section, there was an increasing probability that the granules would be retained and the elastase antigen would be prevented from being extracted and translated over the section.

Another feature of the fully-hydrated cryoultramicrotomy section, is that it is easily damaged by dessication. If the final sealing film of methyl cellulose is too thin, drying artefacts can result from the "explosion" of some cell components, with damage to the surrounding ultrastructure. Such drying effects may be confused with other influences, such as poor fixation, or a poor knife edge used in sectioning, or an inappropriate density gradient method used in cell preparation, which may all lead to poor ultrastructure. The fragility of PMNs is also an age-related phenomenon; the older the PMN, the more fragile it is. This influences the reproducibility of the results and, taken together with all the above-mentioned factors, means that it is necessary to evaluate a large number of sections, before any conclusions can be drawn.

The final aspect which was not fully appreciated at the outset, was that, for the assessment of the association of proteinases with the surface of PMNs, where a polarised distribution of proteinases occurs only in the direction of movement, the orientation of sectioning is not easily changed for a cryoultramicrotomy block. The orientation of sectioning is critical, however, and should be parallel to the direction of cell movement, in order for any "invasion front" proteinases to be demonstrated. No serial sectioning or changing of the orientation of the frozen block of tissue on the microtome stub is possible in cryoultrasectioning, however, unlike resin sectioning. For this reason, a great many sections were evaluated before the results presented here were recorded.

For a number of reasons, therefore, coupled with the technical difficulty of the technique *per se*, the cryoultramicrotomy approach was possibly the most difficult approach that could have been used for the studies reported above. On the other hand, it has led to insights which could not have been achieved with any other technique.

4.8.3 Immunofluorescent labelling

For the assessment of the general distribution of an antigen, immunofluorescent microscopy is a valuable method, giving a greater appreciation of the overall features of cells, due to the low magnification at which the labelling is visible, while giving greater sensitivity of detection than is possible with other light microscopy immunolabelling approaches. A microscope, equipped with an epifluorescence attachment, only became available in the closing phases of the studies reported here, however.

Similarly, adequate facilities for culture of human cell lines were also acquired only in the closing phases. Cell culture has many advantages, allowing the age and population type and state of differentiation of the cells to be controlled, and eliminating the effects of cell isolation procedures. The availability of facilities for fluorescent microscopy and for cell culture was exploited in the studies on U937 cells, reported in this chapter, and in the studies on human breast epithelial cells, reported in Chapter 5.

4.8.4 Conclusion

The studies reported in this chapter were undertaken largely to develop methods, and leucocytes were initially chosen simply as a model of invasive cells. Nevertheless, in addition to the methods-development, exemplified by the section on fixation, a number of novel findings of biological significance have emanated from the present study. These findings were dependent on prior methods development, since they were only made possible by the development of an adequate fixation protocol, and include:-

- i) the demonstration of elastase, cathepsin G and cathepsin D on the surface of *in vivo* f-MLP activated- and/or *in vitro* activated PMNs.
- ii) the demonstration of the effect of exposure of PMNs to different levels of the activating agent PMA,
- iii) the demonstration of a novel secretory body in activated PMNs,
- iv) the ultrastructural demonstration of cathepsin B and procathepsin B in fMLP-activated peripheral blood monocytes,
- v) the demonstration of elastase and cathepsin D in plasma cells,
- vi) observations on the interaction of activated PMNs and splenic endothelial cells, specifically the uptake of elastase from the splenic spaces by the endothelial stave cell.

In addition, immunofluorescence labelling of variously differentiated and undifferentiated U937 cells has shown that these have a particular usefulness in immunocytochemical studies of leucocytes.

CHAPTER 5

PROTEINASE DISTRIBUTION IN "NORMAL" AND *ras*-TRANSFECTED BREAST EPITHELIAL CELL LINES5.1 Introduction

The study reported in this chapter represents a brief contribution to the overall aim of the studies reported in this thesis, which was to discern which proteinases may be involved in the invasive movement of tumour cells and peripheral blood cells. During the course of the study on peripheral blood leucocytes, the advantages of working with a cell line became apparent. It was realised that it is far easier to examine individual, well-defined cell populations, in culture, rather than to isolate cells or search for appropriate cells in tissue samples.

This is especially true of tumour tissues, where the presence of a heterogeneous population of uncharacterised tumour cells, and many other cells, may confuse the results. Moreover, tumour tissues are not easily accessible. For this reason, it was always intended that preliminary studies on human tumours would be performed on tumour cell lines. For the culture and handling of human tumour cell line, however, at least a Class II laminar flow bench and a functional, humidified, CO₂ incubator are required. The brevity of this part of the study was due to the fact that both the equipment necessary for the culture of human tumour cell lines and the MCF-10 cell lines were acquired very late in this study.

5.1.1 The MCF-10A and MCF-10A-Neo-T breast epithelial cell lines

The MCF-10A and MCF-10A-Neo-T-c-Ha-*ras* human cell lines are a unique, newly characterised, pair of cell lines, comprised of an adherent, immortal, "normal" breast epithelial cell line (designated MCF-10A) and a tumour cell line equivalent (designated MCF-10 A-Neo-T, or simply Neo-T), derived from the MCF-10A cell line, by transfection with the *ras* oncogene. These cell lines were developed at the Michigan Cancer Foundation (hence, "MCF"), and were provided to this laboratory by Dr Bonnie Sloane, of Wayne State University, Detroit.

The MCF-10 cell line is an oestrogen receptor-negative, spontaneously immortalised breast epithelial cell line, derived from mastectomy tissue from a 36-year-old parous, premenopausal woman with fibrocystic disease (Tait *et al.*, 1990;

Soule *et al.*, 1990). This cell line is unique as it is the first spontaneously immortalised line of breast epithelium, for which there is direct evidence that the normal diploid chromosome pattern, of the original explanted tissue, was retained for over a year in culture. Transformation to the aneuploid or near diploid karyotype, characteristic of an immortal cell line, moreover, took place spontaneously, with minimal chromosome rearrangement, whereas in other currently available breast epithelial cell lines, immortality was induced by treatment with carcinogenic drugs, or the cell lines were transformed using the SV40 virus and still harbour some transforming SV40 genetic information (Soule *et al.*, 1990).

The parent MCF-10A cell line, therefore, represents the nearest to “normal” breast epithelial cell line available, allowing the effect of a single oncogene transfection to be more easily discerned. Transformation with the *ras* oncogene is, however, especially interesting. *ras*-Transformation of human and murine fibroblasts has been shown to cause alkalinisation of the lysosomes (Jiang *et al.*, 1990). Alkalinisation of acidic compartments, such as endosomes and lysosomes, in turn, causes disturbances in proteinase processing and secretion (Nishimura *et al.*, 1988). As mentioned in Chapter 1, default secretion may be due to a lack of acidification of the late endosome, preventing dissociation of receptors and ligands. This would result in immobilisation of mannose-6-phosphate receptors in swollen, late endosomal bodies, effectively preventing them from recycling and participating in proteinase trafficking (Brown *et al.*, 1986). This could cause proteinases to enter the constitutive, default, secretory pathway.

The acquisition of the MCF-10A and MCF-10A-Neo T cell lines, provided a unique opportunity to study the effect of the transforming c-Ha-*ras* protooncogene on the distribution of proteinases in a defined breast tumour cell line, in comparison to its “normal” counterpart. This study is especially relevant, as overexpression of the c-Ha-*ras*-specific protooncogene mRNA and its protein product, p21, is reported to occur in 60-70% of human primary breast tumours (Ciardiello *et al.*, 1990). Consequently, it was felt that some immunocytochemical studies on these cells should be reported here, even though these are at a very preliminary stage.

5.1.2 Confocal microscopy and electron microscopy on Lowicryl resin embedded sections

As indicated in the previous study on leucocyte proteinases, fluorescent microscopy is often of unique value when an “overall” impression of the distribution

of a specific antigen is required. Immunofluorescence combines a high sensitivity of antigen detection with the low magnification of light microscopy.

The epifluorescence microscope, mainly used in this study, was acquired late in the time frame of the studies reported here. However, during the course of this study, a unique opportunity arose to use a "state of the art" confocal microscope. Access to a Zeiss Laser Scanning confocal microscope, equipped with three independent lasers, a Silicon Graphics Workstation and Voxelfview software, was afforded by an invitation, from Dr B. Sloane, to visit her laboratory in the Department of Pharmacology, Wayne State University, Detroit, Michigan.

The confocal microscope allows low light video detection and digital image analysis. It can be used to quantitate the spacial distribution of fluorescent probes with a resolution of approximately 0.2 μm in the x and y axes and 0.8 μm in the z axis. By comparison, conventional light microscopes capture images with a depth of field, at high magnification, of between 2 to 3 μm (Dr Sonia Wolfe-Coote, pers. comm.). By focusing the laser beam to a point, which is scanned back and forth, "optical sectioning" (confocal microscopy) through a cell is possible. The plane of optical sectioning can be altered in stepwise fashion and the resulting series of images can be collected and stored in a digitised form, allowing subsequent 3-D image reconstruction.

In the present study, the 3-D reconstruction facility was not used, but the "optical sectioning" capability was used, in some cases, to discern whether labelled compartments were distributed on the apical or baso-lateral surface of the cell, i.e. to reveal any differences in proteinase distribution in the "z"-dimension within the MCF-10A and Neo-T cells.

Reporting results in this study required the selection of the most representative images to illustrate the findings. Conclusions were drawn after long and careful examination of many fields of fluorescently labelled cells, though, often, no one cell is truly representative of the overall impression gained. Although one may strive to be as objective as possible, there appears to be no way of avoiding some subjectivity in the interpretation of immunofluorescent labelling micrographs, a question discussed by Griffiths (1993).

Some preliminary immunolabelling studies, using Lowicryl embedding and progressive low temperature embedding, are also included. The facility to do this

was established in our laboratory by the time this investigation was undertaken and afforded the opportunity to immunolabel MCF-10 cells for examination by electron microscopy, and to explore resin embedding as an alternative to the "cryo" approach for certain studies.

5.2 Distribution of cathepsins B, H, L and D, in MCF-10A and MCF-10A-Neo-T cells using confocal and conventional immunofluorescence microscopy

In the immunolabelling studies reported here, the antibodies previously used in the cryoultramicrotomy studies, reported in Chapter 4, were again used. Double and triple immunofluorescence labelling, however, requires the use of antibodies of different species. This study was facilitated by the fact that a unique portfolio of anti-cathepsin polyclonal and peptide antibodies (described in Section 3.3), raised in different species of experimental animals, had already been assembled in this laboratory.

The anti-cathepsin B polyclonal antibody previously used in the studies reported in Chapter 4, and the peptide antibody raised against residues 192-201 of the mature human liver cathepsin B sequence (Section 3.8) did not prove satisfactory in immunofluorescence labelling. This study, however, shows successful immunolabelling for cathepsins H and L and procathepsin B, using peptide antibodies.

This study concentrates on cathepsins H, L and D, though some results on cathepsin B are reported. Some of this work was done in Detroit, using the confocal microscope, and some was done in this laboratory, using conventional epifluorescence microscopy. Consequently, the results reported here, are comprised of a mixture of conventional and confocal fluorescent micrographs.

5.2.1 Reagents

Immunofluorescence labelling reagents, were as reported in Section 4.7.1. Primary antibodies used, however, were i) the polyclonal IgY anti-porcine cathepsin D (306 µg/ml), ii) sheep anti-human liver cathepsin B (300 µg/ml), iii) anti-ppB22-36 anti-procathepsin B (730 µg/ml) and anti-ovalbumin-conjugated B192-201 (anti-mature human liver cathepsin B) peptide antibodies (739 µg/ml) (characterised as described in Section 3.8), iv) the rabbit anti-sheep cathepsin L (386 µg/ml), and, v) rabbit anti-human cathepsins L- and H-ovalbumin-conjugated peptides (equivalents

of peptide B192-201 of human liver cathepsin B - see Fig. 11 and Section 3.3) (394 µg/ml and 412 µg/ml, respectively) [characterised by Coetzer *et al.* (1991) and Coetzer (1993), respectively].

Other rabbit antibodies used were: the anti-bovine 215 kDa mannose-6-phosphate receptor antiserum (used at 1/20) (from Dr B. Hofflack, EMBL, Heidelberg), and the secondary antibodies, rabbit anti-IgY serum (used at 1/200) and rabbit anti-sheep IgG serum (used at 1/150), prepared in this laboratory by Dr Theresa Coetzer. The fluorescent conjugates used were a green fluorescent, DTAF-labelled, affinity purified, donkey anti-rabbit conjugate, pre-adsorbed against bovine, goat, horse, human, mouse, rat and sheep IgG, to eliminate cross-reactivity (used at 1/40), a red fluorescent, CY3™-labelled, affinity purified, rabbit anti-chicken, not adsorbed against IgGs of other species (used at 1/60) and a Texas Red-labelled donkey anti-rabbit antibody (pre-adsorbed as described for the DTAF-labelled antibody and used at 1/40). These antibodies, and a normal donkey serum, used for blocking, were obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. Mouse anti-β tubulin (used at 1/40) was from Boehringer Mannheim, Germany.

Cell culture components, including insulin, hydrocortisone, Penicillin G/Streptomycin (10 000 units and 10 mg/ml, resp.), Dulbecco's Minimal Essential Medium (DMEM): Hams F-12 Medium, trypsin-EDTA solution (X1) and Hanks balanced saline solution (HBSS) were from Sigma Chemical Co., St Louis, Mo.; Fungizone (250 µg/ml) and horse serum were from Gibco, Paisley, UK, and epidermal growth factor (EGF) was from Upstate Biotechnology Incorporated, Lake Placid, NY.

MCF growth medium. The DMEM:Hams F-12 medium (1 l) was supplemented with horse serum [5% (v/v), 52.6 ml], EGF (20 µg, 0.43 ml), insulin (10 mg, 4.2 ml), antibiotics (100 mg Streptomycin and 100 000 units Penicillin G, 10 ml), Fungizone (0.25 mg, 1.1 ml) and hydrocortisone (0.5 mg, 2.14 ml).

MCF serum-free growth medium. Serum free growth medium was prepared as described above, but the serum was omitted.

5.2.2 Procedure

MCF-10A and Neo-T cells were grown to near confluence in MCF growth medium, in a humidified atmosphere, containing 5% CO₂, at 37°C. Cells were washed with HBSS, trypsinised with 1X trypsin-EDTA solution, and seeded (at approx.

1×10^4 cells/ well) into duplicate 12-well Nunc Multiwell plates, one plate containing a 15 mm sterile glass coverslip, in each well. When cells were near confluence, they were washed with HBSS and incubated overnight in MCF serum-free medium before being immunolabelled.

Immunofluorescent labelling was performed as described in Section 4.7.2. Sections were blocked with 5% normal donkey serum, made up as described for the foetal calf serum described in Section 4.7.2. Fluorescent conjugates, diluted to the required dilution (given above) were pre-adsorbed against unfixed, unpermeabilised, monolayers of the MCF-10A and Neo-T cell lines, in dublicately seeded Nunc multiwell plates (30 min, RT), immediately prior to use of the conjugate, to adsorb out any non-specific antibodies.

The confocal mode of the Zeiss Laser Scanning microscope was used to examine some sections. The HENE (543 nm) laser was used for examination of Texas Red and CY³ labelled sections, and phase contrast images, and the Argon (488 nm) laser for DTAF-labelled sections. Otherwise, sections were viewed in an Olympus BH2 microscope, fitted with a BH2-RFC Reflected Light (epifluorescence) Attachment (Section 4.7.2).

5.2.3 Results

The distribution of proteinase labelling was judged, on the basis of the distance of labelled organelles from the outer membrane of the cell, as perinuclear (normal) or peripheral (abnormal). Generally, a more peripheral distribution of proteinases was observed in the Neo-T cells, compared to the MCF-10A cells.

Immunofluorescent labelling for procathepsin B and cathepsin B generally indicates the presence of a greater quantity and a less perinuclear distribution of procathepsin B in the Neo-T cells than in the MCF-10A cells [Compare Plate 16, Figs 1 and 4, (Neo-T cells) with Figs 2 and 3b (MCF-10A cells) labelled for procathepsin B, and compare Figs 7 and 8 (Neo-T cells) with Figs 6 and 5 (MCF-10A cells) labelled for cathepsin B with peptide (Figs 6 and 7) and sheep antibody, (Figs 5 and 8)]. Labelling for cathepsin B with the anti-B192-201 peptide antibody (Plate 16, Figs 6 and 7) and the sheep anti-human (Plate 16, Figs 5 and 8) antibody, was poor, showing a non-punctate, generalised distribution around the nucleus of the cell, with the peptide antibody giving the least labelling specificity, in that nuclear staining is evident.

Immunolabelling for cathepsin D and L generally indicates that labelling is, once again, perhaps, less perinuclear in the Neo-T cells than in the MCF-10A equivalent [Plate 17, for cathepsin D, compare Fig. 1, (MCF-10A cells) with Fig. 2 (Neo-T cells) and Fig. 3a (MCF-10A) with Fig. 4a (Neo-T): for cathepsin L, compare Fig. 3b (MCF-10A) with Fig. 4b (Neo-T)]. Double labelling for cathepsins D and L indicates that organelles labelling for cathepsin D are more numerous, and only in some cases are colocalised with immunolabelling for cathepsin L [Plate 17, Figs 3a and 3b (MCF-10A), and Figs 4a and 4b, (Neo-T cells) labelled for cathepsin D (Figs 3a and 4a) and L (Figs 3b and 4b)].

Plate 18 illustrates how in both the Neo-T and MCF-10A cells, organelles labelled for proteinases (cathepsin L in this case) have a distribution that is orientated along microtubule tracks [Plate 18, Figs 1a and 1b (Neo-T), and 2a and 2b (MCF-10A), show labelling for β tubulin (Figs 1a and 2a) and cathepsin L (1b and 2b)]. The Neo-T cells generally show a more extended microtubule backbone than the MCF-10A cells (Plate 18, Figs 1a and 2a, respectively). The labellings for cathepsin L, in Plate 18, Figs 1b and 2b, and Figs 3 and 4, illustrate immunolabellings performed with the peptide antibody (Figs 1b and 2b) and with polyclonal anti-sheep cathepsin L antibodies (Figs 3 and 4). The polyclonal antibody gave more consistent, and more intense, labelling. However, the peptide and polyclonal antibodies gave similar labelling patterns, thereby validating the peptide antibody labelling.

Immunolabelling for cathepsin H in MCF-10A (Plate 19, Figs A, C and E) and Neo-T cells (Plate 19, Figs B, D and F) shows that the cathepsin H distribution in these two cell lines appears similar, especially when viewed by conventional fluorescence microscopy. Immunolabelling examined using the confocal facility of the Laser Scanning Microscope, to optically section different planes of the MCF-10A and Neo-T cells and identify areas of maximal labelling, indicates that MCF-10A cells (Plate 19, Figs C and E) have a greater distribution of cathepsin H on the baso-lateral surface of the cell, whereas the Neo-T cells appear to have a greater labelling density on the apical surface of the cell (Plate 19, Figs D and F) (The plane of optical sectioning is identified by the "z" co-ordinate). The Neo-T cells also have a more "spread" distribution of proteinases than the MCF-10A cells (Compare Plate 19 Fig. E with Fig. F).

Plate 20 illustrates the proportionally higher amount of immunolabelling for the 215 kDa mannose-6-phosphate receptor seen in MCF-10A cells

(Plate 20, Figs 1a, 1b and 1c) compared to that seen in Neo-T cells (Plate 20, Figs 2 and 3). Not all cells in any one preparation of MCF-10A and Neo-T cells show immunolabelling for the mannose-6-phosphate receptor. If, in a random selection of 10 fields, cells showing labelling are selected and their receptors counted, the Neo-T cells show an average of 3 punctate receptor labellings per cell, whereas MCF-10 cells give an average of approximately 5-8.

