AN IMMUNOCYTOCHEMICAL STUDY OF THE KALLIKREIN-KININ SYSTEM ON THE CIRCULATING NEUTROPHIL

by

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ABSTRACT

Inflammation is the normal biological response to tissue injury, and is characterised by the interactive activation of multiple mediators and cell types. One response to tissue injury is the production of pain, not only by direct trauma to sensory fibres, but also through the release of mediators from sensory nerve terminals. One such mediator is kinin which is a vasoactive peptide considered to play a primary role in inflammation by causing constriction of venules, dilation of arterioles, increasing permeability of the capillary membrane, and interacting with sensory nerve terminal transmitters to evoke pain.The kinin forming enzymes (kallikreins) reach inflammation sites either on the surface of migrating neutrophils or by transudation from plasma. The kininogen molecule which contains the kinin moiety, has been localised on the external surface of the neutrophil, and provides the substrate from which kinins can be cleaved through enzymatic action. The cellular actions of kinins are mediated through B_2 receptors, which are also located on the external surface of the neutrophils. In addition, the induced effects of kinins are regulated by B_1 receptors. The formation of nitric oxide (NO) from arginine released from the kinin C terminus, and receptor membrane signal transduction by nitric oxide following kinin receptor activation is discussed.

A molecular response to cell injury is the formation of chemotactic mediators that attract neutrophils to sites of inflammation. The question whether neutrophils contribute to circulating levels of kinins was examined in infections and inflammatory disorders. This novel hypothesis was tested using circulating neutrophils harvested from patients with tuberculosis meningitis and pneumonia. These neutrophils showed a distinct loss of only the kinin moiety from the kininogen molecule located on the external surface. The confocal

images of fixed, permeabilised neutrophils provided multi-dimensional constructs, and the intensity of fluorescence reflected the relative amounts of the molecule present in both neutrophils harvested from healthy volunteers as well as patient blood. The immunocytochemical labelling experiments using colloidal gold as markers, confirmed, at the ultrastructural level, the presence or disappearance of the kinin moiety from the kininogen molecule on the neutrophil surface.

The cell component of synovial fluid in rheumatoid athritis (RA) consists mainly of neutrophils. This study demonstrates the absence of the kinin moiety from circulating and synovial fluid neutrophils from patients with RA, as well as an increased signal from immunolabelled B_2 receptors in synovial fluid neutrophils. These findings support the hypothesis that in RA, kinins are released during the inflammatory response in the joints, and suggests that there is an upregulation of the B_2 receptor at the site of inflammation. Neutrophils chemotactically drawn to the site of inflammation become activated to release kinin from the kininogen molecule, and thereafter re-enter the circulation where they were harvested systemically. B_2 receptors may be upregulated following activation by kinins or by other mediators present in the inflammatory milieu. Interleukin-1 has been shown to upregulate kinin receptors on human synovial cells.

Anti-peptide antibodies to the loops of cloned B_1 and B_2 receptors have provided powerful probes for the cellular identification of the two kinin receptor families. Mapping of the B_2 receptors showed upregulation on the neutrophils gathered from inflamed joints. However, no activation of the B_1 receptors was observed in normal blood neutrophils as well as those obtained from the different disease states.

DECLARATION

This study represents original work by the author and has not been submitted in any other form to the University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Experimental and Clinical Pharmacology, Faculty of Medicine, University of Natal, Durban, South Africa under the supervision of Professor K D Bhoola.

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PUBLICATIONS ARISING FROM THIS DISSERTATION

Full Publications

1. Naidoo Y, Snyman C and Bhoola KD. Kinins - Pro-inflammatory peptides. *Continuing Medical Education.* 12 (12), 1591-1595, 1994.
2. Rodell T, Naidoo Y and Bhoola KD. Role of Bradykinin in Inflammatory Responses: Prospect for drug therapy. *Clinical ImmunoTherapeutics.* 3(5) 352-361, 1995.
3. Bhoola KD and Naidoo Y. Translocation of the neutrophil kinin moiety and changes in the regulation of kinin receptors in inflammation. *Immunopharmacology,* 31, In press, 1996.
4. Naidoo Y, Naidoo S, Nadar RN, Bhoola KD. Role of neutrophil kinin in infection. *Immunopharmacology,* 31, In press, 1996.
5. Cassim B, Naidoo Y, Naidoo S, Williams R, Bhoola KD. Immunolocalisation of the kinin moiety and BK2 receptors on synovial fluid neutrophils in Rheumatoid Arthritis. *Immunopharmacology,* 31, In press, 1996.

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6. Snyman C, Naidoo Y, Rahn H-P, Kemme M, Müller-Esterl W and Bhoola KD. The cellular visualization of tissue prokallikrein in the human neutrophil, kidney and brain. *Canadian Journal of Physiology and Pharmacology,* 72(2), 40 (P2.4), 1994.

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LIST OF ABBREVIATIONS

TK:	Tissue kallikrein
PK:	Plasma kallikrein
HK:	High molecular weight kininogen
LK:	Low molecular weight kininogen
HF:	Hageman factor
PPK:	Plasma prekallikrein
NO:	Nitric oxide
TNF:	Tumour necrosis factor
IL:	Interleukin
PMN:	Polymorphonuclear
PAP:	Peroxidase-antiperoxidase
PBS:	Phosphate buffered saline
BSA:	Bovine serum albumin
WORM:	write once read many

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 *Historical Overview*

Advances in biochemistry and molecular biology in recent years have established the chemical structure and molecular sequences of the various components of the kallikrein-kinin system. The essential significance of the kallikrein-kinin system is of widespread interest to the cellular basis of medicine, in particular, many inflammatory disorders.

Initial observations of the protein later called kallikrein were made by the surgeon, E.K. Frey in 1926 when he injected human urine into dogs, and observed a hypotensive response (Frey and Kraut, 1928). He attributed this effect to a substance that he considered to be a hormone. This substance was later called kallikrein - derived from the Greek synonym for pancreas, where it was present in high concentrations (Kraut et al., 1930), and therefore thought to be its place of origin (Bhoola et al., 1992). The substance protein kallikrein, responsible for the observed hypotensive effect was isolated and subsequently shown to be a non-dialysable, thermolabile substance of high molecular weight (Frey and Kraut, 1928). These observations were followed by many publications by the German biochemist, Eugen Werle, and co-workers (Werle et al., 1937) who demonstrated that kallikrein was present in blood, pancreas and salivary glands (Frey and Kraut, 1928). Werle, in 1948, further went on to study the biochemical properties of kallikrein, and subsequently discovered that kallikrein was a proteolytic enzyme which released a smooth muscle-contacting substance from an inactive precursor (kininogen). Further, it was observed that the new activity increased and then rapidly disappeared. The new, biologically active molecule was called kallidin (Werle and Berek, 1948) and, unlike kallikrein, it was dialysable and thermostable,

contracted the isolated guinea-pig ileum and showed marked hypotensive activity (Werle and Grunz, 1939). Both kallikrein and kallidin showed different responses in inhibition studies; aprotinin inhibited kallikrein but not kallidin. Furthermore, kallidin was irreversibly inactivated by proteases in plasma and tissue extracts, and this inactivation was initially attributed to degradation by peptidases called kininases. Hence, in 1948, kallidin was the first of the "*kinins*" to be named as such.

About this time, in 1949, Brazilian pharmacologists, Beraldo, Roche e Silva and Rosenfeld discovered a kinin (Roche e Silva et al., 1949), which was also released from plasma proteins by trypsin and snake venom proteases. They called this new kinin, bradykinin, because it produced a slow contraction of the isolated guinea-pig ileum. Later, Werle et al. (1950) showed that both kallidin and bradykinin were released from the same precursor proteins, later called the high and low molecular weight kininogens (Fritz et al., 1988). Pharmacologically active peptides similar to kallidin and bradykinin have since been discovered in insect venoms (Schachter et al., 1978). In 1960, Bhoola et al. discovered the presence of a kinin-like peptide in the venom of hornets, and this venom also contained high concentrations of acetylcholine and histamine. Over the years an in depth understanding of the kallikrein-kinin system has been acquired especially knowledge of the two different kallikrein-kinin systems, plasma and tissue.

1.2 *Turnover of kinins*

1.2.1 **Kallikreins**

The kallikreins are a group of serine proteases that are found in glandular cells, neutrophils and biological fluids (Bhoola et al., 1979). On the basis of their molecular weight, isoelectric points, substrate specificity, immunological characteristics and type of kinin released, the kallikreins are designated into plasma and tissue kallikreins.

1.2.1.1 **Tissue kallikrein (TK)**

Although tissue kallikrein was previously discovered in the pancreas, salivary glands and kidney by Werle and his colleagues (Frey and Kraut, 1928), it was only recently discovered in the neutrophil (Bhoola et al., 1992). Initial characterisation of TK was achieved in the porcine (Fritz et al., 1967), rodent and canine pancreas (Hojima et al., 1975). However, early purification of the enzyme was carried out from the salivary glands of the rat (Brandzaeg et al., 1976), cat (Moriwaki et al., 1975), pig (Lemon et al., 1979), mouse (Porcelli et al., 1976) and guinea pig (Fiedler et al., 1983). The discovery, isolation and purification of this enzyme in the various tissues has enabled an extensive study of the biochemical, immunological and enzymatic properties of tissue kallikrein.

TK was localised in the granules, tubules and striated duct cells of submaxillary glands (Hojima et al., 1975; Orstavik et al., 1976), in the rat transplantable acinar cell carcinoma of the pancreas (Berg et al., 1985), in the connecting tubule cells of the kidney (Tomita et

al., 1981), in the colonic mucous cells (Schachter et al., 1983), in the epithelial cells lining the lumen of the coagulating gland and prostate (Schachter et al., 1978), in the glands of the trachea (Bhoola et al., 1989) and nasal mucosa (Baumgarten, et al., 1989), ependymal cells lining the third ventricle and cell bodies of the arcuate, supraoptic, paraventricular and ventromedial nuclei of the brain (Simson et al., 1985), prolactin secreting cells of the anterior pituary (Vio et al., 1990), and human circulating (Figuerao et al., 1990) and synovial fluid neutrophils (Rahman et al., 1994). Recent immunocytochemical and *in situ* hybridisation studies in this laboratory should advance knowledge with regard to the storage and synthesis sites of tissue kallikrein.

TK is an acidic glycoprotein with molecular weight, depending on the site of origin, ranging from 30 to 45 kDa, and the isoelectric point ranging from 3.5 to 4.4. The enzyme from the different tissues is similar in enzymatic, immunological and chemical properties. Similar to other serine proteases of the trypsin, chymotrypsin and elastase group, the serine residue in TK forms a triad with histidine and aspartic acid, and this spatial arrangement forms the catalytic triad necessary for its enzymic activity. Glycosylation sites vary in number and position in different species and cells in which the enzyme is synthesised. One mole of kallikrein has one glycosylation site in the rat and mouse, whereas porcine pancreatic kallikrein has 2 or 3 sites (Fritz et al., 1967), and human urinary kallikrein has 3-asparagine linked sites (Lu et al., 1989) and 3 additional oxygen glycosylation sites linked to 2 serine and 1 threonine residues (Kellerman et al., 1988). Human urinary kallikrein is comprised of 238 amino acid residues with isoleucine at the amino terminus and serine at the carboxyl terminus (Geiger et al., 1979), and the carbohydrate moieties contain fucose, mannose, galactose, N-acetylglycosamine and sialic acid (Moriya et al., 1983). In addition,

sialic acid seems to partially influence the thermal stability of TK because asialic forms of the enzyme are less stable than the intact molecule.

TK is synthesised bound to a signal peptide of 17 amino acids, that is cleaved off to produce an inactive precursor. In addition to the active enzyme, the inactive pro-form is found in varying amounts in most tissue and body fluids. This pro-enzyme can be activated *in vitro* by proteolytic enzymes like trypsin (Kamada et al., 1988). This activation can also be achieved by thermolysin, a bacterial metalloprotease. The cleavage site for the conversion to the active form is between Arg-7 and Ile-8. Thermolysin is four times more effective than trypsin in activating the human urinary proenzyme (Noda et al., 1985). The amino terminus of the pro-enzyme is alanine, and after removal of a seven amino acid peptide, isoleucine forms the new N-terminus (Takada et al., 1985) (fig 1). Since the sequence of the activation peptide is -Ala-Pro-Pro-Ile-Gln-Ser-Arg- (Girolami et al., 1986), the proenzyme can be activated *in vitro* by either trypsin or thermolysin which hydrolyses the Arg-Ile bond. The endogenous enzyme that performs this function physiologically has not been identified, as yet. TK is a well characterised component of saliva (Sakamoto et al., 1980; Gieger et al., 1983) and it has been suggested that the salivary protein is either identical or closely related to the TK found in urine and other biological fluids. Jenzano et al. (1992) showed that there is considerable variation in TK expression in salivary gland secretions. They demonstrated the presence of a tissue prokallikrein in human mixed saliva indicating the possibiltiy of at least two immunoreactive forms of TK in saliva, mature TK and tissue prokallikrein.