5.3 Immunolabelling of Lowicryl-embedded sections

The natural structure and organelle distribution of an adherent cell is best observed on cells which have not been subjected to proteinase digestion and removal from their substratum (Bou-Gharios *et al.*, 1988). One of the resins which has proved most successful for embedding adherent cells is Lowicryl K4M (Bou-Gharios *et al.*, 1988; Griffiths, 1993). A monolayer of cells is grown in a flaskette and embedded using a simple technique (Bou-Gharios *et al.*, 1988).

An excellent review on the Lowicryl resins and their use is given by Griffiths (1993) and a few points from that review will be repeated here. Lowicryl resins are comprised of mixtures of aliphatic acrylate and ethacrylate esters, the side chains of which may be altered to vary the hydrophilicity of the resin, giving the more hydrophilic K4M variety or the more hydrophobic HM20 resins. K4M, the more hydrophilic resin, was chosen for this study.

K4M resin was designed to have a low freezing point, low viscosity at low temperatures and very good sectioning and beam stability properties. It polymerises in the presence of up to 5% water, at a temperature of down to -40° C, under long wave (360 nm) ultraviolet irradiation (under nitrogen gas, as oxygen inhibits resin polymerisation). These properties enable dehydration and heat polymerisation steps (potentially antigen damaging) to be avoided, while giving excellent preservation of tissue ultrastructure and antigenicity. A refinement of room temperature embedding procedures, giving the best preservation of ultrastructure, is the "progressive lowering of temperature" (PLT) technique, in which dehydration takes place in an ascending series of ethanol concentrations, with descending temperatures (from 0°C to -35°C), in a specially designed refrigerator, equipped with a "heat sink" and nitrogen gas supply, as described by Griffiths (1993). The Lowicryl PLT system was recently established in this laboratory by Philip Fortgens, who did the embedding for the studies reported here.

A procedure modified from that used by Bou-Gharios *et al*, (1988), using a Nunc Lab-Tek Tissue Culture (Multi-) Chamber Slide Vessel, with a glass slide on the under side, was used, as one of the difficulties encountered in embedding a monolayer in Lowicryl is that Lowicryl adversely affects the polystyrene tissue culture flasks in which the cell line is usually grown. Use of the Multiwell Slide Chamber overcomes this difficulty and allows many small blocks to be embedded.

5.3.1 Procedure

MCF-10A and Neo-T cells were grown and seeded in a Nunc Lab-Tek Tissue Culture (Multi-) Chamber Slide Vessel, as described in Section 5.2.2 (without coverslips in the wells). Cells, grown to confluence as described previously (Section 5.2.2), were fixed in 2% PFA, containing 0.2% glutaraldehyde, in HEPES buffer (Section 2.3.2) (15 min, RT) and PLT embedding was performed as described by Griffiths (1993). Sections were cut at right angles to the slide surface, and collected on formvar-coated nickel grids (Hayat, 1986), as described in Section 2.5.2.2.

Immunolabelling, using the sheep anti-human cathepsin B and chicken anti-porcine cathepsin D antibodies (characterised as described in Section 3.8) was performed, essentially as described in Section 4.4.2, except that incubations in gold probes were prolonged to 1 h. Sections were counterstained in uranyl acetate and lead citrate and viewed as described previously (Section 2.5.2.2).

5.3.2 Results

Plate 21, Figs 1 and 2, show sections of Neo-T and MCF-10A cells, respectively, sectioned perpendicular to the slide plane, the basal surface of the cell being indicated with arrowheads. The MCF-10A cells show a greater number of tonofilaments (electron-dense filaments seen in Plate 21, Fig. 2 lower and upper cell, large arrows) than the Neo-T cells. Non-adherent and semi-adherent cells show a greater number of electron-translucent vesicles and "autophagic vacuoles" than adherent cells (Plates 21 and 22) and the Neo-T cells contain more electron-translucent vesicles (Plate 21, Fig. 1) than the MCF-10A cells (Plate 21, Fig. 2).

"Autophagic type" vacuoles, in both the MCF-10A and Neo-T cells are seen to be a complicated conglomerate of membrane-bound, electron-translucent swollen vesicles, which label for cathepsins B and D (Plates 23 and 24). Immunolabelling for these enzymes show that they occur in more electron-dense

aggregates, within the electron-translucent vesicles (Plate 24, Figs. 1 and 2). Double labelling indicates that some electron-translucent vesicles label for both cathepsins B and D, some for only one of these enzymes, while some label for neither (Plate 24, Figs 1 and 2). In a few instances, Neo-T cells are seen to produce protuberances which contain electron-translucent granules containing cathepsin B, and microvilli with cathepsin B associated with their external surface (Plate 25).

5.4 Discussion

5.4.1 MCF-10A and MCF-10A-Neo-T cells

In contrast to some breast tumours and breast tumour cell lines, mortal and immortal MCF-10 cell lines lack amplification, rearrangement or mutational activation of cellular protooncogenes on chromosome 17 (*c-erb/HER-2/neu* and *c-erbA-1*), or chromosome 11 (*int.-2* and *c-Ha-ras*). Such mutations or alterations may, conceivably, have occurred in the MCF-10A immortalisation process, but did not in this case.

Also, using restriction fragment length polymorphism analysis and DNA sequencing, no activating missense mutations in the common activation sites of *Ha-ras* oncogenes have been found, and at least 99% of the *Ha-ras* alleles in the MCF-10A cells were found to be normal in codon 12/13 and 59/61/63 regions (Soule *et al.*, 1990). Analysis for the presence of other *H-ras* mutations, prior to the transfection of the *ras* protooncogene, was also performed on the original MCF-10A cells. This was important as the *ras* protooncogenes have been shown to block calcium-induced senescence in keratinocytes and to initiate epidermal cell neoplasia. The *v-Ha-ras* oncogene has been shown to be both a potent initiator of mammary neoplasia in transgenic mice and to be activated in nearly all rat mammary tumours induced by nitrosomethylurea (Soule *et al.*, 1990).

However, no alterations of any of the above protooncogenes were found to be involved in either the immortalisation or loss of calcium-induced senescence, in the MCF-10 cells. In all obvious respects, therefore, the MCF-10A cell line is the ideal "normal control" and the lack of *ras*-related mutations offers a unique opportunity to study the phenotypic effect of the transformation induced by *ras*-oncogene transfection.

c-Ha-*ras*- transformation of MCF-10A cells, giving rise to the Neo-T phenotype, was found to result in:-

- i) increased expression of transforming growth factor- α (TGF- α) mRNA and secretion of TGF- α ,
- ii) the ability to grow in soft agar and enhanced growth in serum-free medium and,
- iii) reduced mitogenic response to exogenously added EGF and TGF- α (Ciardiello *et al.*, 1990).

All of these are indicative of the acquisition of the transformed phenotype. As reported in human and murine fibroblasts (Jiang *et al.*, 1990), preliminary studies by Sameni *et al.*, (1993) indicate that, upon *ras*-transformation of MCF-10A cells, a slight alkalinisation of the cytoplasm pH was also induced.

These epifluorescence studies indicate that alkalinisation is accompanied by a more peripheral distribution of lysosomes, indicated by labelling for the LAMPS-1 antigen, and cathepsins B and D. Cathepsins B and D are perinuclear and colocalised in the MCF-10A cells, but are only colocalised in the perinuclear area of the NeoT cells (Sameni *et al.*, 1993). This lack of colocalisation possibly reflects a difference in trafficking of cathepsins B and D, which may correlate with the acquisition of the invasive phenotype.

The results of immunolabelling, in the present study, are the first immunolabelling results for cathepsins H and L reported on these cells. The distribution of cathepsin B was difficult to assess, due to the poor quality of the available antisera, but procathepsin B, and cathepsins D, H and L, do seem to show a more peripheral distribution. No membrane-bound cathepsins were evident, in this study.

The results reported here suggest, however, that it might be profitable to investigate, using the confocal Laser Scanning Microscope, whether a more apical distribution of proteinases is a consistent feature of Neo-T cells as compared to a more baso-lateral distribution in MCF-10A cells. A more apical distribution of proteinases, in the Neo-T cells, is consistent with a pattern induced by alkalinisation of endosome/lysosome compartments, in normal epithelial cells exposed to an alkaline pH (Parton *et al.*, 1991) Also, down-regulation of M-6-P receptors seems to parallel transformation.

5.4.2 Antibodies used in immunofluorescence labelling

Immunofluorescence labelling, using the anti-B192-201 peptide antibody and the sheep anti-human liver cathepsin B antibody, proved to be unsatisfactory due to its generalised, non-discrete nature. Similar non-discrete immunolabelling has previously been observed, with other antibodies, where the antibody was used at a dilution greater than optimal. This suggests that a higher concentration of the cathepsin B antibodies in question may give a more specific, punctate labelling pattern.

Immunolabelling of permeabilised cells, in general, seems to require higher concentrations of antibodies than any other sort of labellings. Control immunolabellings, in which non-immune IgG of the same species and at the same concentration as the test, is substituted, however, indicate that the maximum concentration level for which specificity is still possible has almost been exceeded at the test levels. Anti-cathepsin B antibodies proved, once again, to be the most refractory, both to raise and use. A less perinuclear distribution of cathepsin B labelling in Neo-T cells, compared to MCF-10A cells, reported by Sloane *et al.* (1990), however, is indirectly verified by immunolabellings, in the present study, with the procathepsin B peptide antibody.

Though peptide antibodies generally gave weaker reactions, requiring the use of high concentrations of IgG (approx. 700 µg/ml), better quality peptide antibodies, such as the anti-cathepsin L and H antibodies, could be used at lower concentrations (approx. 400 µg/ml). These antibodies gave labelling comparable to that of polyclonal antibodies and proved to be very good reagents.

5.4.3 Electron microscopy of Lowicryl-embedded cells

Griffiths (1993) has cautioned against over-interpretation of fluorescent labelling results. For this reason, confirmatory electron microscopy immunolabelling studies were undertaken.

The reliability of results obtained on the MCF-10A and Neo-T cell lines, depends upon proper characterisation of the ultrastructural features that identify the cells as epithelial and breast in nature (Tait *et al.*, 1990), in the first place, and on the continued monitoring of these features during the course of the study, to ensure that no phenotypic drift has occurred. Upon establishment in our laboratory, this was a

cause for concern, as both the MCF-10A and the Neo-T cell lines, showed some evidence of aberrant morphology.

The source of the aberrant intracellular vesicles seen in both the MCF-10A and Neo-T cells is not clear at this stage. The cells may be showing signs of stress, due to a fault in the CO₂ supply, in the incubator used for the cell culture, at the time. The abnormal vesicles may be a result of alkalinisation of the culture media, due to the low CO₂ levels prevailing at the time of culture. The effects of low CO₂ levels appear to be similar to those of lysosomotropic agents (reagents that increase the pH of acidic cellular organelles) (Brown *et al.*, 1986). Organelles seen here are distended, as previously recorded in other cell lines exposed to lysosomotropic agents (Brown *et al.*, 1986). As mentioned previously, the pH of the external medium is known to affect the pH of internal organelles such as lysosomes and endosomes (Tarnock and Rotin, 1989; Jähde *et al.*, 1989).

Another possibility, however, is that these cells may be expressing more of the MCF-10F, floating cell line phenotype, as it is noteworthy that only the non-adherent cells contain these vesicles. Of the two cell lines that arose from the mortal MCF-10M cell line, the MCF-10A line grows as attached cells in normal concentrations of calcium whereas the MCF-10F grows as free-floating cells in media containing high levels of calcium (Soule *et al.*, 1990). MCF-10F cells have been described as exhibiting the "formation of intracellular lumens.....or channels similar to those reported in numerous normal as well as neoplastic cell types" (Tait *et al.*, 1990), that may be comparable to those seen in this study. Secretion of electron-dense material into these lumens (as seems to have occurred here also - Plate 22, Fig. 2 and Plate 23, Fig. 2) is reported to indicate that the MCF-10F cells, "have preserved their secretory function, although with changed polarity". Immunolabelling studies were temporarily suspended until the origin of the abnormal vesiculation was determined and the true phenotype of these cells was established.

In agreement with the findings from the immunofluorescence labelling studies, however, cathepsins B and D do not appear to be commonly associated with the membrane of these cells, under the conditions under which they were grown. Cathepsin B was found to be associated with the surface of only a very few Neo-T cells in I.E.M. studies (Plate 25).

The preservation of ultrastructural detail and tissue antigenicity, seems to be adequate using this fixation, PLT and Lowicryl embedding technique. The use of PLT and Lowicryl embedding will be useful for future studies on the distribution of multiple antigens. This technique not only allows 3-D reconstruction of cells, by serial sectioning, but also allows alteration of the block orientation. The *in situ* embedding procedure, modified from that of Bou-Gharios *et al.* (1988), as outlined above, also overcomes the otherwise undesirable necessity to trypsinize cells, which may generate artefacts. This technique will, therefore, complement future electron microscopy studies using the "cryo" approach.

The combination of the MCF-10 cell lines and Lowicryl embedding appears very promising for future studies. The MCF-10 cell lines afford the best opportunity yet for unravelling the processes of oncogenic transformation that lead to the development of the malignant phenotype. They are unique in not having been affected by viral or chemical agents (Ciardiello *et al.*, 1990; Soule *et al.*, 1990; Tait *et al.*, 1990), and the MCF-10A line provides the ideal "control" for all future work.

5.4.4 Conclusion

Membrane association of proteinases does not seem to occur upon *ras*-transformation of MCF-10A cells. The more peripheral distribution of proteinases and decreased levels of M-6-P receptors in the Neo-T cells may, however, relate to increased secretion or "directed" secretion, such as seen in osteoclasts (Eeckhout, 1990), and may, therefore, be relevant to invasion.

This question could be addressed by observing cells in the process of invasion and, to this end, *in vitro* assays of invasion are presently being set-up. In these assays, cells are chemotactically activated to invade through a barrier matrix, in an invasion chamber. Immunolabelling of the "invasion front" of cells, in the process of invading, should provide the most direct evidence of the mechanism of invasion and for the involvement of specific proteinases. The matrix could be fixed and processed for "cryo"-immunolabelling (Parton *et al.*, 1991), or, if sections of cells in different orientations are required, the membrane could be embedded in Lowicryl resin.

CHAPTER 6

GENERAL DISCUSSION

The invasive movement of cells, involving a combination of chemotactic activation and diapedesis, and requiring the use of adhesion molecules, hydrolytic enzymes and directed cell movement, is a fascinating subject for study. That it is a phenomenon common to both malignant tumour cells and normal macrophages and PMNs (Terranova *et al.*, 1986; Condeelis *et al.*, 1992), is also a somewhat sinister concept.

Elucidation of the processes of invasive movement has emerged from a blend of techniques drawn from the disciplines of cell biology, biochemistry and molecular biology. Studies of this kind have been spearheaded by microscopy studies, including immunofluorescence studies at the light microscope level and immunocytochemistry at the electron microscope level, and have been considerably assisted by the use of well-characterised, genetically manipulated, cell lines (Ciardiello *et al.*, 1990).

The studies reported in this thesis were aimed, ultimately, at elucidating which proteinases might be involved in invasion, and they have two aspects, which, for ease of discussion, might be designated "technical" and "biological", respectively, though, in places, these tended to overlap. Since development of techniques and equipment, of necessity, preceded the acquisition of biologically significant results, technical aspects of the study will be discussed first.

The central technique used in the present investigation was the "Tokuyasu" technique, of immunolabelling and EM-viewing of fully-hydrated sections, cut using a cryoultramicrotome. For brevity this is called the "cryo" technique. The strength of the "cryo" technique is that results may be generated very rapidly. A tissue may be fixed and processed for cryoultramicrotomy, in the morning; cut, labelled and viewed and the micrograph obtained by the evening of the same day. This is not possible in under 3 days, with alternative systems. This makes the "cryo" technique very suitable for trafficking studies, especially on cell culture systems, where cultures may be easily manipulated to explore different effects. The "cryo" technique is also very suited to the optimisation of fixation and immunolabelling parameters, as it involves only mild tissue processing, which allows the use of weak fixatives, and short fixation regimes. Additionally, it is the only system in which fixation is the only potentially antigen-damaging tissue processing step (Griffiths, 1993).

Having said that, two shortcomings of the technique became obvious in this study: the near-impossibility of replicate sectioning, for comparative purposes, and the fact that the orientation of a block may not be radically changed to allow sectioning in a different plane. This shortcoming became obvious during immunolabelling studies on activated cells, where, in order to demonstrate immunolabelling on the "leading edge", the plane of sectioning must be orientated along the axis of movement.

As is apparent from the structure of this thesis, much method and reagent development was necessary, prior to the application of the "cryo" technique. At the outset, a well-equipped Electron Microscope Unit was accessible and a cryo-ultramicrotome was soon acquired. Modification of some existing equipment, was also necessary, as cryoultramicrotomy and immunocytochemistry were not previously established here. Further items of equipment were obtained in the course of these studies. The order in which some investigations were undertaken, therefore, was dictated largely by the availability of different items of equipment, at different times in the investigation.

The cutting of acceptable frozen sections, in a cryoultramicrotome, is only possible with an exceptionally sharp glass knife. A knife, sharper than is required for cutting sections from plastic-embedded tissues, is essential for cutting frozen blocks. The making of glass knives for cryoultramicrotomy requires skills that are becoming uncommon, as the trend, for sectioning of plastic-embedded material, is towards the use of diamond knives. In this study it was soon learned that, in addition to skill, a precision-built knifemaker is required; one with closer tolerances than are found in all commercially-available glass knife makers, even the latest. Consequently it became necessary to rebuild a knife maker, essentially to eliminate all sources of "play" in the mechanism. The modifications, reported in Chapter 2 and by Morewood *et al.* (1992), increased the success rate, in making acceptable knives, by at least 100%, 9 to 12 knives being selected from a single piece of glass (16 squares).

In practice, it was found that the breaking pins regularly need repolishing, as they develop "flat spots" which disturb the evenness of the breaking stresses. The fact that metal parts expand and contract with the ambient temperature also necessitates adjustment of the machine almost every time a new batch of knives is cut. It was consequently found best to produce a large number of knives at any one time. The quality of the final knife-edge, and its durability in storage, is improved by metal-coating the newly-made knife, *in vacuo*, with a thin layer of tungsten.

Metal-coating, *in vacuo*, is not to be confused with "glow-discharging", *in vacuo*. In the present study, it took some time to find out that "glow-discharging" means ionising the surface of the carbon-coated grid so that the hydrated tissue section will be more likely to adhere. Glow-discharging is achieved by placing the grids in a "Crooke's dark space" (Benade and Pokorny, 1990) which develops in a discharge between two electrodes, closely spaced in a vacuum.

Besides the necessary equipment, a key essential for immunocytochemistry is the availability of adequate antibodies. The production of such antibodies may be considered, at once, both "technical" and "biological". For the production of polyclonal antibodies targeting human antigens, there are three possible approaches, i) to isolate the immunogen from human tissue, ii) to isolate the immunogen from animal tissue, thereby relying on between-species immuno cross-reactivity, or, iii) to use peptide antibodies. All three approaches have been successfully applied, in this laboratory. In the case of cathepsin B, however, the peptide antibody approach was largely used here, with polyclonal anti-human cathepsin B being obtained by collaboration with other workers.

Peptide antibodies (Coetzer *et al.*, 1991) [and the production and characterisation of gold probes (Elliott and Dennison, 1990)], were the subject(s) of the early part of this study. In the course of these studies, much was learned about peptide antibodies. The first, and conceptually most tantalising aspect, concerned the selection and production of peptide antibodies, and the faith that can be placed in peptide selection programmes. Eight peptide antibodies have now been raised in this laboratory, the raising and characterisation of four of these being reported in this thesis. It now appears that this is a costly approach, with useful peptide antibodies being elicited in about 50% of the cases, if only the criteria of hydrophilicity (Hopp and Woods, 1981; 1983) and flexibility (Westhof *et al.*, 1984), are used for the selection of peptides. With the knowledge gained from this study, however, it is considered that this success rate could be significantly improved.

Successful peptide antibodies are worth the outlay expended in their production. Peptide antibody technology obviates the time-consuming difficulties of protein isolation, required for the production of conventional polyclonal antibodies. Peptide sequences selected from the sequences of human antigens may be used to produce "whole protein-reactive" anti-human antigen antibodies, where human tissues are not available for protein isolation and immunogen preparation. Peptide

antibodies can also be made to target regions on the antigen that would not naturally be targeted.

The peptide sequence chosen must fit well into the paratope of an **existing** clone of an antibody-producing cell to elicit high avidity antibodies (Larsson, 1988a). Since the immune competence varies from animal to animal and species to species (Larsson, 1988a), there is no way of predicting this. This is why it is best to inoculate more than one test animal with each immunogen and why the production of useful antibodies, using any criterion for peptide selection, is not assured.

Peptides are highly mobile in solution, only about 1 in $10^4 - 10^5$ peptides adopting the same conformation as in the native protein (Todd *et al.*, 1982). This very mobility makes the different conformations more likely to find "fitting" paratopes, eliciting a "cocktail" of weakly-reacting, conformation-specific, antibodies that may or may not recognise the conformation in the native protein. For this reason, also, an antibody that recognises a protein antigen in a western blot of an SDS-PAGE gel may not recognise the same antigen in fixed tissues and *vice versa*.

The inoculation of "free" peptides appears to produce the poorest antibodies, possibly because the peptide must be more mobile than in a conjugate. Peptides appear partially restrained by conjugation to a carrier protein, as, in general, conjugated peptides elicited the production of higher titre, more avidly-binding antibodies than free peptides, implying the production of the major portion of the constituent antibodies in the polyclonal antibody, against one main, restrained epitope. Choice of the protein conjugate is critical, however. Conjugation with keyhole limpet haemocyanin (KLH) should be avoided, as anti-KLH antibodies are difficult to remove and cause cross-reactivity with many epitopes in tissues. Conjugation to ovalbumin proved a good choice, here.

In this study, it was realised that selection of sequences that will elicit "protein-reactive" antibodies are best made by considering the protein's 3-D structure and by choosing sequences that will be accessible for antibody binding. It is almost impossible to predict "protein-reactive" sequences on the basis of primary sequence only. This is because most "protein-reactive" antibodies recognize conformational epitopes which are themselves difficult to predict from the primary sequence. In a retrospective analysis of the results of this study, the programmes that appear most successful in predicting appropriate sequences were the Chothia accessibility plot (Chothia, 1976), the Welling antigenicity prediction plot (Welling *et al.*, 1985), which predicts protein-

reactivity on the basis of the relative occurrence of amino acids in known antigenic sites, and the T-cell epitope predictive programme (Margalit *et al.*, 1987), which predicts "amphipathic" sequences.

As the T-cell epitope programme predicts, and the flexibility plots of Karplus and Schultz (1985), indicate, some non-mobility in the chosen peptide sequence is advantageous. This is confirmed by the Hopp and Woods (1981) plot, which indicates that immobilisation is best effected by the presence of some hydrophobic region in the peptide sequence.

Perhaps inclusion of the results of the cathepsin B peptides, biases the statistics for the success rate of peptide antibody production, as it has always been difficult to produce anti-cathepsin B antibodies. The best anti-(mature)-cathepsin B peptide antibody has been successfully used on western blots but was not successful in electron microscopy studies and only successful to a limited extent in immunofluorescence studies. This is not surprising, when even the best polyclonal anti-(whole protein) human liver cathepsin B antibody, available at the time, was unsuccessful and proved difficult to use in electron microscopy studies. The procathepsin B peptide antibody, anti-ppB22-36, was, however, very successful and useful.

Peptide antibodies may contain cross-reactive antibody species, since they are comprised of a polyclonal mixture of antibodies, against the different conformations which the peptide can adopt. Cross reactive epitopes may be changed in western blotted proteins compared to tissue samples and cross-reactivity may disappear, and hence go undetected. Adsorption controls, similarly, have the peptide in solution and conformations causing cross-reactivity would simultaneously be adsorbed out along with the specific antibody (Larsson, 1988a).

From the experience gained in this study, two controls, are now considered the minimum acceptable, and a third is considered highly desirable for absolute proof of the validity of immunolabelling observed. The first is a check on the specificity of the antibody using a western blot of an SDS-PAGE gel which has been overloaded with 10- to 100-fold more than optimal crude and purified protein antigen (Larsson, 1988a) and probed with the antibody preparation and a non-immune control IgG preparation, with the IgG at the level which is to be used in the immunolabelling. This control indicates non-specific IgG binding due either to the loading of too much protein or use of too high a level of antibody, both of which will be indicated by similar labelling of the control and test. Use of high levels of antibody, to probe the western-blotted

protein, also favours the interaction of low affinity cross-reacting antibodies and will indicate their presence. Antibody levels as high as possible should, therefore, be employed, when testing antibody specificity.

The second vital control, for EM-level immunolabelling, involves the substitution of a non-immune IgG control. The IgG preparation must be used at the same concentration as the immune IgG preparation, to check for non-specific IgG-binding proteins and protein-protein binding interactions. IgG preparations of antibodies are preferred, therefore, as it is easier to use an IgG control preparation with the equivalent amount of IgG present, than with a pre-immune serum. The last control involves the comparison of immunolabelling results with results achieved in another system, preferably a biochemical analytical system.

"Good" antibodies, either peptide or polyclonal, generally give positive labelling results after labelling is attempted using a reasonable range of antibody concentrations (10-250 $\mu\text{g}/\text{ml}$, for "cryo"/immunogold and 200-750 $\mu\text{g}/\text{ml}$ for immunofluorescent labelling) (the first figure being for polyclonal antibodies and the second for peptide antibodies). Provided a relatively short, weak fixation regime is used, a short incubation period (generally between 30-60 min, RT), is generally adequate for immunoreactivity. Poor, low affinity and low avidity, antibodies are easily recognised as they do not produce immunolabelling results easily. Usually they require adjustments to the labelling regime, such as length of time for which the tissue is exposed to the primary antibody, and adjustments of fixation regimes. Generally, in the experience gained from this study, such antibodies are not worth the expenditure of any further effort. A key lesson learned has been that it is important to establish quickly whether the antibody is "good" or not, and only to expend effort on good, high affinity, high avidity, antibodies.

The peptide antibodies used in this study were raised with a view to producing antibodies that would be not only protein-reactive and useful in immunolabelling studies, but also as reagents that would potentially be immunoinhibitory; possibly finding use in the treatment of tumour invasion. Mouse sequences were compared with human sequences (Chapter 3), due to the availability of a mouse model system in which the efficacy of immunotherapy treatments may be tested. The BL6-B16 melanoma cell line, inoculated into the foot-pad of a nude mouse, provides a useful model for studying metastasis, as the melanoma cells metastasise to the lung. Colonies are easily enumerated after the lungs are removed and cleared in glycerol, as colonies form black foci that are easily seen.

Immunolabelling studies (not reported in this thesis) indicate that the BL6-B16 mouse melanoma cell line secretes cathepsin B and has membrane associated cathepsin B, both phenomena that may be relevant in the invasive activity of this tumour. Peptide antibodies produced against the peptide sequences in or near the active site of cathepsin B, proved to be non-immunoinhibitory, however, and immunolabelling with the peptide antibody at the electron microscope level, was unsuccessful. For this reason, the immuno-therapy aspect of this study was suspended, pending the finding of a suitable immunoinhibitory antibody.

Access to equivalent antibodies, raised here in a number of different species, was useful for immunofluorescence studies, due to the availability of species-specific detection systems. Rabbit and chicken antibodies are the most easily produced, but antibodies raised in rabbits are the most commonly produced. For EM-studies, the protein A gold system has advantages, in its stability compared to immunogold probes, and the fact that double and triple labelling is easily performed using only rabbit antibodies. Multiple labelling, using protein A, with intermediate fixation, has the advantage that one detection system type can be used for different antigens, using different sizes of gold probe. Protein A does not react with chicken, IgY, antibodies, but a linking rabbit anti-chicken antibody enables protein A to be used and, simultaneously, gives amplification of labelling.

In the confocal and epifluorescence study reported in the last chapter, it was found that labelling of permeabilised cells seems to require the use of very high levels of antibody compared to other techniques using non-permeabilised cells. This seems to be the most stringent test to which antibodies could be subjected. In such labelling protocols, antibodies raised against free peptides had to be used at a much higher concentration than antibodies raised against their conjugated peptide equivalents, verifying their generally poorer quality. Immunofluorescence, therefore, constitutes a good screening method, for selecting antibodies on which to invest further effort.

Immunofluorescence also gives a better "overall impression" and is, therefore, also best for preliminary assessment of the distribution of antigens in tissue sections or cell culture preparations. Immunofluorescence studies should, therefore, ideally, precede electron-microscope level immunocytochemical studies.

With the hardware and suitable antibodies to hand, the next technical question concerns tissue fixation methods. From the experience gained in this study, it is possible to recommend a simple approach to fixation, which should be widely

applicable. When beginning a labelling study of a new antigen, for which a suitable tissue fixation regime is unknown, fixation of tissue with either 4% or 8% paraformaldehyde (PFA) or 2% PFA plus 0.2% glutaraldehyde (10 min), should be adequate to allow the survival of the antigenicity of almost any antigen (this was true of at least 8 antigens examined in this study). Such a fixation regime should allow the optimisation of the level of antibody to be used in subsequent immunolabelling. If this approach fails, it is now believed, the suitability and quality of the antibody should be questioned. Once successful labelling has been achieved, the fixation protocol may be modified to achieve optimal preservation of tissue ultrastructure.

One of the biggest fixation challenges with "cryo" sections of tissues, such as PMNs, filled with large, highly extractable material, is that, as the sucrose used to retrieve the section from the knife falls away from the grid-adherent section, when it is floated out onto buffer, the granule contents and other extractable components may fall away with it. Observations made during double immunolabelling procedures, where primary antibody and residual protein A gold reactivity were blocked by an intermediate fixation step (Slot *et al.*, 1991), suggested a solution. When such an intermediate fixation of the section was used, greater immobilisation of granule contents was observed. This suggested that direct flotation of newly-cut sections onto fixative might improve granule retention. This was found to be true and lateral fixation of the sections, prior to immunolabelling, greatly improved granule retention.

Further improvements were made using primary fixation strategies. In these studies on fixation, it seemed that the contents of the granules were not being adequately fixed, perhaps, due to the "nut-shell" effect of fixation strategies that were being used at the time; diffusion of fixative from the cell periphery inwards possibly resulting in premature fixation of the outer membrane of the granule, preventing proper fixation of the contents of the granule. This problem was largely overcome by a combination of the pH and concentration shift strategy protocols reported here and by the use of lateral fixation. Up to now, azurophil granule fixation seems to have been a problem to many researchers, seemingly irrespective of the fixation regime used (Hibbs and Bainton, 1989; Damiano *et al.*, 1986; Cramer *et al.*, 1985; Ganz *et al.*, 1985).

The study on the fixation of elastase in PMNs, has also opened up prospects for further studies on PMNs. The novel approaches to fixation of these cells, reported here, will allow multiple immunolabelling studies that were previously frustrated by

translation of elastase over all the different granule populations. Double labelling of azurophil granules for cathepsin D and elastase, already performed in this study, seems to endorse the existence of previously speculated subpopulations of azurophil granules (Damiano *et al.*, 1988). Double labelling will also assist in the identification of the origin of the novel "secretory body" identified in PMNs. Perhaps this may also be a "thread-like" lysosomal body whose ultrastructure may be improved by a modification of the protocol used here.

A question of central importance to studies on proteinases, using immunocytochemistry, concerns the possible induction of artefactual organelles by fixation protocols, i.e. whether the structures subsequently observed are real or fixation artefacts. For example, fixation appears to have a marked effect on the observed morphology of some organelles, especially lysosomal bodies. These have been shown, in unfixed or extremely rapidly fixed tissues (Swanson *et al.*, 1987; Robinson *et al.*, 1986; Robinson and Karnovsky, 1991), to be naturally **tubular** in nature. The stability of these organelles, in their natural, tubular form, in turn, seems to depend on the stability of microtubules (and possibly actin filaments) (Swanson *et al.*, 1987; Sakai *et al.*, 1989).

Most studies of these organelles have been in macrophages, in which a highly dynamic network of thread-like lysosomal organelles, which participate in autophagy, heterophagy, and pinocytosis, has been revealed. Sakai *et al.* (1989) observed that a characteristic change in macrophage lysosomes was induced by pinocytosis. Long "thread-like", lysosomes (nematolysosomes), capable of dynamic shape changes and movement, were induced in the cytoplasm of such cells. Movement of all types of lysosomes was found to be actin-, microtubule-, and energy dependent. Similar observations have been made in other cell types (Robinson *et al.*, 1986; Robinson and Karnovsky, 1991).

Disagreement seems to exist on how these nematolysosome structures are best preserved. Aldehyde fixation and 5% sucrose was used in the studies of Sakai *et al.* (1989), whereas in the studies of Robinson and Karnovsky (1991) ultrarapid freezing is advocated, followed by freeze-substitution. It seems, however, that calcium and magnesium-containing buffers may be required for the maintenance of these structures (and possibly also the presence of 5% sucrose), as Buckley (1973) reported that similar structures observed in chick embryo cells were preserved by fixation using glutaraldehyde and a calcium-rich, hypertonic, buffer containing 0.3 M sucrose.

In the present study the requirement for calcium-containing buffers may be substantiated by an example of an image captured during confocal observation of immunofluorescent labelling of the MCF-10 cell lines for M-6-P receptor (Plate 20, Figs 1 a and 1b), in which calcium and magnesium-containing buffers were used. In the lower right-hand corner of these figures, an extensive tubular network, possibly a lysosomal body, is visible in an MCF-10A cell. (M-6-P receptor does not occur in lysosomes). These organelles were seen in both cell lines.

Optimisation of fixation for the preservation of these tubular organelles is, perhaps, critical to studies on the distribution of proteinases in normal and cancer cells. Tubular lysosomal bodies were not observed in fMLP-activated cells, in this study, but have been observed in PMA-stimulated peritoneal monocyte/macrophages (Swanson *et al.*, 1987). This suggests that a PMA-activated U937 cell line might be an ideal model for a systematic study of fixation conditions aimed at preserving these tubular bodies.

The pH prevailing during incubation of cells may also have a marked influence on the apparent distribution of lysosomes and on their shape. In cell culture of macrophages, Heuser (1989), reports that alkalinisation of the medium causes the redistribution of lysosomes to the perinuclear area. In epithelial cells, alkalinisation causes the redistribution of late endosomes to the apical surface of the cell (as may be the case in alkalinisation caused by *ras*-transfection in the Neo-T cells), whereas, acidification has the opposite effect (Parton *et al.*, 1991) and causes secretion of mature enzymes (Krepela *et al.*, 1989). Different cells may thus behave differently and unpredictably at different pH values. During fixation, also, pH changes and the absence of microtubule and actin-filament-stabilising ions, such as calcium and magnesium, might change the distribution and shape of the observed organelles. The effect of pH changes, and cytoskeleton-stabilising ion concentrations in fixation protocols should, therefore, be investigated.

Fixation conditions optimised in this study, however, have proved adequate for immunolabelling purposes, and these studies have led to several novel findings, including the observed labelling for cathepsin D and HLE in plasma cells and HLE in eosinophils. The finding of elastase in cells other than PMNs is unexpected, but would serve to explain why more elastase may be isolated from splenic tissue than is commensurate with the number of PMNs present.

The *in vitro* activation study on PMNs and the *in vivo* study of the spleen, are the first cryoultramicrotomy studies of their kind. They are also the first studies that have demonstrated cathepsins G and D and elastase on the surface of activated PMNs, and the first to note the different effects of PMN activation with PMA at different levels. The reported study on endothelial stave cells was the first such study on the lysosomal enzyme content of endothelial stave cells and the first report of their possible secondary function in regulating proteinases. There would appear to be much promise in extending these studies.

For the future, similar studies are planned on U937 cells, differentiated to become PMNs or monocytes/macrophages, and on the MCF-10 cell lines, using a gelatin-agarose slide well diffusion chamber method (Wilkinson, 1986). In fact, the stage is set for many future studies, using cell culture. For example, using Wilkinson's system, it would be interesting to see what happens to the distribution of cathepsin D in fMLP-stimulated monocytes. Monocyte/macrophages are enigmatic cells in which the effects of fMLP-activation do not seem to have been studied. The differentiation-related differences in cathepsin D distribution have not been previously demonstrated in U937 cell lines. Electron microscopy studies on this and on the differentiation-related distribution of cathepsin B would be unique and highly instructive.

In the present study, both cathepsin B and procathepsin B were demonstrated, in monocytes, for the first time, at the electron microscopy level. These studies were made difficult, however, by the relative scarcity of monocytes in peripheral blood cell preparations. The availability of U937 cells, which can be differentiated to the monocyte phenotype, will facilitate such studies and studies on peripheral blood monocytes could be used for result confirmation only.

Studies on the trafficking of cathepsin B were delayed when the anti-ppB64-77 antibody proved unsuitable for immunolabelling. This proved a set-back to planned studies to demonstrate the presence, or absence, of the truncated form of the precursor enzyme in tumour and normal cells. The prospects for useful studies on the differently processed forms of cathepsin B have, however, now been significantly improved as Dr Mach has not only supplied a better anti-mature human liver cathepsin B antibody than that used in this study, but has also supplied two more anti-human cathepsin B peptide antibodies, one against a 9-residue C-terminal extension and the other against a 12-residue N-terminal extension of mature human liver cathepsin B (Mach *et al.*, 1993).

These antibodies will detect an intermediate processing form, between the precursor and mature sequence, that is believed to be found in secretory vesicles and late endosomes only (Dr L. Mach, pers. comm.). Labelling with these peptide antibodies may help define the relative proportion of enzymes entering the secretory pathway, as opposed to the normal lysosome route, in normal and cancer cells. Good model systems for these studies will be the MCF-10, and U937 cell lines, and normal peripheral blood monocytes. These antibodies only became available, however, during the write-up stage of this thesis, when Dr Mach visited this laboratory, for a three-month period.

In the question of which proteinases are involved in the invasive movement of invading cells, some of the most significant results obtained here have been those from *in vitro* activation studies on isolated and activated PMN fractions. Chemotactically activated PMNs were shown, in this study, to exhibit movement-related association of elastase, cathepsin G and cathepsin D with the "invasion front" of activated cells. All of these enzymes have now been shown to be azurophil granule-associated proteinases and are known to be some of the most proteolytically active proteinases present in PMNs. Their association with the invasion front upon activation seems to suggest strongly their relevance in the invasive activity of PMNs. MCF-7 cells have been shown to be capable of chemotactic movement and, although, in the present study, no strong evidence of the association of cathepsin B with the surface of unactivated, *ras*-transformed Neo-T cells has been found, similar movement-directed association of proteinases may occur in chemotactically activated Neo-T cells. Labelling may now be performed with the anti-cathepsins B, H, L and D antibodies and the procathepsin B antibodies optimised in the immunofluorescence studies on the MCF-10 cell lines.