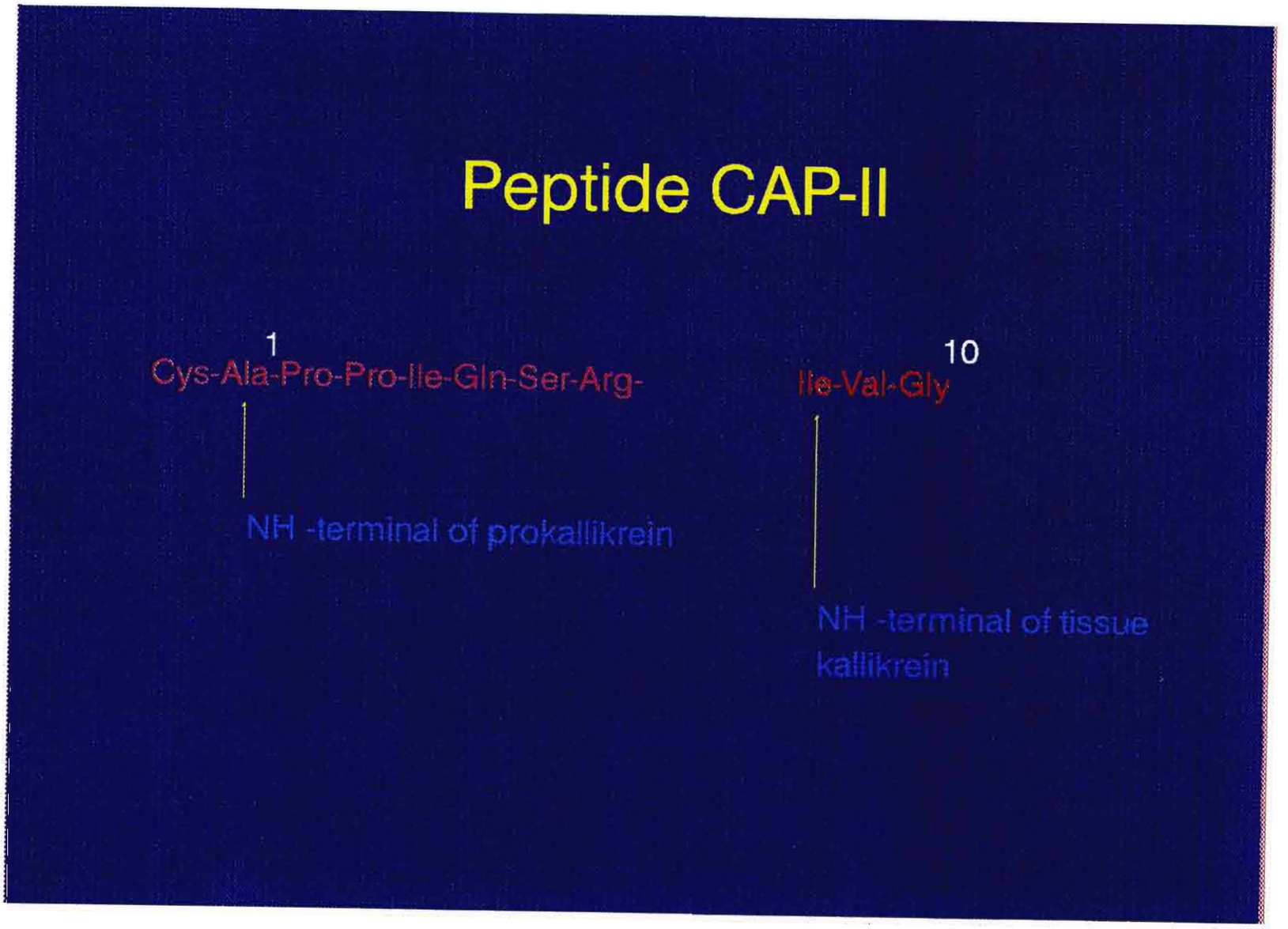


Figure 1: Amino acid sequence of prokallikrein showing amino termini of prokallikrein and TK.
Peptide CAP-II represents prokallikrein and is derived from the first letter of the first three amino acid residues of the amino terminal of prokallikrein.

TK forms kallidin (Lys-bradykinin) from the preferred substrate low molecular weight kininogen, but *in vitro* TK is considered to release kinins from both low (LK) and high molecular weight (HK) kininogens (Iwanaga et al., 1977). The process of forming kallidin by TK involves the cleavage of a Met-Lys bond at the N-terminus in the kinin sequence of the kininogen molecule (Pisano et al., 1974). TK of most mammalian species is unable to form bradykinin because of its inability to accommodate the Lys-Arg-Pro sequence for hydrolysis of the Lys-Arg bond on the amino terminus of the peptide.

Human kallistatin is a newly discovered protein, and is a tissue kallikrein inhibitor. Chao and Chao (1995) purified and cloned human, rat and mouse kallikrein binding proteins. The reactive site of kallistatin has a unique sequence at P_2-P_1-P_1 of Phe-Phe-Ser. Site directed mutagenesis was carried out to determine the structural and functional properties of kallistatin. Chao et al. (1995) showed that P_1 Phe is a crucial specificity determinant, and P_2 Phe is important for the hydrophobic environment of the kallikrein-kallistatin interaction. P_{10} and P_{12} Phe residues are important for maintaining kallistatin conformation. Enzyme linked immunosorbent assays (ELISAs) and *in situ* hybridisation studies have shown that there is a wide distribution of kallistatin in blood cells, body fluids and tissues of healthy and diseased individuals. Plasma kallistatin levels are altered in patients with liver disease, sepsis and pre-eclampsia. The expression of kallistatin in the rat is downregulated during acute phase inflammation, and is upregulated by sex hormones. Chao et al. (1995) found that kallistatin levels were reduced in genetically hypertensive rats, and in the arterial hypertensive rat model induced by the inhibition of nitric oxide synthesis.

Today, techniques determining concentration for TK in tissue extracts and in biological fluids are diverse. Methods include kininogen as the substrate, in which kinin is released and the free kinin estimated by bioassay (Bhoola et al., 1962), radioimmunoassay (Miwa et al., 1968) or enzyme linked immunosorbent assay (ELISA) (Geiger et al., 1986), and high pressure liquid chromatography (Kato et al., 1985). Another method is the hydrolysis of the selective, synthetic substrates (Lu et al., 1989) by which the activity of TK may be determined, and the parent molecule identified using a monoclonal antibody (Takahashi et al., 1988). In addition, antibodies to TK can be used to specifically inhibit the enzymic activity of TK. In order to predict the amount of proenzyme in a sample, synthetic substrate assays are performed before and after trypsin or thermolysin activation and, in this way, values for both active and inactive components can be determined.

Using these techniques, it has recently been established that circulating neutrophils contain a TK (Bhoola et al., 1992). TK in circulation could be affected by degranulating neutrophils. It has been suggested that *in vivo*, the liver is the main organ that clears TK from circulation. Although the accepted property of TK is to form kallidin, other enzymic functions have been attributed to TK, and these include conversion of inert, precursors of enzymes and hormones into biologically active molecules (Bhoola and Dorey, 1971; Bothwell et al., 1979; Mason et al., 1983). The finding of immunoreactive TK in the cells of pancreatic islets (Pinkus et al., 1983), and in the lactotroph cells of the rat (Vio et al., 1990) and human anterior pituitary (Noda et al., 1985) suggests that the functional role of this enzyme may be specific to cell types.

1.2.1.2 **Plasma kallikrein (PK)**

Plasma kallikrein occurs in zymogen form, and differs from TK in its biochemical, immunological and functional characteristics. PK is synthesised as a zymogen in hepatocytes, secreted into the bloodstream where it circulates as a heterodimer complex bound to its substrate, high molecular weight kininogen (HK). The PK-HK complex, and Hageman factor (HF) are involved in the activation of the complement system. Further, the complex plays an important role in the surface-dependent activation of HF that results in coagulation of blood, formation of kinins, regulation of vascular tone and fibrinolysis. The proenzymes involved in clotting include Factor XII (HF), Factor XI (plasma thromboplastin antecedent) and plasma prekallikrein (Fletcher factor). Once the cascade is triggered, clotting occurs to initiate thrombus formation together with the formation of active PK and the release of kinins on endothelial and subendothelial surfaces. The clotting cascade is initiated by the conversion of inactive Factor XII into a two chain active HFa by either exposure to macromolecular anionic surfaces or by enzymatic action of PK (Cochrane et al., 1973). Active HFa comprises a heavy chain of 50 kDa, which has the binding site for the attachment to anionic surfaces during activation, and a light chain of 28 kDa linked together by a disulfide bond (Revak et al., 1978; Revak et al., 1977). The light chain has the active site (Revak et al., 1976; Kaplan and Silverberg, 1987) for converting the plasma prekallikrein (PPK) zymogen into an active form (Miller et al., 1980; Silver et al., 1980; Siverberg et al., 1980). The cascade progresses rapidly as soon as a sufficient amount of PK is formed. PK forms more HFa and drives the reaction forward to activate Factor XI, that enhances clotting, and plasminogen that initiates fibrinolysis. With rising levels, PK enzymatically cleaves HK from which bradykinin is released.

A single gene codes for PPK which is synthesised in liver hepatocytes and secreted as an inactive molecule. HK is also synthesised in the liver and when both HK and PK are secreted into the circulation, they form a complex with Factor XI of the intrinsic clotting system. This complex circulates bound to the outer surface of the human neutrophil (Naidoo et al., 1994a). PPK exists as a single chain glycoprotein with an isoelectric point of 8.9. The enzyme exists in two forms of 85 and 88 kDa and both forms are present in plasma (Talama et al., 1969). The mature human enzyme is made up of 619 residues with 371 residues at the amino terminus linked to a catalytic chain of 248 residues. HK is the prefered substrate for PK which releases bradykinin from it by hydrolysis of Lys-Arg and Arg-Ser bonds to yield the nonapeptide with Arg at both amino and carboxy terminals.

In spite of the fact that LK is a poor substrate, PK does form bradykinin from LK in the presence of neutrophil elastase. This is possible because neutrophil elastase cleaves a fragment from LK from which PK readily releases bradykinin (Kitamura et al., 1985). It is possible that this reaction occurs *in vivo* for kinin generation from low molecular weight kininogen, and this activity has important implications because LK has been localised on the external membrane of the neutrophil. PK has a significant effect on PMN leucocytes, and therefore, is considered to play an important role in inflammation (Henderson et al., 1994).

1.2.2 **Kininogens**

The endogenous protein substrates for TK and PK are kininogens, which by proteolytic cleavage form the vasoactive peptides - kinins. Cysteine proteinase inhibitor domains also

exist in kininogen molecules, and may therefore act as cofactors for inhibition of lysosomal enzymes released during inflammation. Hepatocytes play a role in the synthesis and release of kininogens into circulation (Kitamura et al., 1985; Müller-Esterl., 1988). Current studies have shown that HK and LK are not restricted to extracellular fluids, but also occur in several cell types. Kininogens have so far been identified in the collecting ducts of the human kidney (Proud et al., 1891; Figueroa et al., 1988), platelets (Schmaier et al., 1983; Schmaier et al., 1986), sweat glands (Poblete et al., 1994), endothelial cells (Schmaier et al., 1988) and human neutrophils (Gustafson et al., 1989). Figueroa et al. (1990) identified HK on the external surface of human neutrophils, and described for the first time the presence of LK on these cells. In contrast, it was observed that when platelets were activated, HK was translocated from the granules to the external surface of the cell membrane, where the binding of HK to the membrane probably involves specific acceptor proteins exposed on the outer surface of cells. Binding sites for HK which are specific, saturable and reversible have been identified on the cell membranes of platelets (Greengard and Griffin, 1984; Gustafson et al., 1986; Meloni and Schmaier, 1991), cultured human endothelial cells (Gustafson et al., 1986; van Iwaarden et al., 1988) and neutrophils (Gustafson et al., 1989).