In future studies, it is planned to examine these cells for the association of proteinases with the "invasion front", in the gelatin-agarose chemotractant chamber of Wilkinson (1986). The migrating cells will be fixed *in situ* in the gelatin-agarose and orientated for cryoultramicrotomy and labelling, in blocks cut from the matrix.

Activation studies on PMNs have also shown the presence of swollen organelles which may be related to the activation-related tubular lysosomal bodies that have been observed in macrophages. PMN azurophil granules are known to be the equivalent of lysosomes in other cells, and the presence of elastase in these swollen tubular structures suggests that these may be lysosome-like structures. As studies reported here suggest, these may be secretory bodies which may form transient

connections with the cell surface, integrating their membrane with the external surface, while emptying their contents of enzymes onto the surface of the cell in a non-specific fashion or in a directed fashion. This may be the mechanism by which surface association of proteinases occurs. Discharge of proteinases into an invasion "pocket" may also occur, under certain conditions of activation.

Lysosome associated membrane proteins (LAMPS) have similarly been seen to be associated with the surface of more highly malignant metastatic cancer cells (Hefferman *et al.*, 1989). Together these observations suggest that similar phenomena, as seen in PMNs, may occur in metastatic tumour cells: the non-specific fusion of lysosomal tubular structures with the surface of "activated" tumour cells, or the directed discharge of proteinases into an invasion "pocket" or association of proteinases with the cell surface.

It is difficult to say whether membrane association of proteinases may be a generalised phenomenon in invasive cells. It does seem possible that most chemotactically activatable cells may follow this model. fMLP-activated macrophages have been shown in this study to have no membrane associated pro- or mature cathepsin B associated with the cell surface, but only immunolabelling for cathepsin B was performed. Though membrane expression of proteinases does not seem to be a generalised consequence of malignant transformation, tumour cells may, additionally, require chemotactic activation, for this to occur.

The distribution of enzyme-labelled organelles indicated by immunofluorescent labelling, needs to be confirmed at the electron microscopy level, preferably using a new fixation protocol which will preserve the integrity of any tubular lysosomes present, and a labelling system which will allow a low magnification overview of the immunolabelling. For this purpose, it is planned to use Lowicryl-embedded or "cryo" sections of cells grown on membrane filters and a peroxidase-diaminobenzidine electron-dense detection system (Parton *et al.*, 1991) to give low magnification resolution of labelled organelles.

With regard to the original aims of this study, using the criterion of a surface disposition of the enzymes, it would appear that cathepsins G and D and elastase could play a role in the invasive action of PMNs. In activated monocyte/macrophages, and in *ras*-transformed Neo-T cells, however, none of these proteinases (including cathepsin B which is absent in PMNs), could be detected on the surface. This raises a

question as to whether, in fact, a surface disposition of proteinases is required for invasion, since macrophages, at least, are known to have invasive properties.

Another model that warrants investigation, is the "extracellular lysosome" model, in which it is envisaged that lysosomal enzymes are secreted into an acidified "pocket", formed between the invading cell and the surface being invaded. Immunofluorescence studies on cathepsins B, H, L and D in MCF-10 cells suggest that, in *ras*-transfected cells, organelles containing these enzymes become more peripherally distributed subsequent to *ras* transfection. This phenomenon may be an expression of the malignant phenotype, favouring the secretion of proteinases. The more peripheral distribution of lysosomal enzymes, in transformed Neo-T cells, compared to their normal counterparts, therefore, could be indicative of cells more "prepared" for exocytosis of lysosomal enzymes, and, thus potentially more invasive.

The relative decrease in M-6-P receptors observed in Neo-T cells, suggests a third possibility; that proteinases may enter the default secretion pathway, due to a deficiency in M-6-P receptors.

The question of which model actually applies could, perhaps, best be addressed by observing cells in the process of invasion and, to this end, assays of invasion through a basement-membrane-like substitute, called matrigel, are presently being set-up. Fixation, embedding and labelling of this matrix, as the cells invade, should show - by the most direct means possible, the observation and labelling of the "invasion front" - how invasion is achieved and which, if any, of the proteinases are relevant in cancer cell invasion. It is anticipated that the defined, normal, MCF-10A cell line and its Neo-T, *ras*-transformed, equivalent will be especially useful in these studies.

Future studies on the trafficking of proteinases in the MCF cell lines should also be highly instructive. Dynamic tubular lysosomes may be relevant to the trafficking of proteinases and invasion process in PMNs, other leucocytes and tumour cells. Research into the possible presence, preservation, and function of these tubular structures is, therefore, considered a priority. Fixation studies aimed at ensuring the survival of these organelles are, therefore, planned. These would, perhaps, best be undertaken in PMA-activated U937 cells (monocyte phenotype), in which these lysosomal bodies should be present, as they have been reported to occur in PMA-treated monocytes.

In retrospect, the studies reported in this thesis can be seen to have been a long and, at times, frustrating, climb up a fairly steep learning curve. Nevertheless, the study has been rewarding since, along the way, much has been learned of a technical nature and some biologically relevant findings have been made. Again, in retrospect, these studies can be seen to have constituted a necessary preface to future studies. Only now that the technical problems have been mastered, and the biological questions have become more focussed, can envisaged future experiments be planned and executed with confidence. From this vantage point, therefore, it appears that the most exciting phase lies yet ahead.

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PUBLICATIONS

Short Technical Note

Further modification of the LKB 7800 series KnifeMaker for improved reproducibility in breaking 'cryo' knives

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KEY WORDS. KnifeMaker, reproducibility, 'cryo' knives.

SUMMARY

This note describes further modifications to the LKB 7800 series KnifeMaker to improve the precision of scoring of glass strips and the consequent reproducibility of breaking of 'cryo' knives, by largely eliminating play in the mechanism, and by accurate adjustment of the cutting wheel position.

INTRODUCTION

The principles of knifemaking for cryoultramicrotomy have been discussed by Griffiths *et al.* (1983). Without modification, our LKB 7801B KnifeMaker was found to be incapable of reproducibly making knives acceptable for cryoultramicrotomy. When modified as suggested by Stang (1988), the performance of the KnifeMaker improved, but it was still unable reproducibly to cut to within 0.1 mm of the corner of a 25-mm square, resulting in rejection of a large proportion of the knives.

The principal cause of the irreproducibility was the cumulative effect of a number of manufacturing tolerances, and/or wear, in the clamping head and cutter wheel holder assembly which, together, allowed the cutter to move sideways over a distance of 0.34 mm. Clearly with this amount of play in the mechanism, cutting to within 0.1 mm of the corner was largely a matter of chance. This note describes modifications made to improve the accuracy of the mechanism, as well as other modifications, which have led to a marked improvement in the reproducibility of knifemaking, and a corresponding increase in the proportion of acceptable knives produced.

MODIFICATIONS

The KnifeMaker was first modified according to Stang (1988). This involved manufacture of the aluminium T-shaped holder, and machining of the '38' cam lobes of the scoring selector, to reduce the score length to 12 mm. This 12-mm score was used

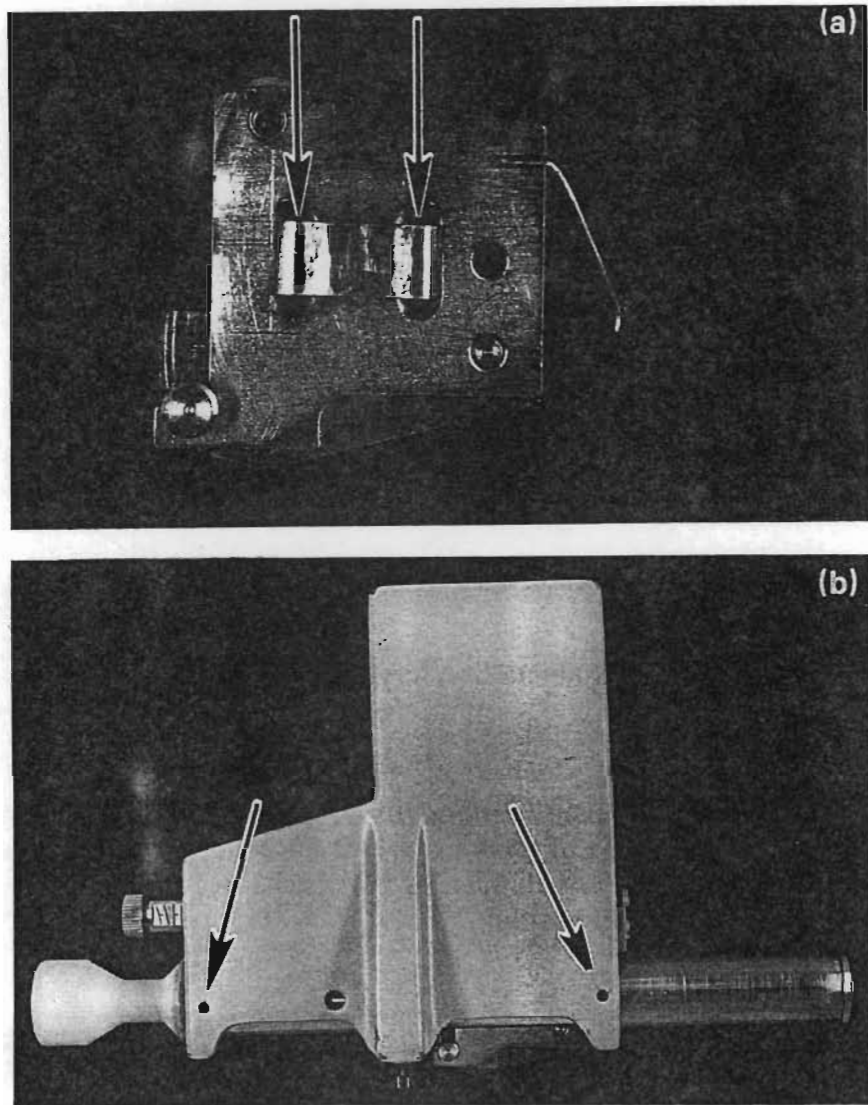


Fig. 1. (a) Illustration of the beryllium-copper spring inserted to eliminate play between the cutter wheel holder bracket (LKB part No. 24-10 688) and the scoring shaft. The arrows indicate the two lines of contact between the spring and the scoring shaft. Note the clearance between the end of the spring and the end of the machined recess. (b) The holes drilled and tapped into the clamping head to accommodate grubscrews. The arrows indicate the position of the two holes.

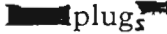
for all glass pieces, including long pieces. In addition, the following modifications were made.

(a) The play between the cutter wheel and the cutter wheel holder bracket was eliminated by removing the cutter wheel and bending the side plates of the cutter wheel holder bracket inwards, by about 0.01 mm at a time, until only a very slight interference fit was perceptible between the cutter wheel and the holder bracket, which nevertheless still allowed free rotation of the cutter wheel.

(b) Similarly, the play between the cutter wheel holder and the cutter wheel holder bracket (LKB part No. 24-10 688; originally 0.06 mm) was eliminated by bending the side plates towards each other, by about 0.01 mm at a time, until only a very slight interference fit was perceptible.

(c) The play (originally 0.11 mm) between the cutter wheel holder bracket and the scoring shaft was eliminated by machining a recess 1.3 mm (deep) \times 6.5 mm \times 15 mm

into the cutter wheel holder bracket and inserting a beryllium copper spring, 0.5 mm thick, bent to form two lines of contact with the scoring shaft, for extra stability (Fig. 1a). This spring must not press too firmly against the scoring shaft, or the cutter wheel holder bracket will not retract after leaving the cam.

(d) The play (originally 0.06 mm) between the scoring shaft and the clamping head was eliminated by drilling two 2.5-mm holes, into the clamping head (Fig. 1b), and tapping these with a 3-mm I.S.O. thread. Small Teflon  plugs were inserted into the holes, followed by grubscrews that were tightened to an extent which just allowed free travel of the scoring shaft.

(e) The breaking pins, which apply the breaking force to the glass, were removed, smoothed in a lathe with fine water paper and buffed to a polished finish.

(f) The play between the scoring shaft guide and the scoring shaft itself was eliminated by bending the outer 'forks' of the scoring shaft guide outwards, a small amount, until a slight interference fit was achieved in the scoring shaft groove.

The angle at which the glass breaks, which should be 90° to the glass surface, is determined by the exact positioning of the cutter wheel scoring edge over the domed breaking pins. Consequently, it is necessary that the cutter wheel is accurately centralized and locked between the support studs to ensure that the score is directly over a line joining the axes of the breaking pins. The holes in the scoring shaft guide are larger than the screw diameters and so the position of the scoring shaft guide can be altered. Movement of the scoring shaft guide causes rotation of the scoring shaft and thus lateral displacement of the cutter wheel. The necessary adjustment can be made, without waste, by test breaks while cutting the glass strips into 50×25 -mm rectangles. If the cutter wheel is off-centre, the glass will not fracture at 90° to the plane of the glass. If this is so, the shaft guide screws may be loosened slightly, the shaft guide plate moved in the appropriate direction to compensate, and the screws retightened. Further test cuts may be made and small corrections made by loosening the shaft guide screws, tapping the plate left or right and retightening the screws. This procedure is repeated until the break in the test piece is true and vertical. When the cutter wheel is properly centred, the KnifeMaker will break optimal knives, with cutting edges at 90° to the plane of the glass. Conversely, if the cutter wheel is off-centre, the resulting knives will have suboptimal, out-of-square cutting edges. Proper adjustment in this respect greatly improves the accuracy of knife breaking.

Accurate and reproducible positioning of the glass pieces is also important. The rear and front glass holders have tongues which slide in slots in the base of the KnifeMaker. On our KnifeMaker the clearance, between the tongues and the slots, was about 0.11 mm, which enabled each glass holder to move left or right by about 0.08 mm, and this affected the reproducible positioning of the glass. The play was consequently eliminated, by peening the end of each slide tongue on the *inner* side and filing the raised section to a slight interference fit, using a no. 4 needle file. While this approach proved adequate, the same effect might be better achieved using appropriately inserted captive balls and springs.

In addition to the modifications and adjustments to the KnifeMaker itself, it was necessary to orientate the glass strips and glass pieces in the KnifeMaker in a specific manner. The glass strip should be orientated on the KnifeMaker with the scored long edge break downwards and the clean edge break upwards. When using 25-mm strips, the strip is first cut into 25×50 -mm rectangles, marked as shown in Fig. 2(a) with a felt-tipped pen and cut into two 25-mm squares. The resulting squares are orientated in the KnifeMaker, for breaking, as shown in Fig. 2(b). It is important to check that the glass strips used have consistent dimensions: this was not true of glass strips from all manufacturers.

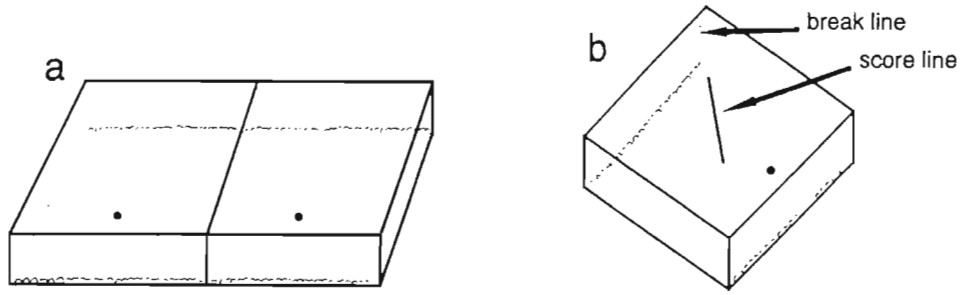


Fig. 2. Orientation of glass blocks for cutting 45° knives, from a 25-mm strip. (a) Marks are made on the glass rectangle as shown, using a felt-tipped pen, before it is cut into 25-mm squares. (b) The 25-mm squares are orientated as shown, before being broken into knives.

CONCLUSIONS

Analysis of the performance of an LKB 7800 series KnifeMaker revealed that its random failure was largely due to an accumulation of manufacturing tolerances, and of wear in the mechanism. After the play was eliminated it was still necessary to adjust the cutter wheel position accurately in order to obtain acceptable knives. With the modifications and adjustments outlined above, the number of acceptable knives that could be obtained from one strip of glass was improved by at least 100%.

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FIXATION AND POST-LABELLING PROCEDURES FOR IMPROVEMENT OF TISSUE STRUCTURE FOR ENZYME IMMUNOLOCALISATION FOR CRYOULTRAMICROTOMY

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The choice of fixative, and fixation conditions, for immunocytochemistry, must primarily be aimed at preservation of antibody-antigen recognition reactions. Tissue integrity, though of secondary importance, must also be adequately preserved. Fixation must allow definition of organelles and prevent target antigen extraction or translocation. With labile antigens such as the enzymes in human leucocytes and spleen, the fixative which met these criteria most successfully, consisted of a high concentration of paraformaldehyde (8% v/v), used at pH 8,0 and for short times (15 min). Paraformaldehyde (8%), in combination with post-labelling lipid-stabilizing procedures¹, considerably improved tissue structure and antigen retention.

Human splenic tissue (2 x 2 mm) and Ficoll-Hypaque® gradient fractionated human polymorphonuclear leucocytes (PMNs) and macrophages, were fixed in either 0,1%, 0,2%, 0,5%, or 1% glutaraldehyde (15 min, RT), in 8% paraformaldehyde (pH 7,2), or 4% paraformaldehyde (pH 7,2) (5 min, RT) followed by 8% paraformaldehyde (pH 7,2) or 8% paraformaldehyde (pH 8,0) (15 min, RT) (all v/v, in either 0,1M sodium cacodylate or 0,2M HEPES). Specimens were washed, cryoprotected, mounted on a copper stub and frozen in liquid nitrogen². Ultrathin sections were cut using tungsten-coated glass knives, using an RMC-MT-600XL ultramicrotome fitted with a CR-200 cryo-attachment. Sections were immunolabelled for the enzymes, elastase, myeloperoxidase and cathepsins B and D, and for lactoferrin², using a goat anti-rabbit (30nm) or a protein A gold probe (4nm). Controls included omitting the primary antibody, substituting pre-immune serum or IgG at the same concentration as the test, and the substitution of pre-adsorbed sera or IgG. After labelling, the section was either directly contrasted by a "positive-negative" contrast procedure using uranyl acetate and methyl cellulose², or post-fixed with 1% glutaraldehyde or with neutral uranyl acetate, followed by the "positive-negative" contrasting procedure.

Fixation with any concentration of glutaraldehyde resulted in a marked decrease or extinction in immunolabelling for elastase. Fixation with 8% paraformaldehyde (pH 8,0) followed by neutral uranyl acetate treatment, before the "positive-negative" contrasting procedure, resulted in the best preservation of ultrastructure and general antigenicity, and retention of antigen *in situ* (figs 4 and 5). Ultrastructural detail was found to be enhanced in the latter, slightly thicker sections (approx. 200nm). Less extraction of cytosolic constituents was evident in tissues fixed in fixatives in HEPES buffers (figs 2, 3, 4, 5 and 6) as compared to those fixed in cacodylate buffers (fig. 1). The occurrence of aldehyde fixation artefacts, such as "blebs" and vesiculation (fig. 6) was also found to be reduced by post-labelling lipid-stabilisation by neutral uranyl acetate, or further glutaraldehyde fixation.

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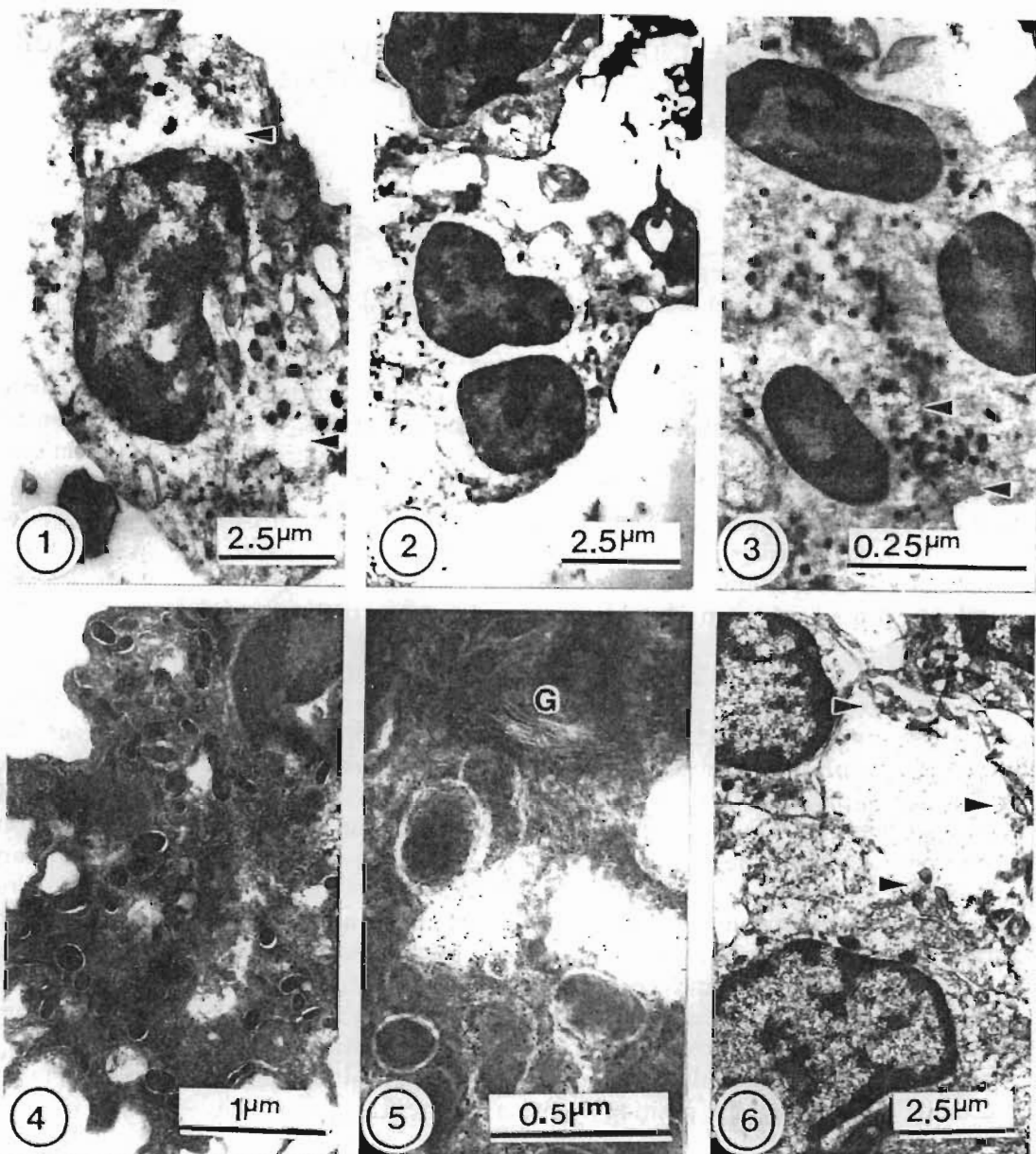


Fig 1. A PMN fixed (15 min, RT) in 8% paraformaldehyde (in 0,1m sodium cacodylate, pH 7,2) and labelled for elastase. Note decreased cytosolic density (arrows).

Fig 2. PMN fixed (15 min, RT) in 8% paraformaldehyde (in HEPES, pH 7,2) and labelled for elastase (without post-fixation). Note poor preservation of ultrastructure.

Fig 3. PMN fixed (5 and 10 min, respectively) in 4% paraformaldehyde followed by 8% paraformaldehyde (in HEPES, pH 7,4) and fixed post-labelling with 1% glutaraldehyde. Note translocation of elastase antigen (arrows).

Fig 4.& 5.

Activated macrophage fixed (15 min, RT) in 8% paraformaldehyde (in HEPES, pH 8,0) post-fixed with neutral uranyl acetate, labelled for pro-cathepsin B. Note improved organelle membrane definition. G = Golgi.

Fig 6. Human spleen fixed (15 min, RT) in 8% paraformaldehyde (in HEPES buffer, pH 8,0) without post-fixation. Note marked membrane "blebbing" artefacts (arrows).

POSSIBLE SECONDARY FUNCTION OF SPLENIC ENDOTHELIAL STAVE CELLS REVEALED BY IMMUNOLABELLING STUDIES

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The reticular meshwork of the cord cells and the phagocytic macrophages in the sinuses of the red pulp of the spleen (fig. 1), are thought to be responsible for the removal of aged erythrocytes from the blood. Phagocytosis, and hence removal of ageing, more rigid, erythrocytes may be favoured by prolonged exposure to the high concentrations of hydrolytic enzymes in extracellular fluids in the sinuses¹. Such exposure may lead to the observed loss of sialic acid residues and the exposure of galactose sugars on outer membranes of ageing cells, possibly leading to phagocytosis by phagocytic cells¹. In the present study, immunolabelling of human splenic tissue for elastase and cathepsin D has revealed that regulation of the levels of extracellular hydrolytic enzymes may be achieved by pinocytosis by splenic endothelial stave cells.

Human spleen (2 x 2 mm) was fixed in 8% paraformaldehyde in 0,1 M sodium cacodylate (pH 8,0, 20 min), cyroprotected by infusion with 2,1 M sucrose (30 min), mounted on a copper stub and frozen in liquid nitrogen². Sections were double immunolabelled for elastase and cathepsin D using 3 nm and 10 nm protein A gold probes. Control labellings consisted of omitting the primary antibody, substituting pre-immune or pre-adsorbed serum or IgG at the same concentration as the test, and performing labellings for one antigen at a time (single labelling). After labelling, sections were contrasted by a "positive-negative" contrast procedure, using uranyl acetate and methyl cellulose².

Cathepsin D has not previously been localised in neutrophils by immunocytochemistry. The present labelling indicates that cathepsin D is present at a concentration approximately ten fold lower than that of the enzyme elastase, and is co-localised with elastase in the azurophil granules of neutrophils (figs 2 and 3). This concurs with biochemical findings. The presence of elastase and cathepsin D, in some pinocytotic vesicles of endothelial stave cells, adjacent to stimulated neutrophils (fig. 3), suggests the co-secretion of these enzymes by neutrophils and simultaneous uptake by endothelial stave cells. The smaller stave cell vesicles, are probably pinocytotic vesicles (figs 2 and 3), due to their size, relatively low cathepsin D content (non-lysosomal nature), and, in the absence of neutrophils, their usually low content of the enzyme elastase. The relatively high content of cathepsin D (usually a lysosomal marker enzyme) in larger stave cell vesicles, present adjacent to the pinocytotic vesicles, indicate that the larger organelles are possibly lysosomes. The uptake of enzymes and hence regulation of the level of enzyme concentration in extracellular fluids in the splenic sinuses, by pinocytosis, may be a mechanism by which endothelial cells may perform a secondary function, the control of levels of extracellular enzymes.

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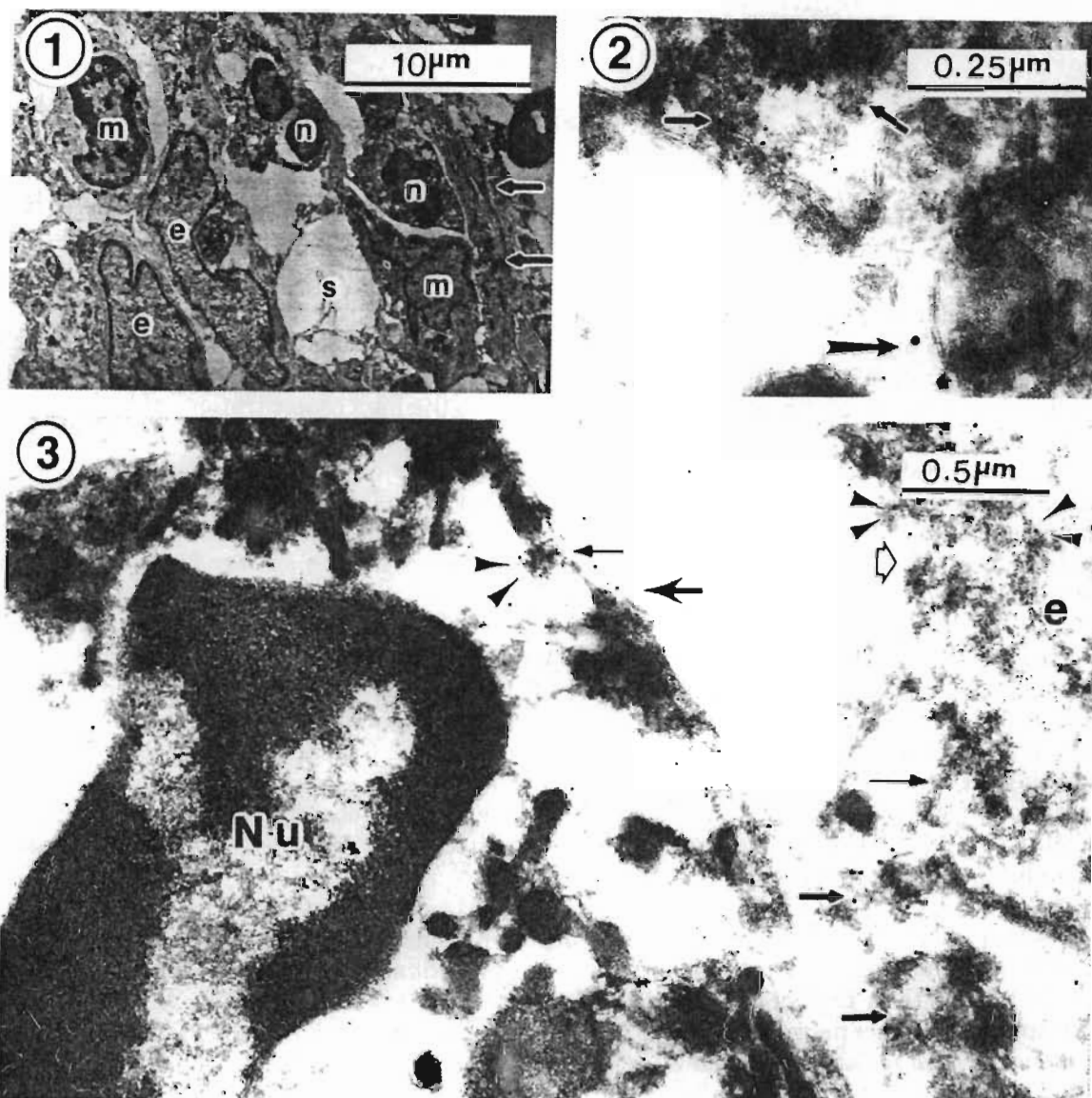


Fig 1. An area of red pulp from a human spleen showing endothelial stave cells (e), macrophages (m), basement membrane (arrows), sinuses (s) and neutrophils (n).

Fig 2. Enlargement of vesicle populations seen in the section of the endothelial stave cell (lower right, fig. 3 below). The smaller, pinocytotic vesicles contain mainly elastase (3 nm gold label, small arrow), while larger vesicles (lysosomes) contain only cathepsin D (10 nm gold label, large arrow).

Fig 3. Neutrophil (Nu) and adjacent endothelial stave cell (e) labelled for cathepsin D (10 nm gold label, large arrow) and elastase (3 nm gold probe, small arrow). Note pinocytotic vesicles (open arrow head) and lysosomal compartments (medium arrow) and co-localisation of elastase and cathepsin D in azurophil granules of Nu and some pinocytotic vesicles (double arrow head).

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Anti-peptide antibodies to cathepsins B, L and D and type IV collagenase

Specific recognition and inhibition of the enzymes

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Anti-peptide antibodies were raised against synthetic peptides selected from the sequences of human cathepsins B and L, porcine cathepsin D and human type IV collagenase. Sequences were selected from the active site clefts of the cathepsins in the expectation that these would elicit immunoinhibitory antibodies. In the case of type IV collagenase a sequence unique to this metalloproteinase subclass and suitable for immunoaffinity purification, was chosen. Antibodies against the chosen cathepsin B sequence were able to recognize the peptide but were apparently unable to recognise the whole enzyme. Antibodies against the chosen cathepsin L sequence were found to recognise and inhibit the native enzyme and were also able to discriminate between denatured cathepsins L and B on Western blots. Antibodies against the chosen cathepsin D sequence recognised native cathepsin D in a competition ELISA, but did not inhibit the enzyme. Native type IV collagenase was purified from human leukocytes by immuno-affinity purification with the corresponding anti-peptide antibodies.

Key words: Anti-peptide antibody; Cathepsins B, L, D; Type IV collagenase; Immunoinhibition

Introduction

Cathepsins B, L and D and type IV collagenase have been implicated in tumour invasion and

metastasis (Liotta et al., 1980; Sloane and Honn, 1984; Denhardt et al., 1987; Spyrtatos et al., 1989). The role of these enzymes in tumour invasion may be explored using specific antibodies and in this context anti-peptide antibodies (Briand et al., 1985) have many advantages. A sequence of ten or more amino acids has a very high probability of being unique to a particular protein and the corresponding anti-peptide antibody is, therefore, also likely to allow highly specific detection of the protein. Moreover, for immunocytochemistry, for example, with polyclonal anti-peptide antibodies against a linear peptide sequence, there is an intrinsically lower probability of the epitope(s) being destroyed during tissue processing, than in the case of a monoclonal antibody which may be targeted at a single, labile, discontinuous epitope.

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Abbreviations: ABTS, 2,2'-azino-di(3-ethyl)-benzthiozoline sulphonic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRPO, horseradish peroxidase; KLH, keyhole limpet haemocyanin; MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; M_r , relative molecular weight; NHMec, 7-(4-methyl)coumarylamide; PBS, phosphate-buffered saline; SBTI, soybean trypsin inhibitor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Z-, benzyloxycarbonyl.

The utility of anti-peptide antibodies may be increased if these are additionally able to inhibit enzymic activity. In the case of the cysteine cathepsins, B, H and L, for example, inhibiting anti-peptide antibodies might constitute tools with a unique ability to discriminate between these enzymes, and might thus aid in their identification. It has also been suggested (Dennison, 1989) that inhibiting anti-proteinase anti-peptide antibodies might be therapeutically useful.

To raise anti-peptide antibodies against the cathepsins, peptide sequences were selected from their primary sequences, mainly by consideration of their 3-dimensional structure, but also with reference to the mobility and hydrophilicity of the chosen peptide sequence. The cathepsins are involved in antigen processing (Takahashi et al., 1989; Van Noort and Van der Drift, 1989) and consequently may be regarded as integral parts of the immune system. The question thus arises as to whether there is any prejudice against production of anti-peptide antibodies to these proteinases, especially against their conserved sequences. As a basis for comparison, therefore, anti-peptide antibodies were also raised against a sequence in a non-lysosomal proteinase, type IV collagenase, similar to that previously shown to successfully elicit anti-peptide antibodies (Höyhty et al., 1988). We report here our observations on raising antibodies to the selected peptides and on the effectiveness of the resulting antibodies in binding to, and inhibiting, the target enzymes.

Materials and methods

Reagents

KLH and MBS were obtained from Sigma. Glutaraldehyde (E.M. grade) and cyanogen bromide were from Merck and ABTS was from Boehringer Mannheim. Human liver cathepsin B was a gift from Dr. D. Buttle, Strangeways Laboratory, Cambridge, U.K. Sheeps' liver cathepsin L was isolated by a modification of the method of Pike and Dennison (1989); chromatography on S-Sepharose, at pH 4.5, being substituted by chromatography on Sephadex G-75. Human spleen cathepsin L was similarly isolated, though in the form of a complex with cystatin, in

a study to be reported elsewhere. Human kidney cathepsin L was purchased from Novabiochem, U.K. Cathepsin D was isolated from human, porcine and bovine spleens by the method of Jacobs et al. (1989). Type IV collagenase was purified from human leukocytes by immunoaffinity chromatography with the anti-peptide antibody immobilised on CNBr-activated Sepharose 4B. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were obtained from Cambridge Research Biochemicals.

Selection of peptides

The peptide sequences from cathepsins B and L (Table I) were selected by considerations of 3-dimensional structure, based on a published structure of the analogous enzyme, papain (Wolthers et al., 1970). The 3-dimensional structures of cathepsins B, H and L have been deduced, from amino acid sequence information, to be comparable to that of papain (Kamphuis et al., 1985; Dufour, 1988).

The sequence selected for cathepsin B, corresponds to residues 13–22 in the structure of human liver cathepsin B (Turk et al., 1986). This sequence is in an accessible position, at one end of the substrate-binding cleft of the enzyme (Wolthers

TABLE I

THE PEPTIDE SEQUENCES SELECTED FOR THE GENERATION OF ANTI-PEPTIDE ANTIBODIES, FROM THE AMINO ACID SEQUENCES OF THE PROTEINASES INDICATED

Peptide	Sequence	Corresponding proteinase
B13–22	Q-C-P-T-I-K-E-I-R-D (+C) ^a	Human cathepsin B
L153–165	E-P-D-C-S-S-E-D-M-D-H-G-V	Human cathepsin L
D112–122	T-K-Q-P-G-L-T-F-I-A-A (+C)	Porcine cathepsin D
COL476–490	M-G-P-L-L-V-A-T-F-W-P-E-L-P-E	Human collagenase IV

^a The selected peptides were modified for synthesis by the substitution of the cysteine residues in peptides B13–22 and L153–165 with α -amino butyric acid and by the addition of an extra cysteine residue to the C termini of B13–22 and D112–122 respectively, in addition to the acetylation of the N terminus of B13–22 and amidation of the C terminus of L153–165.