The kininogens are single-chain glycoproteins that possess the kinin moiety interleafed between the two polypeptides that are bridged by single disulfide loops (Kellerman et al., 1987). The two kininogens differ with respect to structure, size and enzymatic susceptibility. HK is composed of 626 residues and, depending on the species of origin, has a molecular mass of 88 to 120 kDa (Kerbiriou et al., 1980; Jacobsen et al., 1966; Komiya et al., 1974). LK consists of 409 residues and varies from 50 to 68 kDa in size (Kato et al., 1976;

Müller-Esterl et al., 1985). The molecular architecture of these proteins has been analysed by limited proteolysis. The kininogen molecule consists of multiple domains; HK has 6 domains (D1 - D6) and LK has 5 domains (D1 -D5) (Müller-Esterl et al., 1986; Kato et al., 1981) (fig 2).

The heavy chain basic structure of the two kininogens is the same, and forms the 3 amino terminal domains that make up 50-60 kDa. The fourth domain contains the kinin segment followed by a light chain, that varies in the two kininogen molecules (Kellerman et al., 1986; Lottspeich et al., 1985). For HK, the light chain has two segments and a molecular weight of 45 to 58 kDa. In LK the light chain is smaller and single with a sequence of Met-Lys-bradykinin at its amino terminus (Kato et al., 1985). Cysteine proteinases, such as cathepsin B, papain and platelet calpain, are inhibited by domains 2 and 3 of the heavy chain (Ohkubo et al., 1984; Müller-Esterl et al., 1985; Schmaier et al., 1986). The histidine rich region of domain 5 in the light chain of HK it anchors onto anionic surfaces during initiation of the intrinsic clotting cascade. Domain 6 provides a binding site for PPK and the clotting factor XI. Since PPK is coupled to domain 6 of HK, conversion of PPK to active kallikrein on the neutrophil membrane may bring about the release of the kinin moiety, stimulate elastase secretion and induce neutrophil aggregation (Gustafson et al., 1989).

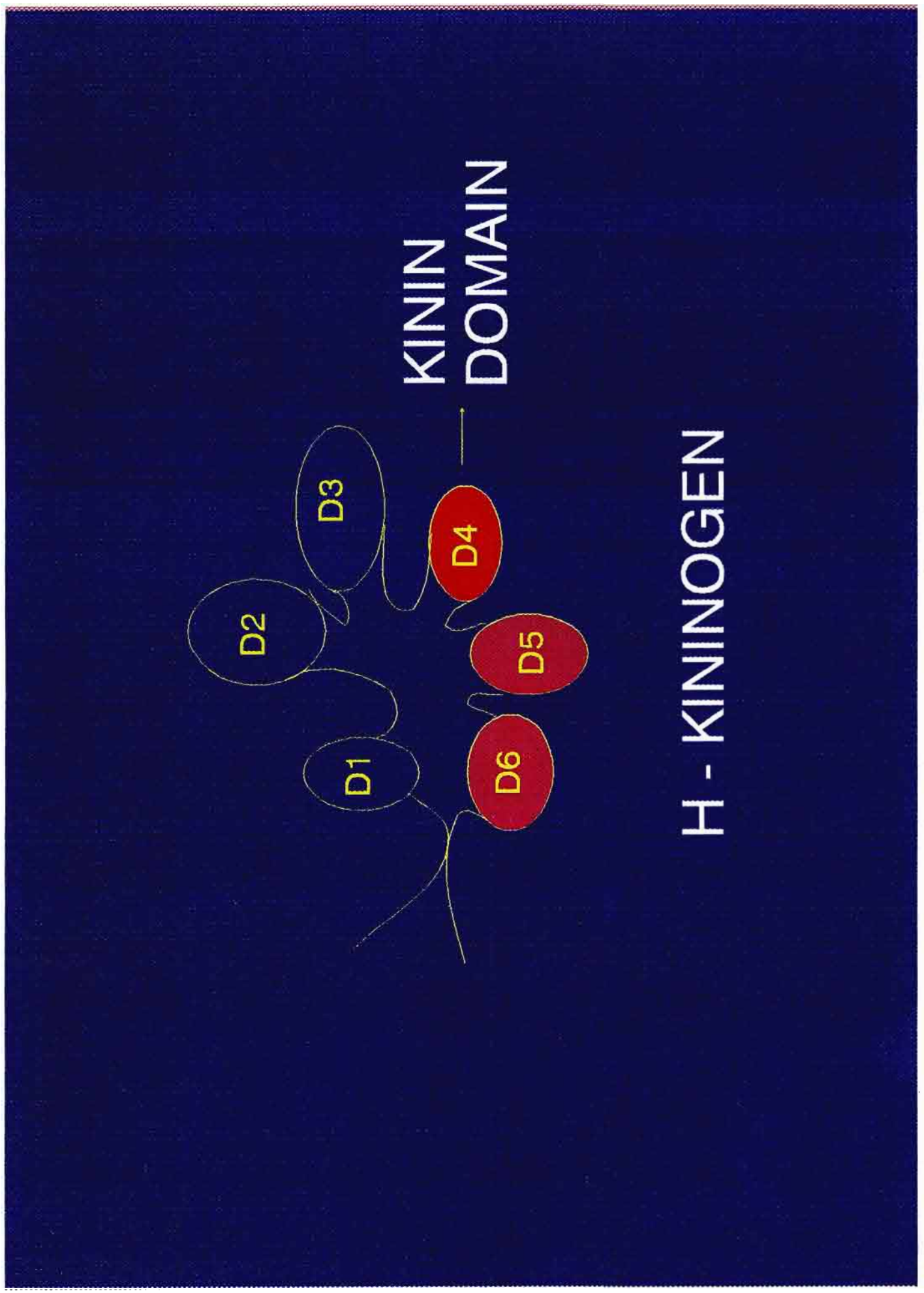


Figure 2: Multiple domains of HK
HK has 6 domains (D1 - D6) and LK has 5 domains (D1 -D5)

Colman (1995) probed the kininogen molecule with monoclonal antibodies, peptides and deletion mutagenesis to elucidate its interaction with other proteins, surfaces and cells. He found that domain 2 inhibits platelet calpain while domain 3 has the ability to block thrombin-induced activation of platelets. Domain 3 also contains a binding site for neutrophils, and this, he postulated, may modulate neutrophil adhesion to artificial surfaces. Domain 5 of HK was found to contain 2 regions which reacted with negatively charged surfaces and cells, one of which interacted with neutrophils and endothelial cells. Domain 6 plays an important role in the cofactor function of kininogen, as this site houses FXII and prekallikrein, the latter of which upon activation causes the liberation of bradykinin from domain 4. In order to determine how kininogens place themselves in their binding sites on endothelial cells, Schmaier et al. (1995) sought to determine the sequences on kininogen that participate in endothelial cell binding and found that domains 3, 4 and 5 all contain binding sites.

1.2.3 **Metabolism and cellular actions of kinins**

Kinins are vasoactive peptide mediators that are formed immediately after a primary injury event. Therefore, they are an important target for therapeutic intervention. Kinins are capable of producing all of the observed cellular effects in inflamed tissues. These include vasodilation, venoconstriction, pain and endothelial cell retraction that result in vascular leakage. Kinins are capable of releasing transmitters such as substance P from nerve terminals, stimulating the synthesis of cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF), inducing the formation of prostaglandins and leukotrienes by activating phospholipase A_2 and releasing endothelium-derived relaxing factor (EDRF; nitric

oxide) from endothelial cells. Injected intravenously, kinins produce a rapid drop in blood pressure with a gradual return to baseline over a period of several minutes. With very high doses, pulmonary and systemic vascular leakage occurs, resulting in cyanosis and oedema. Injection of bradykinin into the carotid artery results in pseudoaffective responses and vocalisation, suggesting severe pain (Ricciopo et al., 1974). It is generally accepted that kallidin (Werle et al., 1961) is formed from LK by TK, and bradykinin from HK by the action of PK (fig 3). Bradykinin is a nonapeptide of 1060 daltons and kallidin is a decapeptide of 1188 daltons.

The conversion of kallidin (Lys-bradykinin) to bradykinin may occur in the circulation and tissue fluids through removal of the amino terminal lysine by aminopeptidases (Rodell et al., 1995). Once produced, bradykinin and kallidin are inactivated by several different kininases, therefore the effects of kinins in biological fluids are often very short-lived because they are rapidly destroyed by potent peptidases (fig 4). Circulating carboxypeptidase N and the membrane bound carboxypeptidase M (kininase I) cleave the C-terminal Arg of bradykinin to produce des-Arg^9-bradykinin. Angiotensin converting enzyme or kininase II cleaves 2 internal bonds, producing completely inactive metabolites. Aminopeptidases (prolidase) remove the N-terminal Arg. In addition, a neutral endopeptidase that occurs on the surface of endothelial cells, and the outer surface of the neutrophil membrane also degrades the molecule.