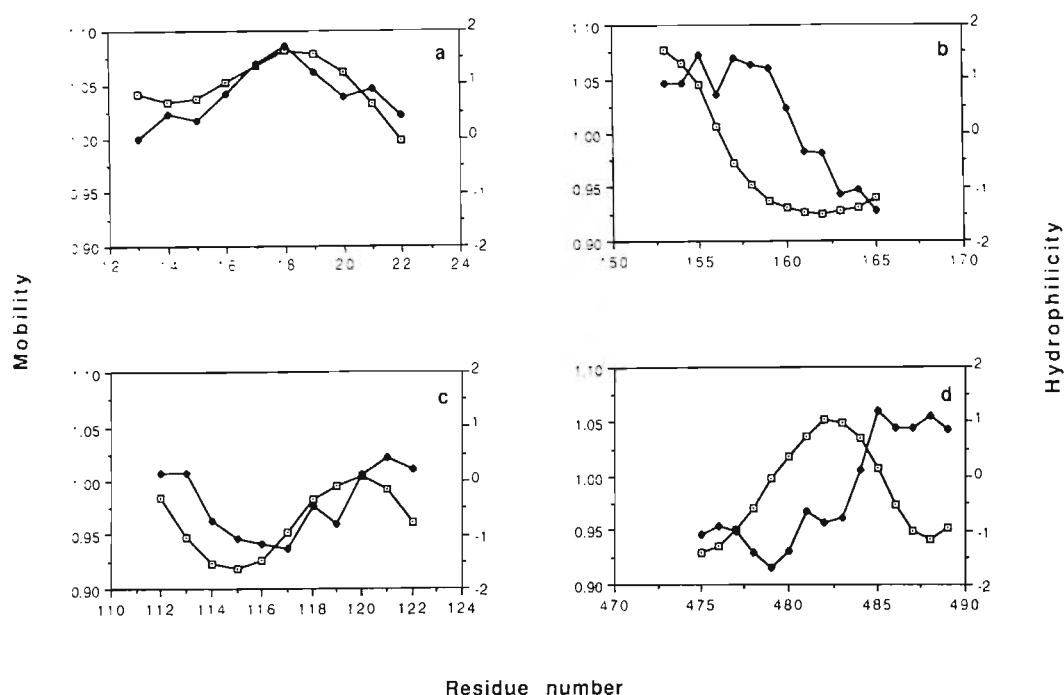


Fig. 1. Hydrophilicity and segmental mobility profiles of the selected peptides. \blacklozenge , hydrophilicity, calculated according to Hopp and Woods (1981, 1983); \square , segmental mobility, calculated according to Westhof et al., (1986). Profiles indicated are for the peptides: (a) B13–22; (b) L153–165; (c) D112–122; and (d) COL476–490.

et al., 1970). It also corresponds to a peak of both hydrophilicity (Hopp and Woods, 1981, 1983) and segmental mobility (Westhof et al., 1984) (Fig. 1a).

A sequence different from that for cathepsin B was chosen for cathepsin L, to potentially maximise the information gained from the experiments. Also, the region chosen for cathepsin B is not a suitable choice for human cathepsin L since the human cathepsins L and H have analogous sequences in this region, with seven out of the 11 amino acids being similar or identical (Ritonja et al., 1988). There is thus an increased probability that an anti-peptide antibody to the sequence in cathepsin L may cross-react with cathepsin H.

By contrast, the loop of amino acids containing the active site histidine is also accessible (Wolthers et al., 1970), and there are marked differences in the sequences in this region between the different cysteine cathepsins. The presence of the active-site histidine in this sequence was also thought to increase the probability that antibodies targeting this region might be inhibitory. The chosen se-

quence corresponds to residues 153–165 in the amino acid sequence of human cathepsin L (Ritonja et al., 1988); in papain the comparable residues are 150–161. The sequence is largely hydrophilic but has a cluster of hydrophobic residues towards its C terminus (Fig. 1b). The L153–165 sequence is also relatively conserved between species and may be expressed as Glu-Pro-Asx-Cys-Ser-Ser-A-Asx-B-Asp-His-Gly-Val, where Asx is either Asp or Asn, A is Glu or Lys and B is Met or Leu (Dufour et al., 1987; Ishidoh et al., 1987; Ritonja et al., 1988). An additional criterion in its selection, therefore, was its potential to target cathepsin L across species.

The sequence chosen for cathepsin D (Table I) was based on the 3-dimensional structure of a related aspartic proteinase, penicillinopepsin (Hsu et al., 1977), since no 3-dimensional structure of cathepsin D has been published. The sequence corresponds to residues 112–122 in porcine cathepsin D (Faust et al., 1985), and corresponds to a loop on the rim of the substrate-binding groove of penicillinopepsin. It has low hydro-

phlicity and mobility (Fig. 1c) and differs from human cathepsin D in a single, conservative, substitution of leucine for isoleucine at position 117 (Faust et al., 1985).

The sequence chosen for human type IV collagenase (Table I) is based on the sequence of a CNBr-generated fragment of this enzyme from human melanoma A2058 cells (CB4 peptide), reported by Höyhty et al. (1988) to elicit antibodies which bind only to type IV collagenase and not to related, secreted, extracellular matrix metalloproteinases, such as interstitial collagenase and stromelysin. The sequence corresponds to residues 476–490 in human type IV procollagenase (Collier et al., 1988) and is hydrophilic towards its C terminus and mobile in its centre (Fig. 1d). In the present study the C terminal Lys was omitted from the CB4 peptide to ensure that glutaraldehyde conjugation was effected exclusively through the N terminus, thereby exposing the hydrophilic part of the peptide.

Synthesis of peptides

The selected peptides were modified, before synthesis, by the substitution of the cysteine residues in peptides B13–22 and L153–165 with α -amino butyric acid and by the addition of an extra cysteine residue to the C-termini of B13–22 and D112–122 respectively. The resulting peptides were custom synthesised by Multiple Peptide Systems, San Diego, CA.

Conjugation

All four peptides were conjugated to KLH, using two different conjugation methods. Peptides B13–22 and D112–122 were conjugated, through their C termini to KLH, using MBS (Robertson and Liu, 1988). The maleimide content of KLH-MBS was determined by the addition of mercaptoethanol and subsequent assay for reduced thiol content (Kitagawa and Aikawa, 1976). Due to their solubility differences, it was necessary to treat B13–22 and D112–122 differently. B13–22 was dissolved in 200 mM sodium phosphate buffer, pH 8.0, and D112–122 was dissolved in the same buffer, but containing 8 M urea, before reduction and conjugation. The method of Sedlak and Lindsay (1968) was used to determine the peptide reduction. Peptides L153–165 and COL476–490 were conjugated to KLH, through

TABLE II
INOCULATION PROTOCOL

Week	Freund's adjuvant	Site	Dose
0	Complete	s.c. ^a	200 μ g conjugated peptide
2	Incomplete	s.c.	200 μ g conjugated peptide
3			Bleed
6	Incomplete	s.c.	200 μ g conjugated peptide
8			Bleed
10	Incomplete	s.c.	200 μ g conjugated peptide or
	-	i.v. ^b	1 mg free peptide
12			Bleed
Monthly boosters as indicated for 10 weeks			

^a s.c. = subcutaneous injection on the back at each of five sites.

^b i.v. = intravenous in marginal ear vein.

their N termini, using 1% (v/v) glutaraldehyde, according to Briand et al. (1985). A carrier protein-to-peptide ratio of 1:40 was used.

Inoculation protocol

For each peptide two rabbits were inoculated with peptide conjugate according to the protocols summarized in Table II. For comparison the protocol of Richardson et al. (1985) was followed, in which conjugate was replaced by free peptide from week 10. B13–22 was only subjected to the latter protocol.

ELISA for anti-peptide antibodies

Wells of microtitre plates (Nunc Immunoplate) were coated overnight at room temperature with peptide solution in PBS, pH 7.2, at 5 μ g/ml (B13–22 and L153–165), 0.5 μ g/ml (D112–122) and 1 μ g/ml (COL476–490). Wells were blocked with 0.5% BSA in PBS for 1 h at 37°C and washed 3 \times with 0.1% Tween 20 in PBS (PBS-Tween). Dilutions of the primary antiserum in 0.5% BSA-PBS were then added, incubated at 37°C for 2 h, and excess antiserum was again washed out 3 \times with PBS-Tween. A 1/200 dilution of sheep anti-rabbit IgG-horseradish peroxidase conjugate, in 0.5% BSA-PBS, was added and incubated for 30 min at 37°C. The ABTS substrate (0.05% in 150 mM citrate-phosphate buffer, pH 5.0, containing 0.0015% H₂O₂) was added and incubated for 15 min. The enzyme reaction was stopped by the addition of 0.1% NaN₃ in citrate-phosphate buffer

and the absorbance was read at 405 nm in a Bio-Tek EL307 ELISA plate reader.

ELISA for immobilized enzyme

The ability of anti-peptide antibodies to cross-react with the respective whole enzymes (not necessarily in their native form) was measured by coating the wells of microtitre plates with either cathepsin B or L (5 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$, respectively, in 50 mM carbonate buffer, pH 6.0, for 3 h at 37°C, followed by overnight at 4°C) or cathepsin D (2 $\mu\text{g}/\text{ml}$ in PBS, pH 7.2, overnight at room temperature). The remainder of the procedure was as outlined above except that IgG was purified from serum, by the method of Polson et al. (1964), to remove serum inhibitors of the enzymes (e.g. cystatin). Species cross-reactivity of anti-peptide antibodies was measured using the same ELISA by coating with cathepsins purified from various sources.

Competition ELISA for native enzyme

The binding of the anti-peptide antibodies to the native cathepsins was tested in an ELISA in which free enzyme was permitted to compete with immobilized peptide for binding to the antibody and thus prevent a fraction of the antibody from being immobilized. Microtitre plates were coated with peptide as described above. Various amounts of antibody (between 10 and 450 $\mu\text{g}/\text{ml}$ IgG) were pre-incubated at 37°C for 30 min with different levels of enzyme (molar ratios of peptide-to-enzyme from 1:24 to 1:0.5), before the incubation mixture was transferred to the peptide coated wells. After a further 1 h incubation at 37°C, the ELISA was developed as described above.

Removal of anti-KLH antibodies

KLH was coupled to cyanogen bromide activated Sepharose-4B according to Kohn and Wilchek (1982). Anti-KLH antibodies were removed from immunoglobulin fractions, purified from serum according to Polson et al. (1964), by passage through KLH-Sepharose.

Immunoblotting

The different enzymes were subjected to reducing SDS-PAGE (Laemmli, 1970), before transfer

to nitrocellulose membranes (Schleicher and Schull, BA 85.0.45 μm) essentially as described by Towbin et al. (1979). Following electro-blotting for 16 h, the nitrocellulose membrane was air dried for 1.5 h and non-specific binding sites were blocked with low-fat dried milk powder (5% in TBS) for 1 h. After this, and at all subsequent steps, the membrane was washed (3×5 min) with TBS. Anti-peptide antibodies, from which anti-KLH antibodies had been removed, were diluted in 0.5% BSA-TBS and incubated with the membrane (2 h), followed by sheep anti-rabbit IgG-HRPO conjugate (1 h). All incubation steps were carried out at room temperature. The HRPO reaction was detected with 0.06% 4-chloro-1-naphthol in TBS, containing 0.0015% H_2O_2 . The reaction was stopped by rinsing in TBS containing 0.1% NaN_3 . Targeting of sheep and human cathepsin L by anti-L153-165 antibodies was also visualised by protein A-gold labelling with silver amplification (Moeremans et al., 1984).

Immunoinhibition assays

Assays for the immunoinhibition of cathepsins B and L were carried out using the substrates Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, respectively, as described by Barrett and Kirschke (1981). Cathepsin B (250 ng) or cathepsin L (25 ng) were incubated at 30°C for 15 min with anti-peptide IgG, or normal rabbit IgG, at the appropriate concentration in 400 mM Na-phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.1% Tween 20. Assays against the Z-Phe-Arg-NHMec substrate revealed that the IgG fractions had intrinsic activity against this substrate, which is probably attributable to contaminating plasma kallikrein which cleaves this substrate (Barrett and Kirschke, 1981). This activity was controlled by the addition of 40 $\mu\text{g}/\text{ml}$ of SBTI, and by subtracting the residual activity in the antibody fractions from the measured cathepsin L activity. SBTI inhibits kallikrein but not cathepsin L. Stopped time assays were carried out over the range of IgG concentrations, and the inhibition by anti-peptide antibodies was calculated in comparison to normal rabbit IgG. Immunoinhibition of cathepsin D was carried out using acid denatured hemoglobin as substrate, essentially as described by Dingle et al. (1971).

Results

Anti-peptide antibody production

All four peptide conjugates elicited antibodies, which reacted with the corresponding immobilized peptides in an ELISA (Fig. 2). In each case, it appears that the antibody titer peaked at about 8–12 weeks. No significant difference could be observed in titer obtained with the two inoculation protocols (using conjugate throughout or changing to free peptide after 10 weeks) when tested against immobilized peptide. Anti-B13–22 antibodies showed a decline after 12 weeks, but this could not be attributed to changing to inoculation with free peptide since anti-D112–122 antibodies, for instance, showed a similar decline in titer after 8 weeks with both inoculation protocols.

Recognition of enzymes coated to ELISA plates

The anti-B13–22 antibodies, although able to recognize the peptide B13–22, were unable to recognize the whole enzyme, coated to a multititer

plate at pH 6.0, 7.2 or pH 9.6 (results not shown). By contrast, anti-L153–165 antibodies were able to recognize both human and sheep cathepsin L, immobilised on ELISA plates (Fig. 3a). They apparently reacted more strongly with the sheep than the human enzyme, from which the peptide sequence was selected, but this may merely be a concentration phenomenon. Human spleen cathepsin L, used in this test, was complexed to cystatin and the measured protein concentration was therefore not a true reflection of the amount of cathepsin L present per se. Anti-porcine cathepsin D, was able to recognize whole human, porcine and bovine cathepsin D enzymes, immobilised on an ELISA plate (Fig. 4). The peptide antibodies, raised against D112–122 (a sequence from porcine cathepsin D), apparently reacted better with human than with porcine or bovine cathepsins D. In the region corresponding to the chosen peptide, the human cathepsin D sequence shows a single substitution of leucine for isoleucine, at position 117, compared to the porcine

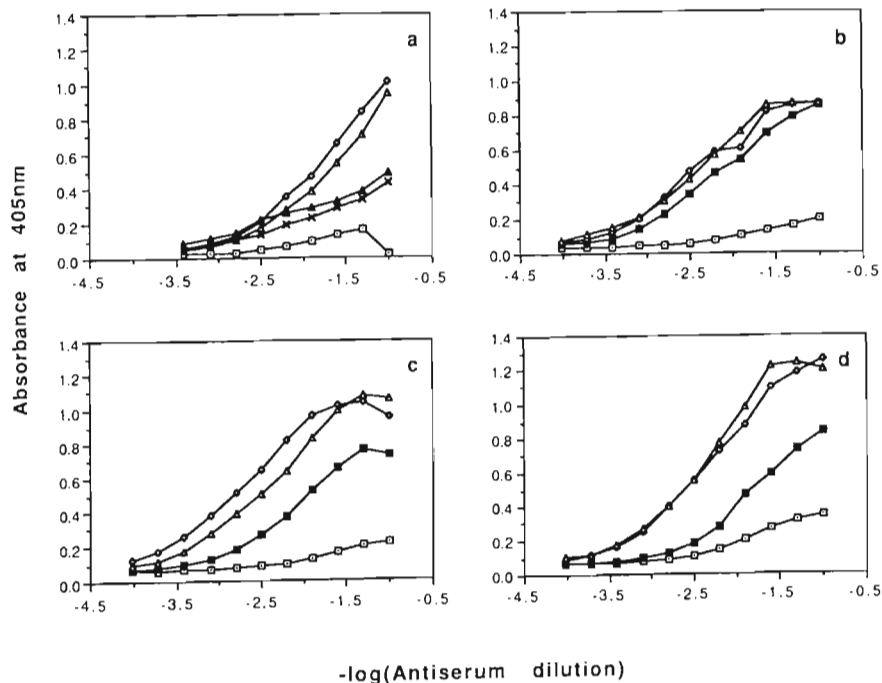


Fig. 2. Progress of immunisation with peptide conjugates as determined by ELISA. Peptides were coated to microtitre plates, (a) B13–22; (b) L153–165; (c) D112–122; and (d) COL467–490 and incubated with serial two-fold dilutions of antisera collected after 3 (■), 8 (◇), 12 (△), 30 (▲) and 32 weeks (×). Normal rabbit serum control (□). This was followed by incubation with HRPO- linked secondary antibody and ABTS as a chromogenic substrate, as described under materials and methods section. Each point is the mean absorbance at 405 nm of duplicate samples.

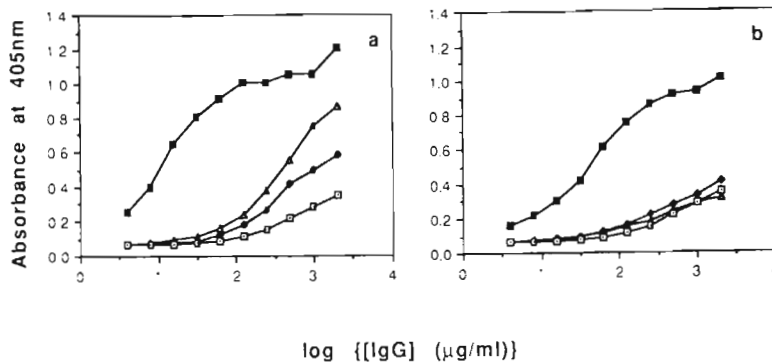


Fig. 3. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin L. Cross-reaction of anti-L153-165 antibodies with human (\blacklozenge) and sheep (\triangle) cathepsin L, and peptide L153-165 (\blacksquare). Normal rabbit IgG (\square). Experimental procedure as in Fig. 2 and in the materials and methods section. (a) anti-L153-165 antibodies elicited by use of conjugated peptide throughout. (b) anti-L153-165 antibodies elicited by use of conjugated peptide followed by free peptide in the inoculation procedure.

enzyme, and it may be inferred from the results that the bovine enzyme must also be very similar in this region. Due to the lack of sufficient enzyme, the anti-COL476-490 antibodies could not be tested against the collagenase IV enzyme, in an ELISA.

Although antibodies raised using the two different immunisation protocols apparently had the same titer against immobilised peptide, a clear difference was sometimes seen in their ability to target the immobilised whole enzyme. In the case of anti-L153-165 peptide antibodies, for example, where conjugate was used throughout, the resulting antibodies cross-reacted with the whole protein to a much higher degree (Fig. 3). This phenomenon was less marked in the case of cathepsin

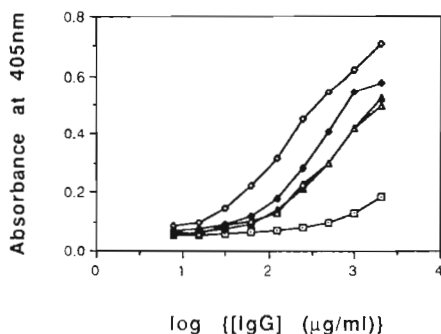


Fig. 4. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin D. Cross-reaction of anti-D112-122 antibodies with human (\blacklozenge), porcine (\triangle) and bovine (\blacktriangle) cathepsin D, and peptide D112-122 (\circ). Normal rabbit IgG (\square). Experimental procedure as in Fig. 2 and in the materials and methods section.

D (result not shown). Anti B13-22 antibodies, tested against whole cathepsin B, did not give a positive reaction at any stage, including at 8 weeks, before the switch to free peptide.

Specificity of anti-peptide antibodies: Western blot analyses

In Western blot analyses it was found that a more specific reaction was obtained if anti-KLH antibodies were removed by passage through a column containing immobilised KLH. Anti-L153-165 antibodies targeted human cathepsin L to a much higher degree than the sheep enzyme and protein A-gold labelling with silver amplification was required to show the targeting of sheep cathepsin L (Fig 5A). The specificity of this targeting was evidenced by the fact that there was no cross-reactivity with human cathepsin B. Anti-B13-22 and D112-122 antibodies did not show any reaction with the corresponding enzymes on a Western blot (result not shown). The anti-COL476-490 antibodies detected a M_r 66,000 band of type IV collagenase purified from human leukocytes (Fig. 5B).