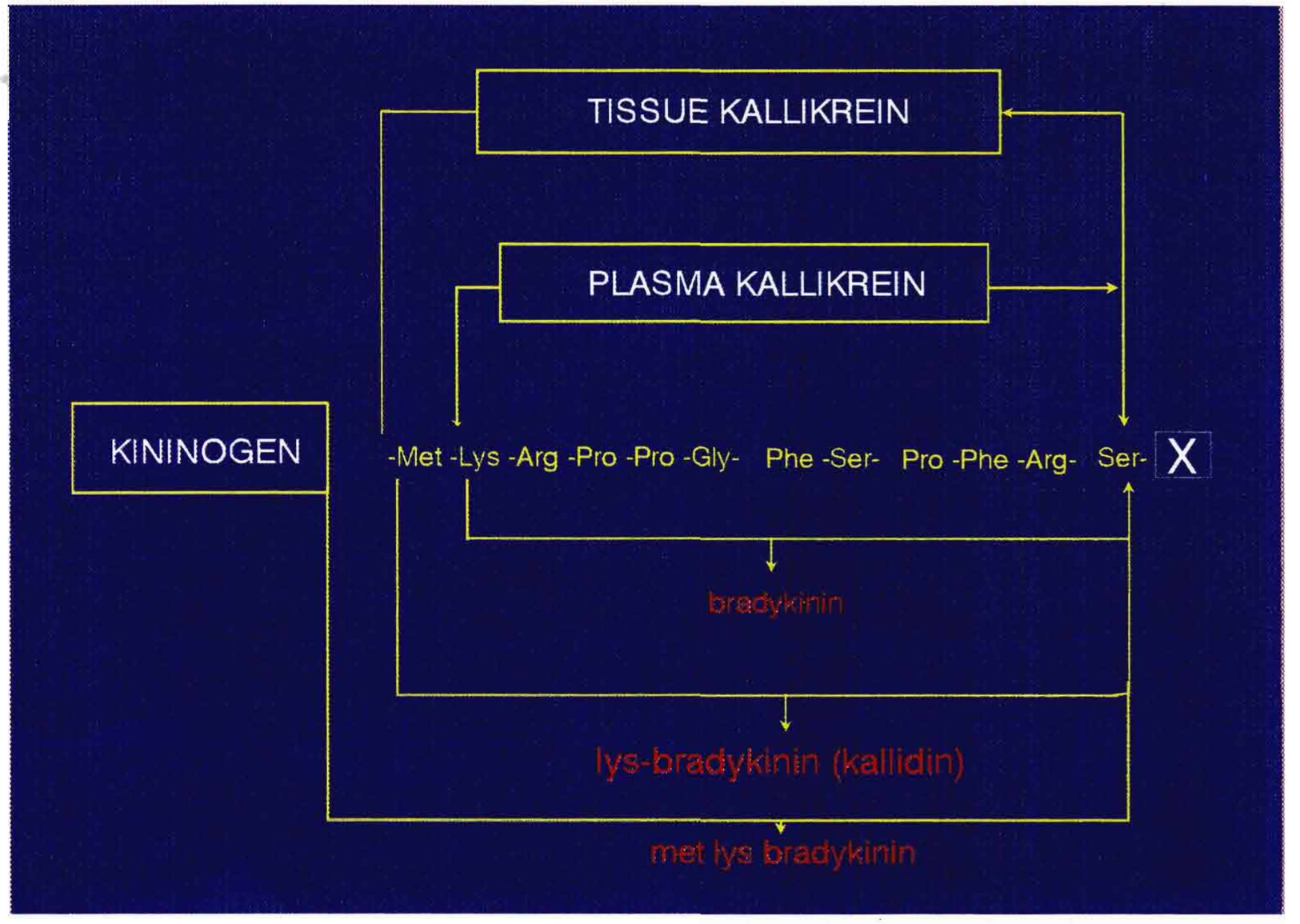


Figure 3: Cleavage sites for TK and PK on the kininogen molecule

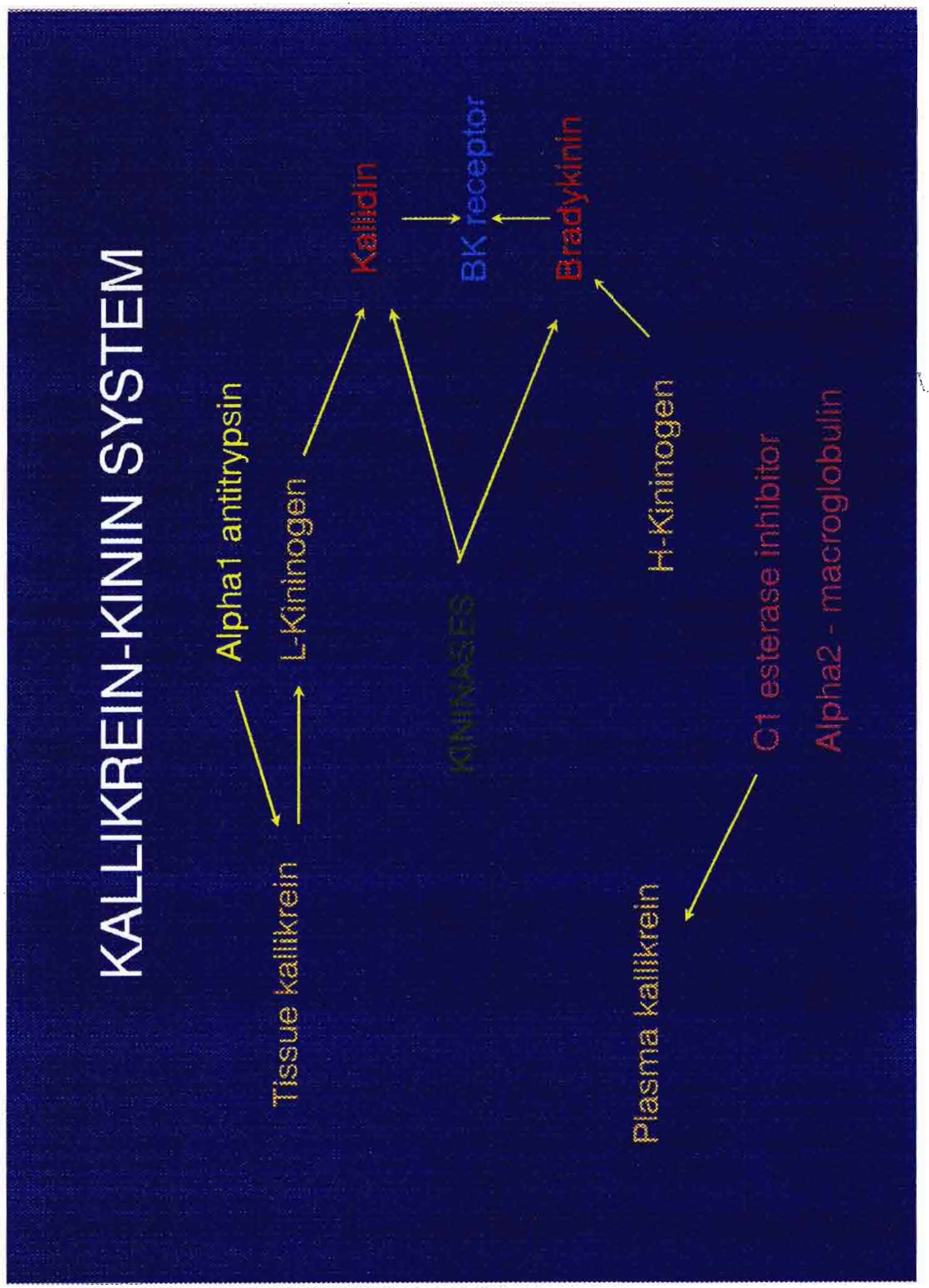


Figure 4: Generation of kinin by kallikreins

In blood, the reported half life of about 30 sec for bradykinin and kallidin requires to be confirmed (McCarthy et al., 1965). Kinins in biological fluids and tissue homogenates are mostly readily measured by radioimmunoassay. The finding of varying tissue levels may be due to the fact that the kininogenases and kininases may not have been adequately inhibited, and a difference in the type of antibody used. More recently, a mean value of 3.8 pg/ml was recorded when syringes containing 0.8 M hydrochloric acid (HCl) were used to withdraw blood samples. Kinins in the urine amount to 24.6 μg/day in samples collected in HCl and pepstatin containing flasks (Scicli et al., 1982). Kinin levels have also been reported in nasal fluids (Shimamoto et al., 1982), bronchoalveolar lavage fluid (Christianson et al., 1987), human (Scicli et al., 1984) and rat CSF (Hermann et al., 1986) and synovial fluid from patients with rheumatoid and osteoarthritis (Rahman et al., 1994).

1.2.4 **Kinin receptors and signal transduction**

The cellular effects of kinins are mediated by two different classes of receptors, namely B_1 and B_2. Studies of the structure and activity of these receptors have been carried out to determine the potency of agonists, differences in activity profile, affinity of competitive antagonists and mechanisms involved in signal transduction. The B_1 (Hess et al., 1994) and the B_2 (McEachern et al., 1991) receptors have been cloned and have been found to belong to the superfamily of G-protein receptors. These receptors have the characteristic of seven transmembrane spaning regions comprising three extracellular and four intracellular loops, that are unique in their sequences (Hess et al., 1994). The B_2 receptor seems to be the physiological receptor, instrumental in most of the reported actions of kinins. The B_1 receptor has attracted interest because of its apparent upregulation following induction of

inflammation (Marceau et al., 1995).

In addition to the B_1 and B_2 receptors, Farmer et al. (1991) proposed an airway B-subtype receptor in the trachea of guinea pigs; however, its significance in humans is still unknown.

Both isolated cell membrane fragments and intact cultured cells have been used to demonstrate the bradykinin receptor coupling to second messenger systems. Although bradykinin increases free cytosolic calcium in many tissues, in vascular smooth muscle, the intracellular calcium is mobilised by receptor coupled activation of phospholipase C, hydrolysis of inositol biphosphate and formation of inositol triphosphate. The coupling of kinin receptors to secondary messenger systems is through a family of G proteins. The particular second messenger system initiated, therefore, may be a function of the receptor-coupled G protein in each cell type, indicating the occurence of B_2 receptor subtypes.

Two B_2 bradykinin receptor subtypes have been described: a smooth muscle and a neuronal B_2 receptor which differ pharmacologically. The B_2 smooth muscle receptor has been cloned from several species, whereas this is the first report of cloning the neuronal B_2 receptor. The B_2 receptor was proposed as an important gene involved in the manifestations of common chronic disorders such as hypertension and ischaemic heart disease, since bradykinin has been implicated in a variety of physiological and pathological processes.

1.3 Role of Nitric Oxide (NO), the second messenger, in sepsis and infections

Neutrophil accumulation in tissue is a characteristic of inflammation, and is associated with a variety of pathological conditions (Spitzer et al., 1994). Bacterial infections are characterised by the selective aggregation of neutrophils in large numbers to phagocytose and kill invading micro-organisms. However, activated neutrophils can also cause injury to tissues. There is a growing body of evidence to suggest that NO regulates neutrophil-endothelial cell interactions, which is a key feature of inflammation and may, therefore, modulate the inflammatory response (Kubes, 1995). It has been postulated that inhibition of NO synthase, the enzyme forming NO through the conversion of L-arginine to L-citrulline, promotes neutrophil adhesion.

Septic shock is the host's inflammatory response to infection. The pathogenesis of septic shock is mediated by a variety of endogenous substances. Some of the major mediators include cytokines, NO and prostaglandins (Castillo and Sanchez, 1993). Management of septic shock is focussed on maintaining haemodynamic stability and an adequate oxygen delivery and utilization. The most important factor to prevent complications and improve outcome is to give careful attention to each organ-system. Endotoxin and other bacterial products induce the release of mediators which alter the circulation and cellular metabolism and one such mediator is NO (Rosenberg et al., 1994). The proposed mechanism by which NO initiates intracellular events is the activation of guanylate cyclase with subsequent biosynthesis of 3'5' cyclic guanosine monophosphate. Tiao et al. (1994) observed that NO production increases during sepsis and endotoxaemia. Inhibition of NO synthetase has been suggested as a therapeutic modality in sepsis and endotoxemia, but in recent reports NO

synthase inhibition increased mortality rate. However, the mechanism of this phenomenon is not known. The detrimental effect of NO synthase inhibition during endotoxemia may be caused by excessive production of tumour necrosis factor (TNF) and interleukin-6 (IL-6).

1.4 Neutrophils and the kallikrein-kinin system

Normal human blood consists of leucocytes of different morphology, and classification is based on the presence or absence of granules staining with acridine dyes in the cytoplasm, as well as the shape of the nucleus. Neutrophils, eosinophils and basophils are the granular leucocytes (polymorphonuclear), and are classified according to specific staining affinities of the intracellular granules. The non-granular leucocytes are divided into monocytes and lymphocytes. Neutrophils, as with all granulocytes, originate from stem cells located in the bone marrow, and the stem cells in turn originate from pluripotential stem cells which are referred to as the colony forming units. The pluripotent cells self-replicate into progenitor stem cells, one of which becomes committed to the formation of granulocytes. Maturation of the neutrophil from the stem cells takes about 10 days, after which the neutrophil remains in the bone marrow for 5 days before going into the circulation, extracellular tissue space and biological fluids.

During immunocytochemical studies on a pyelonephrotic human kidney (Figueroa et al., 1989), it was observed that there was an infiltration of neutrophils, in which TK was discovered as shown by an intense granular pattern of immunostaining. Figueroa et al. (1989) examined, and identified, immunoreactive tissue kallikrein in polymorphonuclear (PMN) leucocytes of normal human blood and bone marrow, specifically in the mature

neutrophils as well as in immature forms such as metamyelocytes and myelocytes. Further, no tissue kallikrein was detected in eosinophils, lymphocytes, macrophages, megakaryocytes and platelets. Additionally, large numbers of neutrophils were observed in the synovial fluid and membranes of patients with rheumatoid arthritis. It is therefore likely that TK from the neutrophils could form kallidin in an inflamed joint. This finding was important since a major role has been postulated for TK in acute inflammation, in which granulocytes are considered to participate in regulating vascular permeability by contributing kinins. Although immunoreactive TK was localised subcellularly in the granules of the neutrophils, it has not been determined whether the enzyme is contained in azurophilic or specific granules.

If TK is indeed synthesised in the neutrophil, its expression would be controlled by a single gene that encodes a pre-pro-enzyme with a 17 amino acid signal peptide, that is subsequently split off during protein translocation within the cells. Anders et al. (1994) demonstrated for the first time that tissue prokallikrein specifically binds to intact human neutrophils and structural features of the zymogen are required for the interaction with unoccupied sites on the neutrophil surface. The question of whether TK is synthesised *in situ* or emniocytosed after binding to its membrane receptor has yet not been answered unequivocally.

Changes in vascular calibre and tone, as well as an increase in vascular permeability leading to the formation of a protein rich exudate, are all part of the acute inflammatory response. Neutrophils participate actively in such a response, initially within vessels where they marginate and adhere to the vascular endothelial cells, and later when they migrate through

the vessel wall to reach the site of inflammation or injury. Macrophages and lymphocytes follow later after the first arrival of the neutrophils which is usually within a few minutes. During the early stages of inflammation, bradykinin, produced by the action of PK on HK, may be an important mediator of changes in vascular permeability or calibre. This property has led to the conclusion that kinins are likely to be formed also in the protein rich exudate present in acutely inflamed areas. Recently, researchers have suggested that during acute inflammatory reactions, locally generated chemotactic factors attract neutrophils to vessel walls, where they play a role in the control of vascular tone and capillary permeability, leading to the protein and fluid exudation (Issekutz, 1984), prior to migrating to the site of inflammation.

Specific, reversible, and saturable binding sites for HK have been previously determined in human neutrophils by Gustafson et al. (1989). Experiments with platelets have shown that when theses are activated, HK is translocated form the alpha granules to the external surface of the cell membrane. Figueroa et al. (1992) showed that, in the neutrophil, immunoreactivity for HK and LK was restricted to the cell membrane, and that it was absent from granules and other organelles. They concluded that clusters of kininogen molecules present on the neutrophil surface represented kininogen bound to specific receptor proteins. Both the kininogens associated with the neutrophil membrane were thought to have originated from the plasma pool although they could be synthesised in the neutrophil and translocated to the cell membrane. With the availability of specific antibodies, it was next possible in this study to demonstrate PK on the outer surface of the membrane.

1.5 Hypothesis

Neutrophils contribute to circulating levels of kinin in infections and inflammatory disorders.

1.6 Aims

The aim of the current study was to demonstrate immunocytochemically, the presence or absence of the different components of the kallikrein-kinin system, and kinin receptors on the human neutrophil in blood from normal, healthy volunteers as well as blood from patients with sepsis and clinical disorders.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Neutrophil isolation*

2.1.1 **Reagent**

Phosphate buffered saline pH 7.2 (PBS)

$NaH_2PO_4.2H_2O$ (3.12 g), $Na_2HPO_4.H_2O$ (11.32 g), NaCl (8.5 g) were dissolved in 950 ml distilled H_2O, titrated to pH 7.2 with NaOH, and made up to 1 litre.

2.1.2 **Procedure**

Whole blood (10 ml) was obtained by venipuncture through a precision glide vacutainer needle (Becton Dickinson Vacutainer Systems, UK) from healthy volunteers and patients presenting with pneumonia, myeloid leukaemia and rheumatoid arthritis at King Edward VIII Hospital, and patients presenting with tuberculosis meningitis at Wentworth Hospital, Durban, Natal. Blood samples were obtained from patients with informed consent. Each 3 ml was drawn into a vacutest tube (Radem Medical, Sandton, SA) containing 0.5 ml of 3.8% (w/v) sodium citrate. The anticoagulated blood was pooled and mixed with an equal volume of phosphate buffered saline (PBS). Neutrophils were isolated by careful layering of blood (7 ml) on 3.5 ml Histopaque 1119/1077 (Sigma, UK), and on centrifugation (15 000 x g, 30 min, 20^0C) neutrophils formed a band. Neutrophils from this layer were harvested with a pasteur pipette, mixed with an equal volume of PBS, centrifuged, and the washing process repeated twice.

Therapeutically aspirated synovial fluid obtained from patients with informed consent was centrifuged (10 000 x g, 7 min, room temperature) to remove cells and debris and the supernatants were thoroughly mixed, dehydrated through a graded series of alcohol (70%, 90% and 100%) and wax embedded. Three micron sections were then cut on a Leica Jung RM 3025 microtome. Samples were stored at 4^0 C.

2.2 *Immunocytochemistry*

2.2.1 **Reagents**

Paraformaldehyde (2%)

Paraformaldehyde (2 g) was added to 80 ml distilled H_2O, heated to clear with 5 drops NaCl (1 M) and made up to 100 ml.

Blocking solution (1% Bovine serum albumin and 1% human IgG in PBS)

Bovine serum albumin (BSA) (0.01 g) and human IgG (10 µl) was dissolved in 1 ml PBS.

Kaiser's jelly mounting medium

Gelatin (10 g) was added to 60 ml distilled H_2O and heated to dissolve the gelatin after which 70 ml of glycerol and 0.25 g of phenol were added. Jelly was heated before use.

Toludine blue

Toludine blue (2%) (5 ml) was diluted to 25 ml using distilled H_2O.

Gluteraldehyde (4%)

8% Aqueous gluteraldehyde was diluted to 4% using PBS.

2.2.2 **Antibodies**

Polyclonal rabbit IgG prepared against purified (recombinant) mature tissue kallikrien (TK) was from Dr. M. Kemme, Technische Hochschule Darmstadt, Germany. Monoclonal IgG against the kinin moiety of the kininogen molecule (SBK1) was from Dr. M. Webb, Sandoz, UK. Polyclonal rabbit anti-human kininogen IgG (I 108) was from Dr. W. Müller-Esterl, University of Mainz, Mainz, Germany. Polyclonal rabbit anti-human plasma kallikrein (PK) was also provided by Dr. W. Müller-Esterl, University of Mainz, Mainz, Germany. Evidence of specificity of antibodies is given by Henderson et al, 1994.

Polyclonal rabbit anti-human B_2 receptor IgG (ED & DTL, 283) was from Dr. W. Müller-Esterl, University of Mainz, Mainz, Germany. CY3-flourochrome labelled sheep anti-rabbit IgG was from Sigma, St. Louis, UK). Polyclonal rabbit anti-human B_1 receptor IgG was from Dr. F. Hess, Merck, Rahway, New Jersey, USA). An anti-peptide polyclonal antiserum against 8 intra- and extracellular domains of the human B_1 receptor was from Dr. W. Müller- Esterl, University of Mainz, Mainz, Germany (Table 1).

Table 1 : Description of antibodies utilised in this study

Antibody	Donor animal	Mono- or polyclonal	Working dilutions	Antibody raised to	Supplier of antibody
Tissue Kallikrein (TK)	rabbit	poly	1:500	recombinant TK	Dr. Kemme (Germany), Professor Bhoola
kinin (SBK1)	mouse	mono	1:500	kininogen molecule	Dr. Webb (Sandoz, USA)
kininogen (I108)	rabbit	poly	1:250	kininogen molecule	Dr. Müller-Esterl (Germany)
Plasma Kallikrein (PK)	rabbit	poly	1:250	plasma kallikrein	Dr. Müller-Esterl (Germany)
B_1 Receptor	rabbit	poly	1:100	Human B_1 receptor peptide loops	Dr. Hess (Merck, USA)
B_1 Receptor	rabbit	poly	1:100	Human B_1 receptor peptide loops	Dr. Müller-Esterl (Germany)
B_2 Receptor	rabbit	poly	1:250	Human B_2 receptor peptide loops	Dr. Müller-Esterl (Germany)

(All reagents not otherwise stated, were of analytical grade, obtained from Sigma chemicals, UK).

2.2.2 **Light microscopy**

2.2.2.1 Tissue preparation

The isolated neutrophils from were pipetted onto a glass slide, air-dried and fixed with paraformaldehyde (2% in PBS, 10 min). The slides were stored at 4^0 C. Cells were immunostained using the peroxidase anti-peroxidase (PAP) method, using the universal PAP kit (Signet laboratories, Dedham, Massachusetts 02026, USA). Wax embedded synovial fluid tissue was rehydrated with xylene and a graded series of alcohol (100%, 90%, 70%) and thereafter immunolabelled with the appropriate antibodies.

2.2.2.2 Immunolabelling procedure

Following blocking of Fc sites with normal human serum, the neutrophil smears were sequentially incubated with the appropriate primary antibody for 18 h, a sheep anti-rabbit immunoglobulin linking agent (50 µl of the anti-species antibody (Signet laboratories, USA) in 0.01 M PBS, 0.1% NaN3, 1% carrier protein) for 20 min, and PAP (fig 5) (50 µl of 15 ml complex in 0.01 M PBS, 1% carrier protein) for 40 min. Visualisation of the immunoenzyme complex was achieved by incubation with 3-amino-9-ethyl-carbazole (Signet laboratories, USA) (AEC, in 1% hydrogen peroxide and 0.1 M acetate buffer) (fig 6).

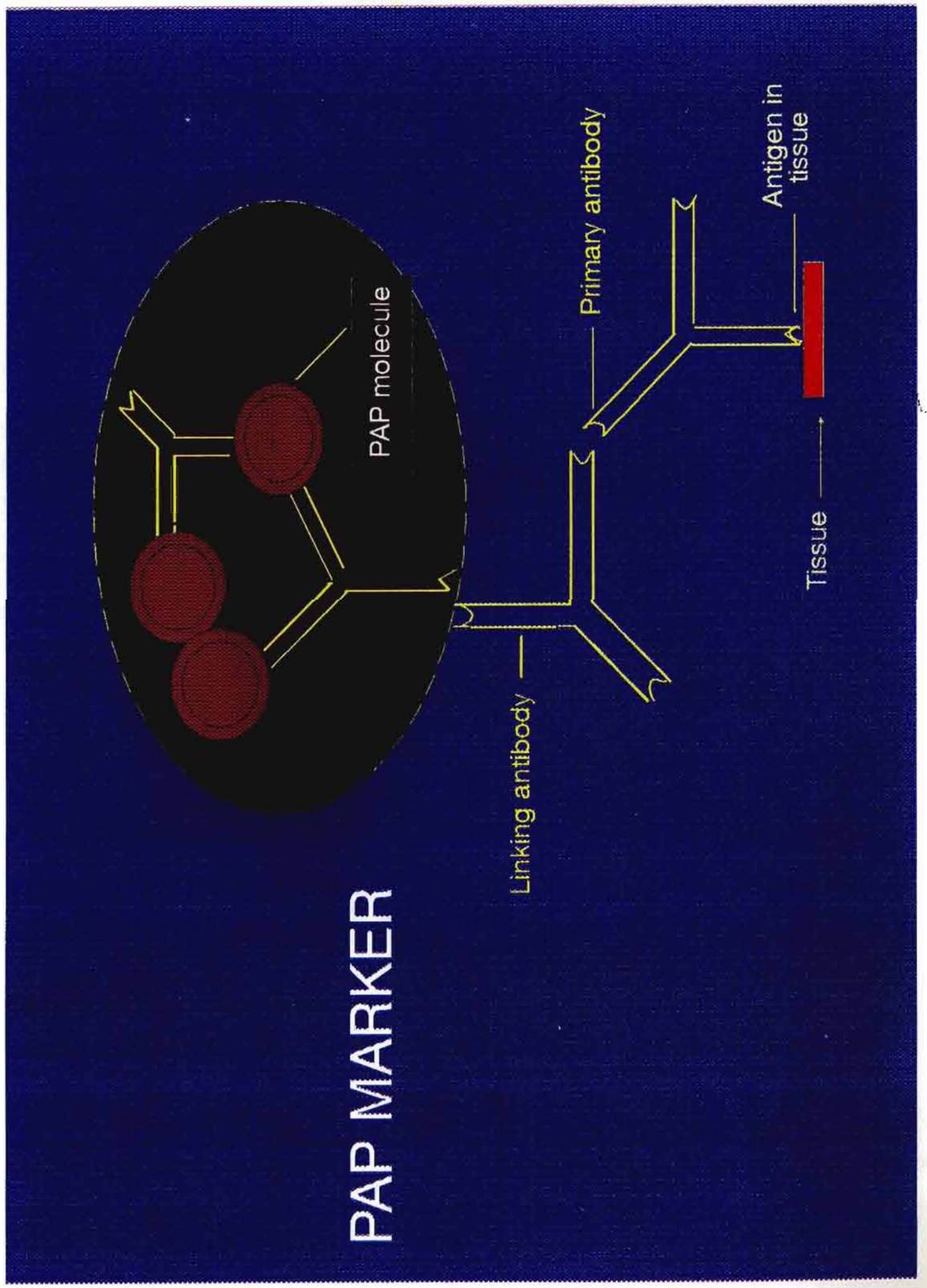


Figure 5: Visualisation of the immunoenzyme complex using the PAP marker
The PAP ring consists of 3 molecules of horseradish peroxidase and two antibodies