Recognition of native enzymes

Anti-B13-22 antibodies did not interact with the native form of cathepsin B when tested in a competition ELISA and immunoinhibition assays, all at pH 6.0 (results not shown). Cathepsin D inhibited the binding of anti-D112-122 antibodies (250 $\mu\text{g/ml}$) to the peptide coated to multititer plates, in a dose-dependent manner, up to 60% at

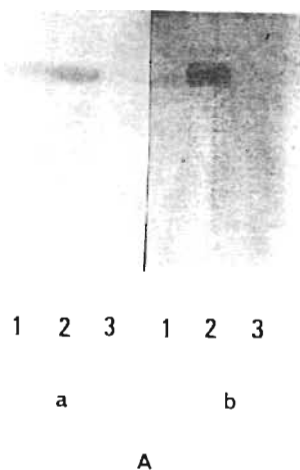


Fig. 5. Targeting of cathepsin L and type IV collagenase by anti-peptide antibodies on Western blots. *A*: samples ((1) sheep cathepsin L; (2) human cathepsin L; (3) human cathepsin B) were subjected to 12.5% reducing SDS-PAGE, electroblotted onto nitrocellulose and then incubated with anti-KLH-purified anti-L153-165 IgG, before developing with (a) protein A-gold with silver amplification or, (b) sheep anti-rabbit-HRPO conjugate as described in the materials and methods section. *B*: human type IV collagenase was electrophoresed on a 7.5% SDS-polyacrylamide gel with reduction, transferred to nitrocellulose and immunologically stained with anti-KLH-purified anti-COL476-490 IgG as described in the materials and methods section.

446 $\mu\text{g/ml}$ (Fig. 6), suggesting that the antibody recognizes the native enzyme. Because of the relatively high concentrations of enzyme required for

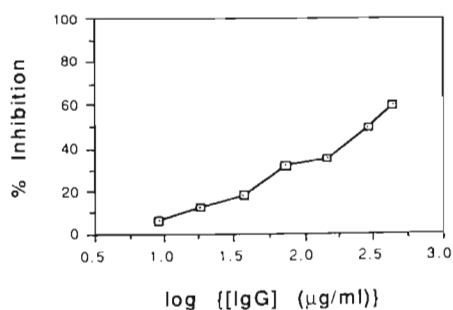


Fig. 6. Competition ELISA for native cathepsin D. The ability of cathepsin D to inhibit the binding of anti-D112-122 antibodies to immobilised D112-122 was measured by pre-incubating various amounts of IgG with different levels of enzyme before transfer of the incubation mixture to peptide coated plates. The ELISA was developed as in the materials and methods section. The percentage inhibition was calculated from control incubations containing either normal rabbit IgG or no competing cathepsin D.

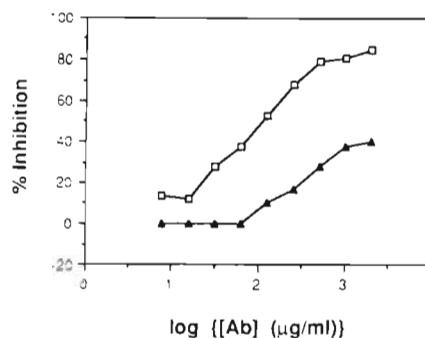


Fig. 7. Immunoinhibition of human and sheep cathepsin L by anti-L153-165 antibodies. Stopped time assays were carried out using human (\square) and sheep (\triangle) cathepsin L as described in the materials and methods section and the percentage inhibition calculated relative to control assays with normal rabbit IgG.

this assay, cathepsin L and type IV collagenase were not included in these tests. Cathepsin D activity was, however, not inhibited by anti-D112-122 antibodies in the enzyme immunoinhibition test.

An indication that anti-COL476-490 antibodies bind to native type IV collagenase is given by their effectiveness in immunoaffinity purification of the enzyme. Type IV collagenase thus purified from human leukocytes showed gelatinolytic activity on a gelatin zymogram (result not shown).

The L153-165 antibodies almost completely inhibited human cathepsin L at high antibody concentrations and inhibition decreased with decreasing antibody concentration until a plateau was reached at low antibody concentration (Fig. 7). Sheep liver cathepsin L was also inhibited, but to a lesser extent than the human enzyme. These results therefore show that the antibody was able to bind to and inhibit native human and sheep cathepsin L. Anti-L153-165 antibodies did not inhibit cathepsin B (results not shown), showing the specificity of this immunoinhibition for cathepsin L.

Discussion

The failure of the anti-B13-22 peptide antibodies to recognize whole human cathepsin B was not expected since the peptide corresponds to peaks of both hydrophilicity and mobility in the sequence

of cathepsin B (Fig. 1a) and, from a consideration of the 3-dimensional structure of papain, it would also appear to be on the surface of the molecule. It has been reported that, in general, segmental mobility is an important criterion for the recognition of the native protein by anti-peptide antibodies (Van Regenmortel, 1988a). This does not appear to hold for the peptide B13-22, and it may be speculated that the presence of a disulfide bridge might, perhaps, constrain the peptide in a particular way in the native protein. There is thus an apparent conflict between the high mobility value assigned to Cys-14, by Westhof et al. (1984) antigenicity prediction profile, and its participation in a constrained disulfide bridge. Comparison with the results obtained for the cathepsin L peptide, L153-165, may be instructive. The peptide L153-165 was conjugated through its N terminus, which is close to the Cys residue involved in a disulfide bridge, and in this case antibodies to the peptide were able to recognize the native protein. In both cases, the Cys residue was substituted by an α -amino butyric residue, but since the peptide L153-165 elicited competent antibodies, this substitution per se is probably not the reason why the peptide B13-22 failed to raise antibodies able to recognize the native enzyme. It may be interesting to examine the possible recognition of the native protein by antibodies raised against B13-22, but conjugated through its Cys residue, or its N terminus.

It must be noted that cathepsin B is generally a refractory enzyme with regard to antibody production and normal polyclonal antibodies, raised against whole cathepsin B, are only able to recognize denatured forms of the enzyme (Barrett, 1973). Monoclonal antibodies against native cathepsin B have been reported (Wardale et al., 1986). Monoclonal antibodies are produced *in vitro*, however, and it may be speculated that there is a prejudice against production of anti-cathepsin B antibodies *in vivo*, due to its involvement in antigen processing. By contrast, antibodies are easily raised against native cathepsin L, and it is interesting to note, in this regard, that Takahashi et al. (1989) have concluded that cathepsin B, and not cathepsin L, might be the major enzyme involved in antigen processing. Therefore, if human B13-22 shares sequence homology with its rabbit

counterpart, any rabbit B cell clones producing anti-B13-22 antibodies capable of recognising native cathepsin B may be suppressed.

The cathepsin L peptide, L153-165, is much less hydrophilic and mobile at its exposed C terminus, than at its N terminus (Fig. 1b), but it was decided to conjugate it through its N terminus so as to expose the active site histidine. This stratagem appears to have been successful in eliciting anti-peptide antibodies able to inhibit native human and sheep cathepsin L (Fig. 7). Due to the specificity of this inhibition, anti-L153-165 peptide antibodies may be useful research tools, since the inhibitors currently in use are unable to discriminate qualitatively between cathepsins B and L (Kirschke et al., 1988). Anti-L153-165 antibodies also discriminate very specifically between cathepsins B and L on Western blots (Fig. 5A), which suggests that they may also be useful in immunocytochemistry. They may also be useful as therapeutic agents in pathologies arising from excessive cathepsin L activity.

A criterion in the selection of the peptide L153-165 was the potential of antibodies to this peptide to target cathepsin L across species. The cross-reactivity between anti-L153-165 antibodies and sheep cathepsin L, immobilised in ELISAs and on Western blots, and in the enzyme immunoinhibition assays (Figs. 3, 5A and 7), confirms this expectation.

Anti-D112-122 antibodies recognized the peptide as well as whole human, porcine and bovine cathepsin D enzymes, immobilised on multiter wells (Fig. 4). Nevertheless, the colour took a relatively long time (about 1 h) to develop in the ELISA assay against immobilised whole enzymes. There is evidence (Van Regenmortel, 1988b) that proteins become partially denatured or undergo conformational changes when adsorbed to solid phases, so the slow colour development may indicate that anti-D112-122 antibodies recognize the partially denatured enzymes only weakly or that only a small percentage of the enzyme adopts a conformation suitable for antibody binding. The antibody clearly recognizes the native form of human cathepsin D, as evidenced from the competition ELISA results (Fig. 6), but does not target the fully denatured enzyme on a Western blot. It may be inferred, from these results, that the epi-

tope in the native enzyme, recognized by anti-D112-122 antibodies, may be a continuous but conformationally specific epitope which is destroyed by reducing SDS-PAGE. Consideration of the 3-D structure of penicillinopepsin reveals a prominent spiral turn in the region corresponding to the D112-122 sequence and suggests that this may constitute such a conformational epitope.

Conjugation of the peptide D112-122, to KLH, was effected through its C terminus, since these residues appear to be less accessible in the native protein; a situation which may therefore be mimicked in the conjugate by the presence of the carrier protein. The more exposed N terminal residues proved to be antigenic and the resulting anti-peptide antibodies were able to bind to the native protein, but were not able to inhibit the enzyme. The paratope-epitope interaction is possibly too distant to occlude the substrate binding cleft.

From a methodological point of view it is of interest that although peptide D112-122 is largely hydrophobic, and was initially insoluble in all solvents tested except 8 M urea, it remained in solution after subsequent removal of the urea and could successfully elicit antibodies able to recognize the native enzyme. The significance of this is that many enzymes apparently have hydrophobic binding sites. From the results obtained using peptide D112-122, it would appear that generation of peptide antibodies against such hydrophobic sites is not impossible.

Omission of the CB4 C terminal Lys-residue (Höyhty et al., 1988), in COL476-490, used in the present study, ensured glutaraldehyde conjugation exclusively via the N terminus. This presentation, exposing the more hydrophilic C terminus, proved to be sufficiently immunogenic to elicit anti-peptide antibodies which are able to interact with native type IV collagenase from human leukocytes in immunoaffinity purification. This result confirms the finding of Höyhty et al. (1988) that the anti-CB4 antibody specifically immunoprecipitated native type IV collagenase from a mixture of metalloproteinases secreted by human melanoma cells and also recognized the denatured proteinase (M_r 68,000) on Western blots following SDS-PAGE. Targeting of a 67,000 band on a Western blot by anti-CB4 antibodies was also

used by Spinucci et al. (1988) to positively identify the proteinase purified from c-Ha-ras oncogene transformed mouse NIH 3T3 fibroblasts. In the present study anti COL476-490 antibodies similarly recognized the denatured M_r 66,000 proteinase from human leukocytes (Fig. 5B). From these results it may be inferred that these anti-peptide antibodies recognize native and denatured type IV collagenase from both normal (leukocyte) and malignant (melanoma) human sources as well as oncogene transformed mouse NIH 3T3 fibroblasts.

From this study it is clear that there is as yet no reliable basis on which to predict which peptides will successfully elicit antibodies capable of recognizing the native target protein. Consideration of the 3-D structure, when this is available, appears to be the most promising approach and was successful with cathepsin L, though not with cathepsin B. It will be interesting, in future, to further explore the structures of cathepsins B and D with a view to finding inhibitory peptide antibodies to these proteinases.

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Analysis of Colloidal Gold Probes by Isoelectric Focusing in Agarose Gels

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Colloidal gold particles of different size (3–20 nm in diameter) were prepared by tannic acid–citrate and citrate reduction methods. From these colloids, different probes were prepared using sheep anti-rabbit antiserum, sheep anti-rabbit IgG, bovine serum albumin, polyethylene glycol, and protein A as the primary stabilizers and polyethylene glycol and/or bovine serum albumin as secondary and tertiary stabilizers, in different combinations. The probes were analyzed by isoelectric focusing in agarose gels, which allow the migration of particles in the size range 3–20 nm. (P. Sewer and S. J. Hayes, 1986, *Anal. Biochem.* 158, 72–78). Isoelectric focusing revealed that the surface charge of colloidal gold probes is dependent upon the size of the gold particle, the reduction method used, the primary ligand, and the pH at which this is adsorbed, as well as upon the secondary and tertiary stabilizers used. It is proposed that such differences in surface charge may underlie the different results which may sometimes be observed in colloidal gold labeling, especially when novel ligands are used. © 1990 Academic Press, Inc.

Colloidal gold particles, commonly with a diameter of about 3–30 nm, to which suitable ligands are adsorbed, may be used as probes, typically for determining the ultrastructural distribution of target molecules of interest (1,2), although they also find application in the analysis of Western blots (3), for example. Most commonly, immunoglobulins are adsorbed to the gold particles, thereby forming immunogold probes (1,4), but other ligands such as protein A (5), lectins (6), peptide hormones (7), etc, have also been successfully used. When optimally adsorbed, the ligand also protects the colloid against salt-induced aggregation and thus constitutes the primary stabilizer (8).

Optimal irreversible attachment of the ligand to the gold particle is thought to require multipoint contacts, which are favored by the use of the minimum concentration of ligand which will nevertheless stabilize the colloidal gold against an electrolyte challenge (8). At this minimum concentration, the ligand might not coat the entire surface of the gold particles and a further protein, such as BSA,² or a polymer such as polyethylene glycol, is required to additionally stabilize the colloid. If they were not quenched³ by the secondary stabilizing agent, exposed portions of the gold surface might also bind non-specifically to components of the sample being probed. Finally, to prevent aggregation of coated particles, especially during centrifugation, the probes are normally stored in a tertiary stabilizing solution, usually of 1% BSA or polyethylene glycol (8).

Although colloidal gold probes have been successfully prepared with ligands of relatively large molecular weight, such as immunoglobulins (M_r 160,000) or protein A (M_r 42,000), we have encountered unexpected difficulties in our (unpublished) efforts to make probes for tissue proteinases, using relatively small proteinase inhibitors (M_r ca. 12,000) as ligands. The proteinase inhibitors successfully stabilized the colloids against an electrolyte challenge, but we were unable to prepare functional, *specific* probes. On Western blots and dot blots, blocked with different agents, probes prepared in different ways either targeted both the proteinase and certain standard proteins (lysozyme and carbonic anhydrase) or failed to target any proteins. The former observation suggested that residual binding sites may be inad-

² Abbreviations used: BSA, bovine serum albumin; PEG, polyethylene glycol; SαR, sheep IgG anti-rabbit IgG; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; IEF, isoelectric focusing; TA, tannic acid.

³ Colloidal gold probes may be used to analyze dot blots and Western blots, for example, and the term "quenching" (9), in reference to secondary stabilization of the colloidal particles, is preferred to the term "blocking," which may be confused with blocking of sites on the blotting membrane.

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equately quenched, while the latter suggested that, by steric hindrance, the primary ligand may be masked or the ligand may be displaced from the gold particle. It must be realized that the stabilizing and quenching proteins are of about the same size as the gold particles (10) and the numbers of each bound may be a function of particle size. In some cases this may lead to inadequate quenching. The relative sizes of the ligand and quenching agents may also be relevant in masking.

In an attempt to explore these observations further, we were motivated to find a way of analyzing differences in the surface properties of colloidal gold probes of different size, prepared and stabilized in different ways, with the ultimate objective of ascertaining whether the conditions of adsorption, stabilization, and storage could be manipulated to produce functional probes with a greater specificity.

Electrophoretic studies on proteins in solution, and bound to colloids, indicate that the charge on a colloid is usually obscured by the adsorbed layer of protein (11–13). Electrophoretic mobility is a function of the charge exposed in the shear layer of adsorbed protein (11). The charge on the underlying colloid may, however, have an indirect influence on electrophoretic mobility by influencing the orientation in which proteins are adsorbed. Isoelectric focusing is a technique commonly used to analyze the surface charge properties of proteins and we report here on an extension of this technique to a study of the surface charge properties of protein-coated colloidal gold probes.

MATERIALS AND METHODS

Chemicals. Protein A (Catalog No. P6031) was obtained from Sigma (St. Louis, MO), chloroauric acid (Catalog No. 1582) and Coomassie blue R-250 from Merck (Darmstadt, West Germany), general chemicals (AR Grade) and polyethylene glycol (PEG 6000 and 20,000) from BDH Chemicals (Poole, U.K.), and bovine serum albumin (BSA, fraction V preparation) from Boehringer (West Germany). Unless otherwise stated, all reagents were made up using water purified by reverse osmosis in a Millipore R/Q water purifier; this is referred to as "distilled H₂O."

Reagents. For the 10% BSA quenching solution, 1 g BSA was dissolved in 20 mM Tris (10 ml), adjusted to pH 8.2 with NaOH, and filtered through a 0.22- μ m filter. For the 1% PEG 20,000 quenching solution, 0.1 g PEG was dissolved in distilled H₂O (10 ml) and pressure filtered through a 0.22- μ m filter.

IgG was isolated from a sheep anti-rabbit IgG antiserum, by a modification of the method of Polson *et al.* (14). PEG 6000 was added, to 15% (w/v), to a mixture of equal volumes of antiserum and pH 8.6 borate buffer. The IgG precipitate was collected by centrifugation (7740g, 10 min, 4°C), redissolved in an equal volume of

0.1 M phosphate buffer, pH 7.0, and reprecipitated with PEG 6000 (15% w/v). The IgG precipitate was dissolved in 10 mM Na-phosphate buffer, pH 6.8, and a total of 39 mg IgG was applied to a hydroxyapatite column (11.0 \times 1.5 cm) and eluted with a gradient of 10–300 mM Na-phosphate, pH 6.8. The major eluted peak was collected, extensively dialyzed against distilled H₂O (4°C), and used for the production of sheep anti-rabbit IgG–gold probes (S α R probes). The absence of PEG in the eluted peak was established using Nessler's reagent (14).

A total of 32 probes were prepared, as listed in Table 1. Gold particles 3.5 nm (2–3.8 nm), 5 nm (4–5 nm), and 10 nm (10–12 nm) in diameter were prepared by the tannic acid–citrate method (15) and particles of 10 nm (8–10 nm), 15 nm (13–18 nm), and 20 nm (20–22 nm) were produced by the modified citrate method (16); these are referred to below as TA probes and Cit probes, respectively.

The titration method of Zsigmondy, as described by De Mey (8), was used to determine the dilution of protein required for probe production. Endpoints of stabilization against salt aggregation were judged visually (17), and were assessed for all probes in one batch procedure.

Adsorption of the sheep IgG anti-rabbit IgG (S α R) fraction to gold particles was effected at pH 7.0 (18), the pH of the colloid being adjusted with K₂CO₃ (25 mM). The determined amount of S α R for probe production was added slowly to the relevant colloid, while mixing. The IgG and colloid were allowed to interact for 2 min and residual protein binding sites on the colloid were quenched with either BSA or PEG 20,000 quenching solutions, added to a final concentration of 1 or 0.05% (w/v), respectively (8). Probes were washed twice in 20 mM Tris, pH 8.2, containing either 1% BSA or 0.05% PEG 20,000 and stored at 4°C (8). Probes 32 and 34 were washed in 0.01 M phosphate buffer, pH 7.2, containing 1% BSA (15), instead of the Tris buffer. "Probes" were also prepared omitting the primary ligand.

Probes were similarly prepared by adsorption of whole S α R serum or BSA (as a primary stabilizing protein) to variously sized particles at pH 7.0 and pH 5.5, respectively. Protein A–gold probes were prepared at pH 7.0 (15). Particles used in the production of protein A probes were washed and size-fractionated in a glycerol gradient using PBS or TBS buffers, containing 1% BSA, pH 7.2.