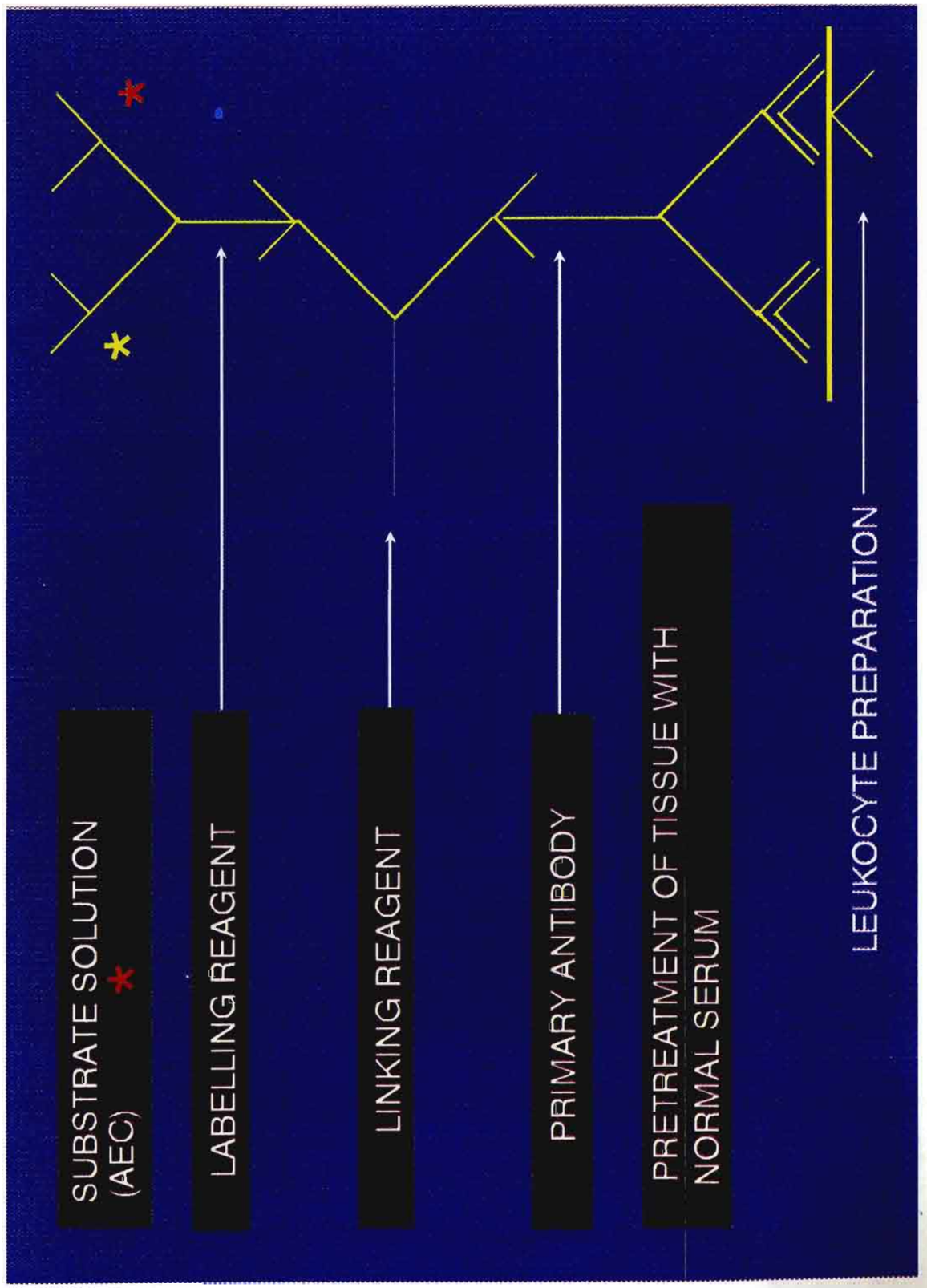


Figure 6: Immunocytochemical bridge by which the immunoenzyme complex enables visualisation of the antigen

2.2.2.3 Counterstaining and viewing

After immunostaining, the smears were counterstained with 2% toludine blue, mounted in Kaiser's jelly and viewed under a Nikon Optiphot photomicroscope.

2.2.3 **Transmission electron microscopy (TEM)**

2.2.3.1 Immunolabelling procedure

The isolated neutrophils were washed with PBS containing sodium azide (0.2%) to prevent contamination in the medium. The suspended cells were pipetted into microfuge tubes and sequentially prefixed with paraformaldehyde (1% in PBS) for 15 min, incubated with the primary antibody (1:500, v/v) for 3 h, and as a blocking step, the cells were washed with PBS containing bovine serum albumin (1%, v/v) and human IgG (1%, v/v), and spun at 300 g for 4 min into a soft pellet. The cells were incubated with an anti-species antibody coupled with 10 nm gold particles (fig 7) for 2 h (Amersham International, Buckinghamshire, UK) and fixed overnight in glutaraldehyde (4% in PBS).

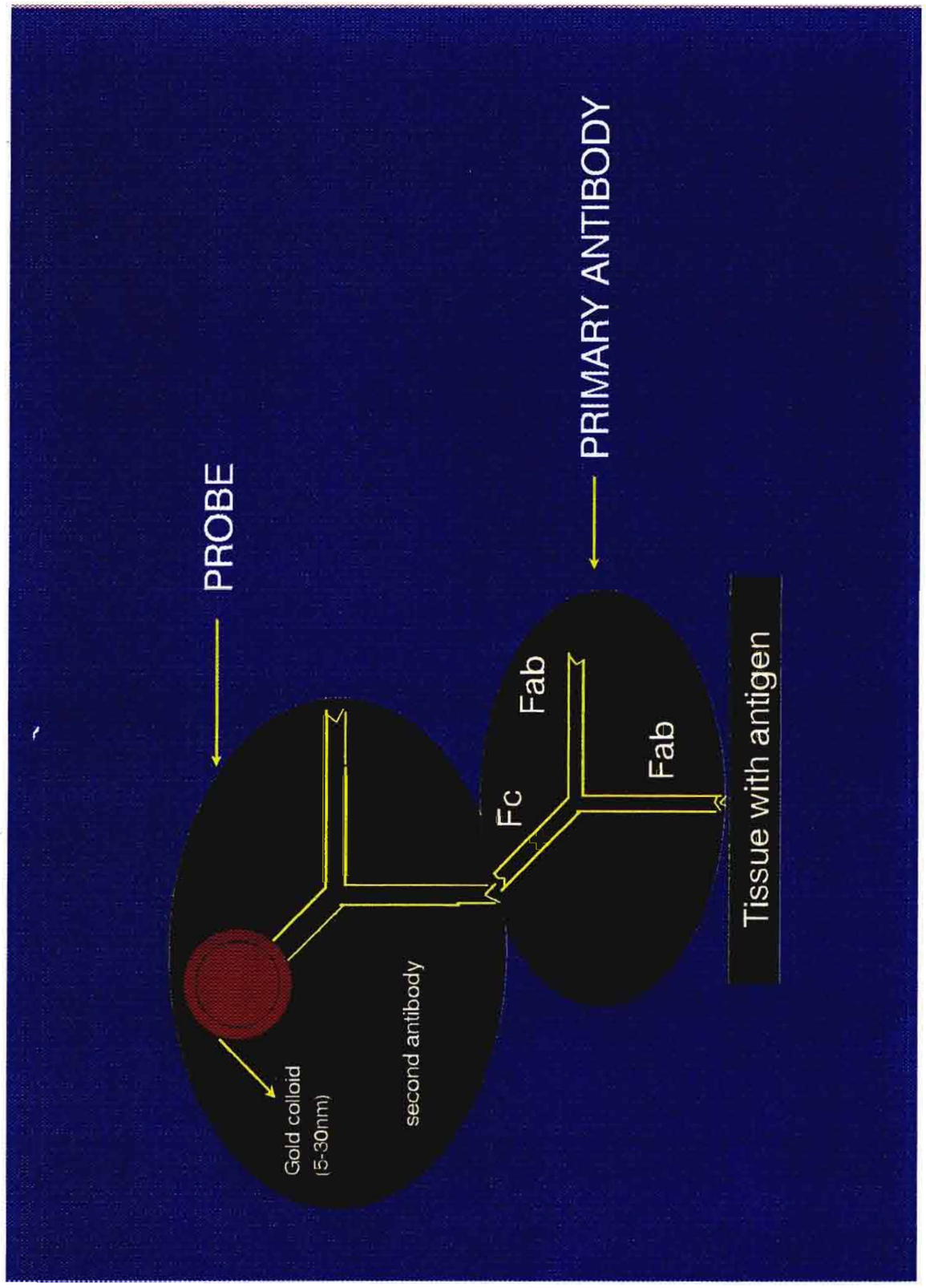


Figure 7: Immunolocalisation of the antigen in tissue using gold colloidal probes

2.2.3.2 Tissue preparation

Pellets were fixed in 4% glutaraldehyde overnight and after a washing in PBS, the pellets were post fixed in 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer for 45 min. Pellets were dehydrated through a graded series of alcohol, embedded in epoxy-araldite resin and cured overnight at 36^0C. Ultrathin sections were cut on a Reichert Ultracut ultramicrotome and picked up on Cu grids (200 mesh), counterstained with lead citrate and uranyl acetate, and viewed in a Joel 100C transmission electron microscope.

2.2.4 **Confocal microscopy**

2.2.4.1 Sample preparation

The isolated neutrophils were pipetted on to a glass slide, air-dried and fixed with paraformaldehyde (4% in PBS, 1 min). The slides were stored at 4^0C until immunolabelled and viewed in a confocal microscope (Leica, Heidelberg, Germany).

2.2.4.2 Immunolabelling procedure

The neutrophil smears and rehydrated wax sections were rinsed with PBS containing bovine serum albumin (1%, v/v) for 20 min, and sequentially incubated with the appropriate primary antibody (1:500) for 3 h, rinsed in PBS and incubated with fluorescein-isothiocyanate (FITC)-conjugated $F(ab)_2$ fragments (fig 8) of antispecies IgG (1:250, v/v) for 30 min.

2.2.4.3 Viewing of specimens

The specific wavelengths of the resulting emitted light beam (485 nm for FITC, 514 nm for CY3) within cells was detected with a confocal microscope equipped with an excitatory argon laser. The argon laser has spectral lines at 488 nm and 514 nm. The FITC probe has an optimal excitation wavelength of 485 ± 11 nm and an optimal emission wavelength of ± 530 nm while the CY3 probe has an optimal excitation wavelength of 546 ± 5 nm and an optimal emission wavelength of ± 590 nm. The microcope optically collects the emitted fluorescent light from sequential positions in a grid format within any focal plane of the objective and reconstructs the reading from these points into a mosiac format, thereby achieving visualisation of the immunostained cell antigens throughout that plane (fig 11).

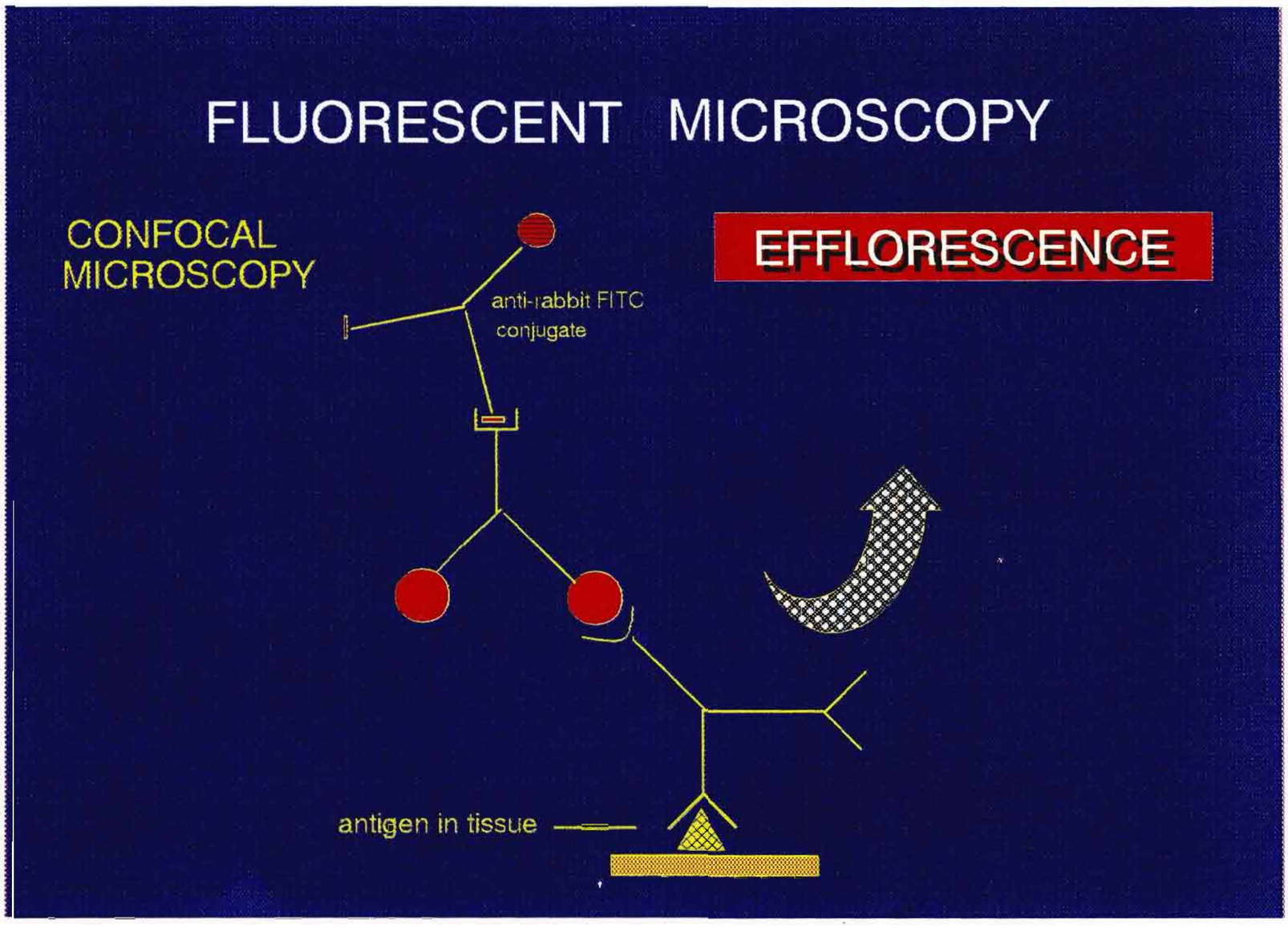


Figure 8: Localisation of the antigen in tissue by fluorescent microscopy

2.2.5 **Immunocytochemical controls**

The immunolocalisation controls included omission of the primary antibody or replacement by non-immune serum to assess the validity of the labelling method. Controls also included replacement of the primary antibody with the diluted, specific antibody preabsorbed with the respective antigen to assess the specificity of the primary antibody. Negative labelling results in both these controls will indicate accuracy of the method and specificity of the antibody.

2.2.6 **Release Experiments**

2.2.6.1 Enzymes

TK was from Dr. M. Kemme, Technische Hochschule Darmstadt, Germany), PK, trypsin and bacterial proteases *Nagarse* and *Serratiopeptidase* were from Sigma, St. Louis, UK.

2.2.6.2 Dose - response

After separation of neutrophils and washing with PBS, the pellets were resuspended in PBS containing 5 mM/l glucose, oxygenated (95% O_2 and 5% CO_2 dispensed from an Afrox, 10 l cylinder) and dispensed into microfuge tubes for dose-response measurements. One ml each, of a 20, 100, and 500 ng/ml enzyme concentration was added to microfuge tubes containing neutrophils, but for controls, neutrophils were incubated without the enzyme. After 20 min incubation, the cells were pelleted (10 000 x g, 5 min, room temperature),

spotted onto glass slides and immunolabelled with the appropriate primary antibody and fluorescent marker. The different dose effects on the kinin moiety contained in the kininogen molecule on the surface of the neutrophil were observed using a confocal microscope.

2.2.6.3 Time - dependence procedure

After separation of neutrophils and washing with PBS, the pellets were resuspended in PBS containing 5 mM/l glucose, oxygenated (95% O_2 and 5% CO_2 dispensed from an Afrox, 10 l cylinder) and dispensed into microfuge tubes for time -dependent measurements. One ml each of a 100 ng/ml enzyme concentration was added to microfuge tubes containing neutrophils but for a control, neutrophils were incubated without enzyme. At 0, 15 and 30 min intervals, the cells were pelleted (10 000 x g, 5 min, room temperature), spotted onto glass slides and immunolabelled with the appropriate primary antibody and fluorescent marker. The effects of different times of exposure of the enzymes to the kinin moiety on the surface of the neutrophil were observed.

CHAPTER 3

RESULTS

3.1 *Controls - Normal neutrophils*

3.1.1 **Light microscopy**

Demonstration of the kinin forming enzymes and their substrates on the normal human neutrophil was achieved at first by light microscopy immunostaining. All components of the kallikrein-kinin system rendered intense labelling results when incubated with the appropriate primary antibody. Immunoreactive tissue kallikrein (fig 9), prokallikrein (fig 10), PK (fig 11) and kininogen (fig 12) were observed as intense red staining imparted by the aminoethylcarbazole (AEC) chromogen. The monoclonal antibody, (SBK1) directed against the bradykinin sequence in the kininogen molecule also immunoreacted intensely with the antigen localised on the surface of the neutrophils harvested from normal blood (fig 13).

An antiserum is specific when it contains primary antibodies reacting only with the antigen to be localised with no cross-reactivity with other compounds. The absorption of an antiserum with the antigen to which it was raised represents a test for such specificity. Inhibition of each label was obtained when the antiserum was pre-absorbed with the specific antigen. A typical result is illustrated in fig 14, in which the antiserum SBK1 was pre-absorbed with bradykinin. Replacement of the primary antibody with the pre-immune equivalent was done only at the beginning of each set of experiments because of the quantity required and the expense and availability of antibodies. In experiments that followed, the primary antibody was replaced with buffer only. In controls where primary antibody was replaced with buffer, no labelling was observed (fig 15).

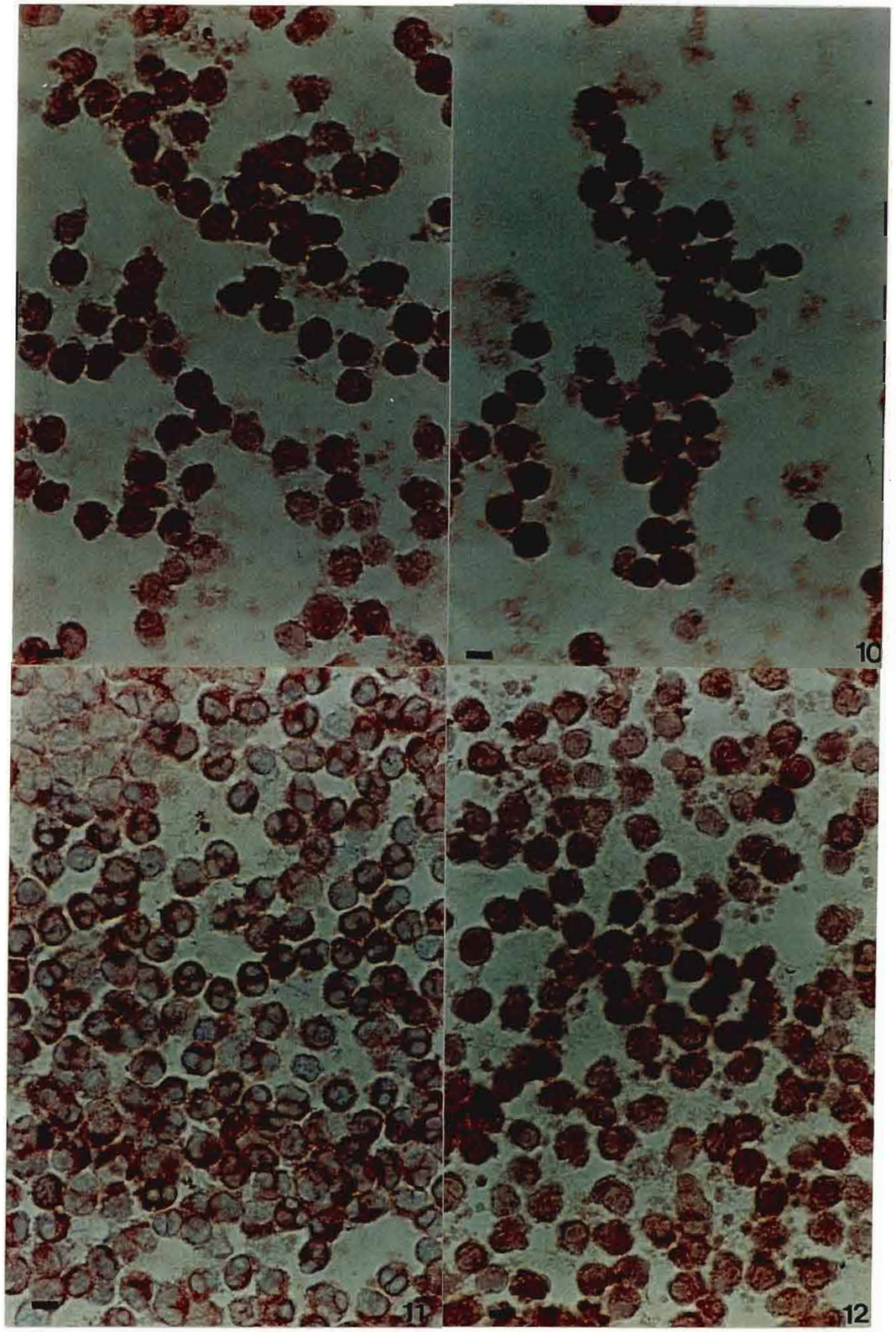
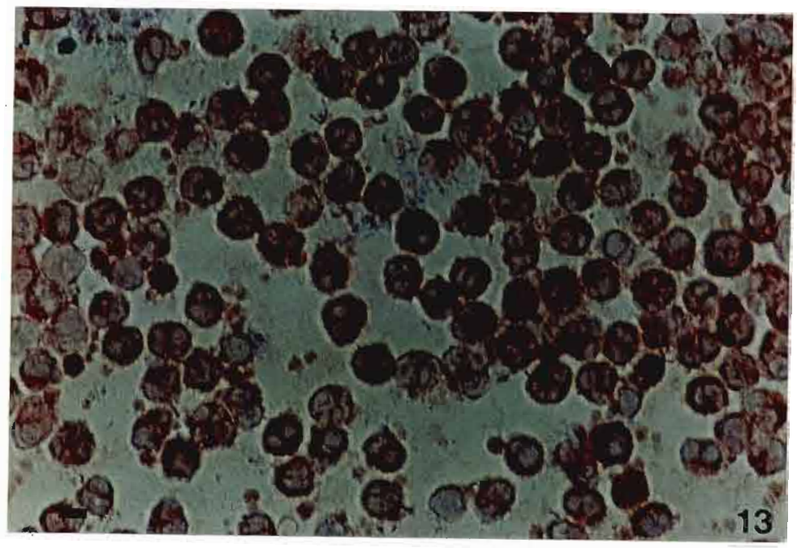


Figure 9: Immunolocalisation of TK in neutrophils isolated from normal, healthy volunteers

Figure 10: Immunolocalisation of proTK in neutrophils isolated from normal, healthy volunteers

Figure 11: Immunolocalisation of PK in neutrophils isolated from normal, healthy volunteers

Figure 12: Immunolocalisation of kininogen in neutrophils isolated from normal, healthy volunteers
(Bar = 10 μ)