Isoelectric focusing. Isoelectric focusing was performed using an LKB 2117 Multiphor system. A 1% agarose (Pharmacia IEF) gel (115 \times 250 \times 1.0 mm) containing Pharmalyte pH 3–10 and 12% sorbitol was cast, according to the manufacturer's instructions, onto a sheet of GelBond, using a sealed mold. The set gel was positioned in the Multiphor apparatus and blotted, and gold probe samples (20 μ l) were pipetted into slots (8 \times 5 mm) in an acetate sheet template placed on the surface of the gel, parallel to the cathode wick and about 20

TABLE I
 Immunoprobes Produced^a

1	S α R	(TA-Au _{3.5})	PEG:PEG				
2	S α R	(TA-Au ₅)	PEG:PEG				
3	S α R	(TA-Au ₁₀)	PEG:PEG	19	S α R	(TA-Au ₁₀)	PEG:BSA
4	S α R	(Cit-Au ₁₀)	PEG:PEG	20	S α R	(Cit-Au ₁₀)	PEG:BSA
5	S α R	(Cit-Au ₁₅)	PEG:PEG	21	S α R	(Cit-Au ₁₅)	PEG:BSA
6	S α R	(Cit-Au ₂₀)	PEG:PEG	22	S α R	(Cit-Au ₂₀)	PEG:BSA
7	S α R	(TA-Au _{3.5})	BSA:BSA				
8	S α R	(TA-Au ₅)	BSA:BSA				
9	S α R	(TA-Au ₁₀)	BSA:BSA	34	PA	(TA-Au ₁₀) _P	BSA:BSA
10	S α R	(Cit-Au ₁₀)	BSA:BSA	32	PA	(Cit-Au ₁₀) _P	BSA:BSA
11	S α R	(Cit-Au ₁₅)	BSA:BSA	33	PA	(Cit-Au ₁₀)	BSA:BSA
12	S α R	(Cit-Au ₂₀)	BSA:BSA				
				31	PA	(Control)	
				35	PA	(TA-Au ₁₀)	BSA:BSA
13	BSA	(TA-Au ₁₀)	PEG:PEG				
14	BSA	(Cit-Au ₁₅)	PEG:PEG				
15	PEG	(TA-Au ₁₀)	PEG:BSA				
16	PEG	(Cit-Au ₁₅)	PEG:BSA				
17	Nil	(TA-Au ₁₀)	PEG:BSA	23	Nil	(TA-Au ₁₀)	BSA:BSA
18	Nil	(Cit-Au ₁₀)	PEG:BSA	24	Nil	(Cit-Au ₁₀)	BSA:BSA
25	S α R _{serum}	(TA-Au ₁₀)	BSA:BSA				
26	S α R	(Control)					
27	S α R _{serum}	(Control)					
28	S α R _{serum}	(TA-Au ₁₀)	PEG:PEG	30	S α R _{serum}	(Cit-Au ₁₀)	PEG:PEG
29	S α R _{serum}	(Cit-Au ₁₀)	BSA:BSA				

^a Abbreviated notation: Probe number-ligand (colloid) quencher:tertiary stabilizer. The notation of Goodman *et al.* (18) is used to denote the gold particle size. Thus Au₁₀ indicates gold particles with a diameter of 10 nm. PA = protein A; S α R (and S α R_{serum}) = sheep IgG (or serum) anti-rabbit IgG. Probes 32 and 34, marked with a subscript P, were made in phosphate buffer (15); all other probes were made in Tris buffer (8).

mm from it. Electrofocusing was effected using 10 W at 10°C, and was completed in about 5–6 h. The template was removed after 35 min. The gels were fixed, washed, dried, photographed, stained with Coomassie blue R-250, and photographed again. Before staining, stable probes appear red while destabilized probes appear a light blue. After staining, proteins not adsorbed to probes appear deep blue, while the stable probes remain red.

RESULTS

“Naked” gold colloids (to which no stabilizing proteins were adsorbed) rapidly become destabilized on exposure to agarose, containing ampholytes. Stabilized gold colloids, however, could be successfully evaluated by agarose IEF and yielded reproducible results for each probe, although the results changes slightly with the age of the probe. The results suggest that the apparent charge on the stabilized probes is influenced by the methods of preparation, stabilization, quenching, and storage of the probes, as well as by the size of the gold particles.

Effect of the size of gold particles. Particle size appears to have a marked effect on the electrophoretic properties of otherwise comparable probes. Smaller gold

probes (3.5 and 5.0 nm) generally focused to a lesser extent (Fig. 1, tracks 2, 3, 11, and 12). Although there are differences between probes prepared by different methods, probes 4–6 (Fig. 1, tracks 7–9) and probes 7–9 (Fig. 1, tracks 11–13) show a trend of greater anodal migration with smaller particle size.

Effect of method of colloid production. The effect of the reduction method used in producing the colloid is shown by comparing probes of a similar size, i.e., probe 3 (Fig. 1, track 4) with 4 (Fig. 1, track 7) and probe 9 (Fig. 1, track 13) with 10 (Fig. 1, track 16). These comparisons reveal some marked differences and that, in general, for 10-nm particles, TA probes give more and more clearly defined bands than the equivalent Cit probes.

The S α R (TA-Au₁₀) probe, quenched and stored in PEG (probe 3; Fig. 1, Track 4), exhibits a definite band at approximately pH 5.8, close to the position of the main band in the S α R IgG fraction used in production of the probe (Fig. 1, arrowheads). Although the equivalent citric acid probe (probe 4; Fig. 1, track 7) gives a very different overall result, a similar, faint band is nevertheless discernible. The electrofocusing patterns for differently produced BSA-quenched probes (probes 9 and 10) (Fig. 1, tracks 13 and 16), however, appear generally similar to each other.

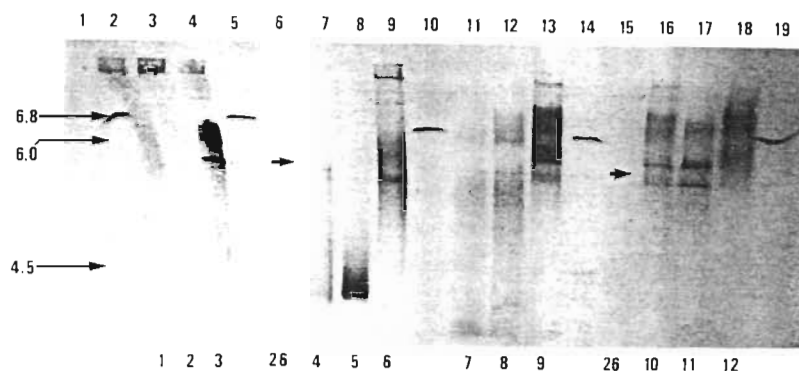


FIG. 1. Effect of particle size, quenching, and stabilizing agents upon agarose gel isoelectric focusing of gold probes. Particles of different size (3.5–20 nm), quenched and stored in either 0.05% PEG 20,000 or 1% BSA, are shown in tracks 2–4 and 7–9 and tracks 11–13 and 16–18, respectively. Protein bands were revealed by subsequent staining with Coomassie blue R-250; for clarity the gel is shown before staining. In this and subsequent figures, the approximate focused positions of standard marker proteins, myoglobin (*pI* 6.8), carbonic anhydrase (*pI* 6.0), and ovalbumin (*pI* 4.5–4.9) are indicated by arrows and the position of the main band of unbound SαR IgG is indicated by arrowheads. Numbers above each figure represent “track” numbers and those below indicate the identity of the probes (Table 1).

Effect of ligand and secondary and tertiary stabilizing agents. SαR–gold probes quenched with PEG 20,000 and stored in 0.05% PEG 20,000 in Tris (Fig. 1, tracks 2–4 and 7–9), show fewer focused bands than those quenched and stored in 1% BSA in Tris (Fig. 1, tracks 11–13 and 16–18). SαR–gold probes quenched with BSA show well-defined bands at approximately pH 6.0 and 5.5 and a faint band at pH 5.8, a band that seems to be common to most of the SαR–gold probes (Figs. 1, 2 and 3). A large diffuse band also occurs on the alkaline side of pH 6.8 in these probes. This band is more defined in the 10-nm TA probes (Fig. 1, tracks 4 and 13) than in the 10- to 20-nm citric acid probes (Fig. 1, tracks 7–9, 16, 17, and 18).

Used as a quenching agent, BSA is usually added at a pH of 9.0, after the adsorption of the primary protein. To gain some insight into the interaction of BSA with gold probes at higher pH, it was added at pH 8.2 to “naked” colloidal gold. The resultant probes (probes 23 and 24) were washed and stored in BSA. The IEF results (Fig. 2, tracks 18 and 14) may be compared to those from the equivalent SαR probes (probes 9 and 10; Fig. 2, tracks 11 and 12). Similar focusing patterns were observed, except that the band in the pH 5.8 region is less dominant than in the SαR–gold probes.

In an attempt to identify the level of influence of the tertiary stabilizers, the IEF patterns of SαR probes (Fig. 2, tracks 3, 4, 5, 6, 7 and 8) and probes without a primary protein adsorbed (Fig. 2, tracks 13 and 14), quenched, washed, and stored with either BSA or PEG, were compared. The substitution of PEG with BSA, as a “storage” or tertiary stabilizer, results in movement of the main group of diffusely focused bands to a more acidic position (Fig. 2, tracks 3–8). Substitution of BSA for PEG as a secondary stabilizer in the BSA-stored probes, in which no primary protein has been adsorbed, has the opposite effect (Fig. 2, tracks 13 and 14).

The effect of BSA, added as the primary stabilizer, may be assessed by comparing probes 13 and 14. In producing these probes, the BSA was adsorbed at pH 5.5, i.e., 0.5 pH unit above its *pI*, and the probes were quenched and stored in PEG. These probes produced bands focused in the pH 4.5–4.7 region (Fig. 3, tracks 2 and 3), corresponding to the region where the unbound BSA protein focused (Fig. 3, track 1). This was markedly different from the pattern produced when the BSA was added to a naked gold probe, at pH 8.2, as a secondary stabilizing agent (Fig. 2, tracks 14 and 18).

Generally, IgG and “serum” probes produce similar patterns. Probes produced from whole SαR serum, (Fig. 3, tracks 8–11) appear to give focusing pattern similar to those produced by the SαR IgG equivalents (Fig. 1, tracks 4, 7, 13, and 16). A different pattern is, however, observed in the focusing of the protein A–gold probes. Though only BSA-quenched and stored probes were made with this protein, the main focused bands (Fig. 3, tracks 13–15) were different from that of the unbound protein A (Fig. 3, track 7). In the citric acid probes only (Fig. 3, track 13 and 14) diffuse bands occurred in the more acidic region. A more dense, diffuse band occurred in the probes in which Tris buffer was used in the final washing steps (Fig. 3, track 14) than observed when PBS was used (Fig. 3, track 13). The tannic acid equivalent, however, shows a large diffuse band alkaline to pH 6.0 (Fig. 3, track 15). These results suggest that both the method of colloid production and the buffers commonly used in the production of gold probes could affect the surface characteristics and hence, possibly, also the binding properties of gold probes.

DISCUSSION

The structure and stability of gold colloids and colloidal gold probes have been discussed by De Mey (8) and

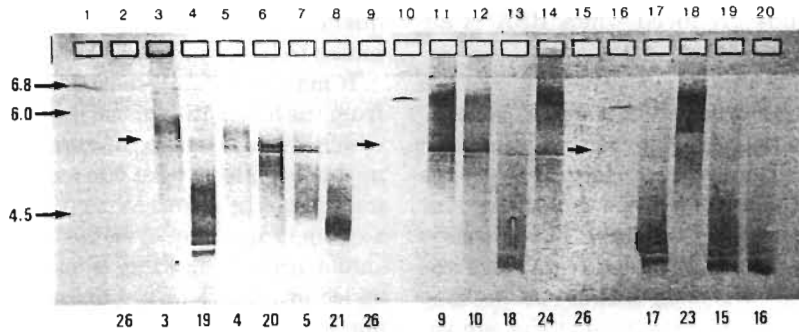


FIG. 2. Agarose gel isoelectric focusing of $S\alpha R$ -gold probes. The effect of the storage conditions (tertiary stabilization) of 10- and 15-nm PEG-quenched probes is shown in Tracks 3-8. The effect of the method of preparation of the colloidal gold (10 nm) is shown in tracks 11 and 12. The effect of secondary stabilizers, PEG vs BSA, applied to "naked" gold probes is shown in tracks 13, 14, and 17-20. Tracks 11 and 12 show the patterns given by probes 9 and 10 when these were 2 months old: comparable patterns, for the same probes at 1 week old, are shown in Fig. 1, tracks 13 and 16. The sample application template was repositioned on the gel before it was photographed, in this case.

Horisberger (19). As demonstrated in this study, isoelectric focusing of colloidal gold probes is able to reveal differences in the interaction of proteins with colloids of different size, and with colloids produced using different methods, at different pH, and in combination with different quenching agents. A knowledge of these differences could provide insight into differences in the labeling behavior of the different probes.

Why do probes focus isoelectrically and what does this indicate? If the interaction of the adsorbed protein with the gold particle was totally random, in terms of the orientation of the adsorbed proteins, it would be anticipated that no focusing would occur. If the number of possible orientations was limited and the number of proteins adsorbed was very small (e.g., one protein mole-

cule per particle), then focusing into a finite number of bands might be anticipated. On the other hand, if the number of molecules adsorbed was small, the charge of the underlying colloid might predominate and give rise to a general anodal drift, without focusing. The exposed parts of the colloidal particles might also adsorb various ampholyte molecules which would cause focusing over a spectrum. If a larger number of protein molecules were adsorbed, focused bands might also be expected if the number of orientations of adsorbed proteins were limited and if all molecules adsorbed to a given gold particle had the same orientation. An interesting question which arises from this is whether all bands would represent functional probes or whether, on some, the ligands might be inappropriately orientated.

In this study, adsorption of proteins to the negative gold colloid seemed to decrease, or leave unaltered, the observed isoelectric point of protein adsorbed at its pI or about 0.5 unit above. The BSA probes show focused bands corresponding to the pI values of the free protein, whereas the pI of adsorbed $S\alpha R$ (IgG) appears slightly decreased. This suggests that most of the charge on the adsorbed proteins is exposed in the shear layer. Experiments with nonionic and ionic surfactants of similar chain length have shown that the compound least soluble and nonionic was bound to colloidal gold in greater proportion (20). If hydrophobic interactions predominate in this way, the bound proteins may be orientated in such a way as to expose their hydrophilic, ionic residues in the shear layer, creating the observed pI . This would explain why the observed pI 's of the adsorbed proteins agree with those of unbound proteins in these cases. In cases where a different pI is obtained upon adsorption, this could be due to a different orientation of the bound protein. Ribonuclease, adsorbed to various negatively charged colloid systems, such as glass, Dowex resins, and Nujol, manifests a pI higher than when unbound (21). Similarly, orientation differences may ex-

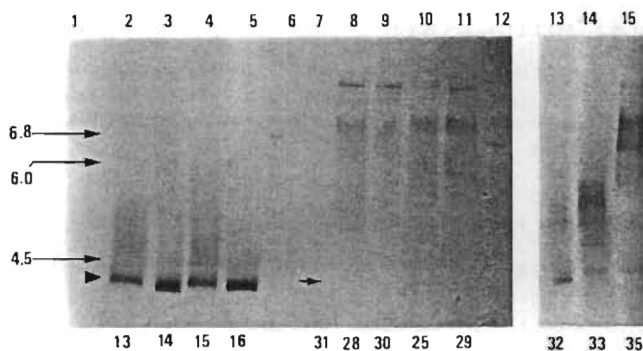


FIG. 3. Agarose gel isoelectric focusing of BSA probes, serum probes, and protein A probes. Tracks 2 and 3 illustrate the effect of BSA adsorbed (at its pI) to gold colloid as a primary stabilizing protein. This may be compared to "naked" colloid, blocked with PEG and stored in BSA (tracks 4 and 5). Results from probes made with whole $S\alpha R_{serum}$ are shown in tracks 8-11, and from protein A probes in tracks 13-15. Leakage of proteins from $S\alpha R_{serum}$ gold probes and BSA gold probes was revealed by subsequent staining of gels with Coomassie blue R-250. Tailless arrowhead indicates focused position of unbound BSA.

plain the different bands produced when BSA is adsorbed at its *pI* or at pH 8.2.

Small gold probes (3.5 and 5 nm) generally focused more diffusely than larger probes. On average, an estimated 1.6 antibody molecules [3.6 nm in diameter (10,11)] may be adsorbed onto the surface of a 3.5-nm gold probe (22). This number of protein molecules may be too small to focus the probe adequately. The presence of BSA, as a quenching agent, also failed to produce the usual distinctive pattern, suggesting that the probes may also be inadequately quenched.⁴ Because of the equivalent size of the small gold particles and the ligand and quenching proteins (10), perhaps only a very few of the latter can bind. Exposed residual binding sites on the colloid surface may interact, with the ampholytes or agarose, giving the diffuse patterns. Such interactions could explain the observed nonspecific interaction of small gold probes with cytoskeletal entities (23). Perhaps the use of smaller quenching molecules may be advantageous with smaller gold particles.

The way in which the gold colloids are produced may affect their surface character and the subsequent focusing of probes. Equivalent probes, with the same protein ligand, made from differently produced colloids, focus differently.⁴ This may explain reports of variations in the nonspecific binding (23) and labeling affinities (19) of gold probes made from differently produced colloids.

The surface structure of gold particles may also be relevant. Colloidal gold particles are roughly spherical, but have surface aberrations thought to result in van der Waal's forces that favor protein adsorption (8). Stepwise irregularities in shape are larger in larger colloids, which may result in greater, and more specific, adsorption of protein to these specific sites. Specifically oriented adsorption, via specific amino acid residues or sequences, may result in more dominant focused bands. The diffuse focusing of smaller probes may, similarly, be explained by their more uniform surface charge, a consequence of their more regular shape. Random adsorption to the regular colloid surface may lead to more diffusely focused bands.

Certain bands seem to recur in the electrofocusing patterns of probes of different size to which different proteins have been adsorbed, suggesting the existence of a common surface feature, expressed more or less in probes of different size. The use of PEG to quench probes, however, seems to obscure differences and results in a smearing of bands. How this correlates with the specificity and labeling characteristics of PEG-

quenched probes, relative to BSA-quenched probes, remains to be established.

It may be difficult to assess which focused bands arise from the adsorption of the primary adsorbed protein and which are the result of interactions of the quenching molecules, either with the colloid itself or with the primary layer of adsorbed protein. When BSA is added as a quenching agent, it seems that it binds directly to residual protein binding sites on the gold probe. BSA, added at pH 8.2, in the presence or absence of the primary adsorbed protein, focuses similarly, suggesting that the BSA binding sites are independent of the primary ligand protein. Interactions of the storage or tertiary stabilization molecule, with the primary and quenching agents, seem to take place in certain instances. BSA, introduced at a tertiary level, seems to change the surface characteristics of PEG-quenched probes and hence may alter their labeling activity. It may therefore, be unwise to dilute PEG-stabilized probes in buffer containing BSA.

There is evidence that -SH and -S-S- groups react with gold surfaces (24). BSA has three main domains containing 9 loops (25) in which 17 disulfide bridges and a free cystine occur (26). BSA has a more extended structure at high pH than at its *pI* (27), and the many bands produced by probes with BSA adsorbed at pH 8.2 (probes 7-12, 23, 24, 28, and 29) may be a reflection of the different ways in which the extended protein may be adsorbed via sulfur-containing residues. A hypothetical, ideal IEF result would have a single, strong band corresponding to the specific ligand and representing an active probe. Such a result may be possible only with non-protein quenching and stabilizing agents specifically engineered for the task.

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⁴ A referee has suggested that the different behavior of colloids produced by the citrate-tannic acid method may be due to the adsorption of residual TA polymers to the gold surface (15). It is suggested that these should be removed by treatment with H₂O₂ before adsorption of the ligand.

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