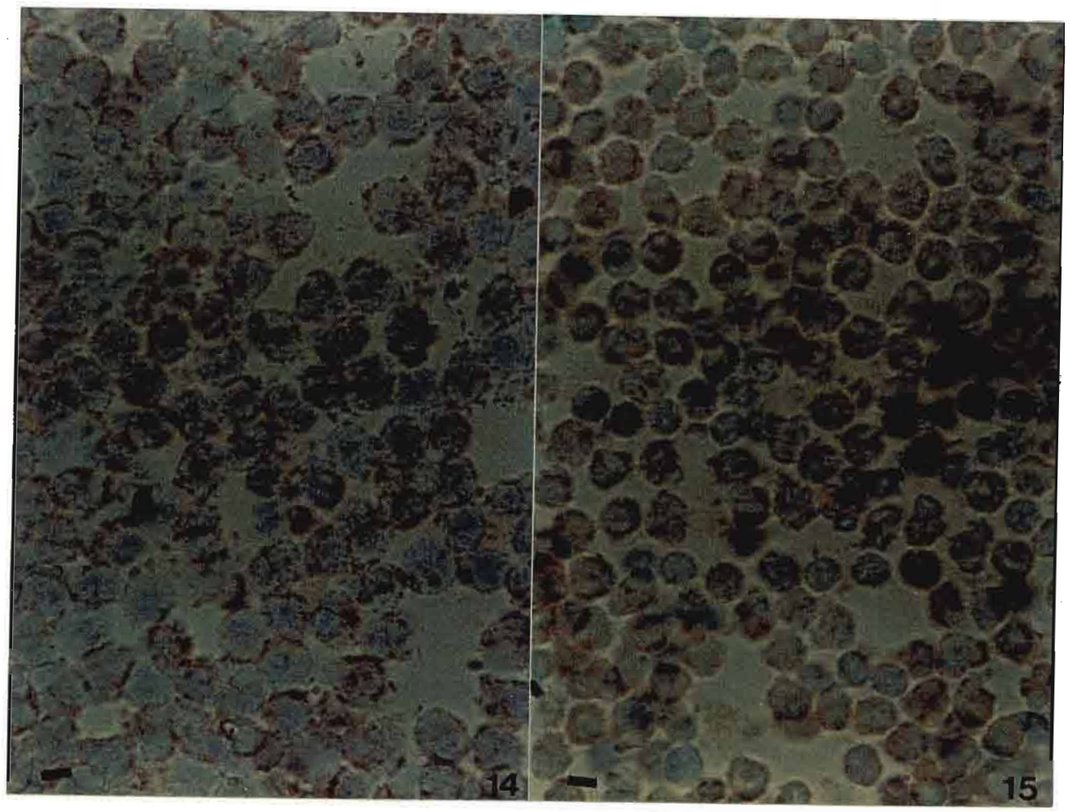


Figure 13: Immunovisualisation of the kinin moiety on the surface of the human neutrophil

Figure 14: Antibody control showing no labelling when the antibody was preabsorbed with the respective specific antigen

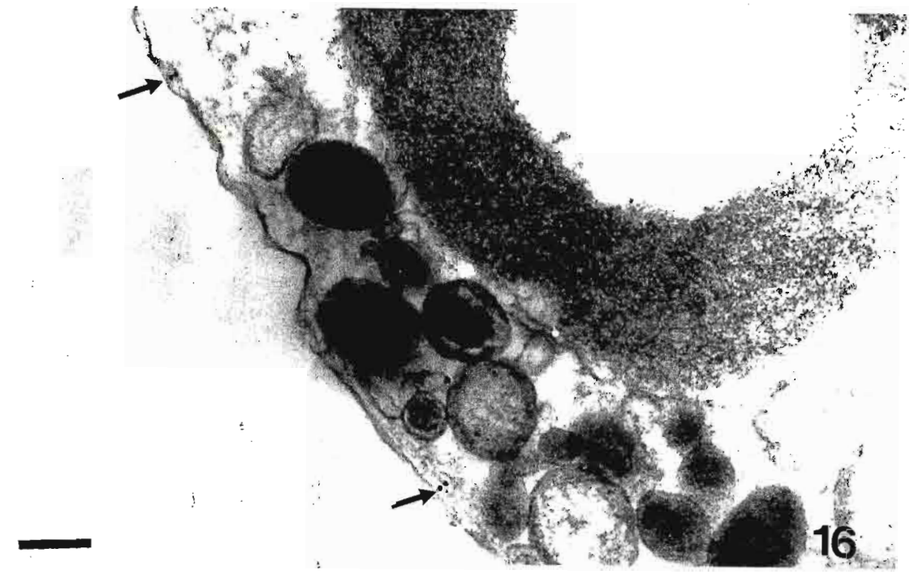
Figure 15: Antibody control showing no labelling when the antibody was replaced with buffer
(Bar = 10 μ)

3.1.2 Transmission electron microscopy

Although labelling was achieved, a more exact localisation within the cell or on its surface was difficult to ascertain with light microscopy. In order to achieve a more accurate cell distribution profile, resin embedded cells were immunolabelled with colloidal gold for visualisation by electron microscopy.

In immunocytochemistry, a compromise must be sought between optimal labelling and retention of ultrastructural detail. For this reason, the suspended cells were post-fixed in 4% glutaraldehyde, which optimally preserves the ultrastructure without destroying antigenicity.

Immunogold labelling for tissue kallikrein showed immunoreactive TK (fig 16) and ProTK (fig 17) within the cell. The kinin moiety was observed as clusters of gold deposits on the surface of the neutrophils isolated from blood of normal healthy volunteers (fig 18). The density of immunogold complexes on the cells differed between cells. Of the various cell types, localisation was observed only in the neutrophils. Where the primary antibody was replaced with buffer, no gold deposits were observed (fig 19). Kininogens, both LK and HK are similarly orientated on the external surface of the human neutrophil (Henderson et al., 1994).



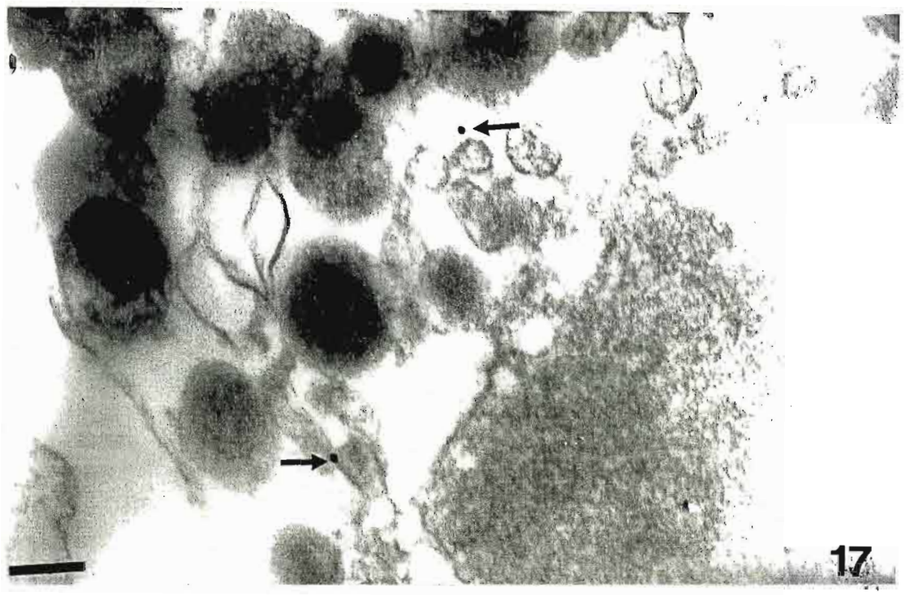
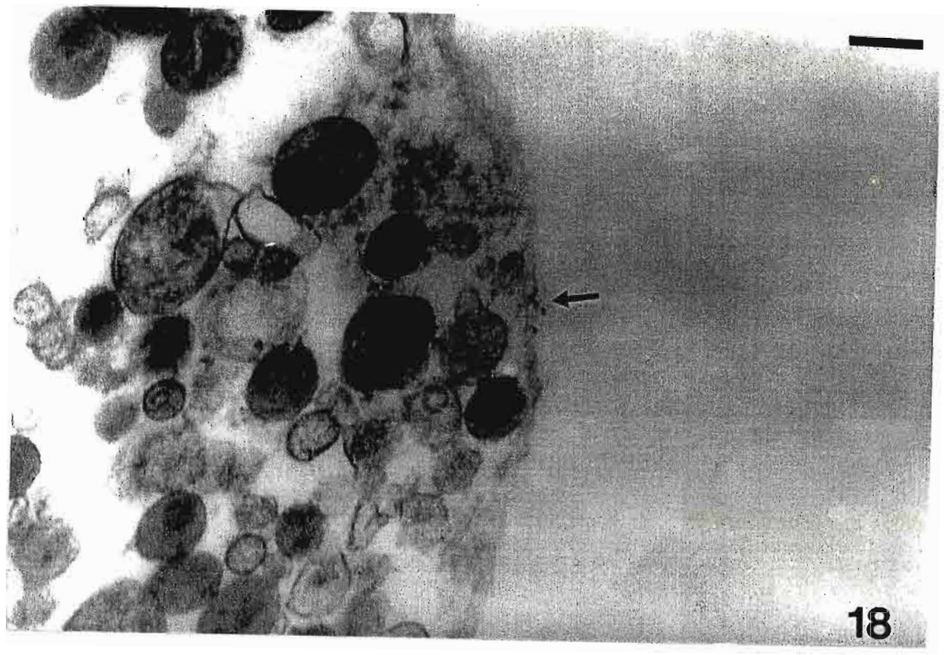


Figure 16: Electron micrograph demonstrating immunoreactive TK
Figure 17: Electron micrograph demonstrating immunoreactive proTK (Bar = 100 nm)



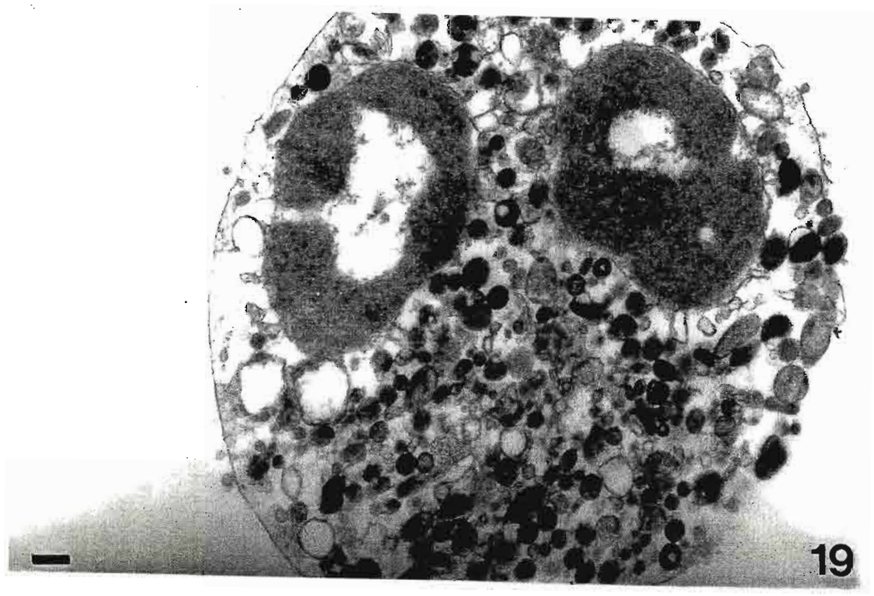


Figure 18: Electron micrograph of a neutrophil isolated from a normal, healthy volunteer exhibiting clusters of gold deposits on the surface

Figure 19: Electron micrograph showing no gold deposits when the primary antibody was replaced with buffer
(Bar = 100 nm)

3.1.3 Confocal microscopy

In order to create multi-dimensional, high resolution optical constructs of the immunolocalised components of the kinin system, on and within the neutrophil, the powerful imaging technique of confocal scanning laser microscopy was used (fig 20). Progressive scanning of the cellular field, from the surface to the deeper layers, provided optical images of high clarity.

The images obtained for immunolabelled TK, proTK, PK, kininogen and kinin from blood of healthy volunteers are illustrated in figs 21, 22, 23, 24 and 25 respectively. Doubts have been expressed as to whether TK is emniocytosed from the circulation by neutrophils or is synthesised *in situ* in neutrophil stem cells. Smears prepared from the blood of patients with chronic myeloid leukaemia revealed intense immunostaining to TK and prokallikrein in the cytoplasmic granules of precursor cells of the neutrophil cell line (fig 26, 27). Myelocytes showed significant immunoreactivity while no labelling was observed in other precursor cells. Furthermore, experiments with the kinin B_1 and B_2 receptors indicated that there was no induction of the receptors in the normal neutrophils. Figure 28 shows the visualisation of the antigen in different optical planes. The image processing programme recorded the fluorescence emitted from the FITC-labelled F(ab)2 secondary antibody, linked to the specific primary antibody, with a pseudocolour gradient that ranged from white-gold (maximal) to brown (nil), giving an estimate of the amount of antigen present in each optical plane.

A particular advantage of a confocal microscope is its ability to do optical sectioning at selectible positions on the z-axis. After having determined the height of the z-axis of the labelled cell, the microscope was focussed halfway down the z-axis on the middle part of the cell. At this point the cell membrane may be visible as a ring structure around the cytoplasm, which eliminates the problem encountered with other microscopes, where labelling which may occur at the top or bottom surfaces may appear as labelling inside the cytoplasm. Labelling which is membrane bound will then be visible as a fluorescent ring.

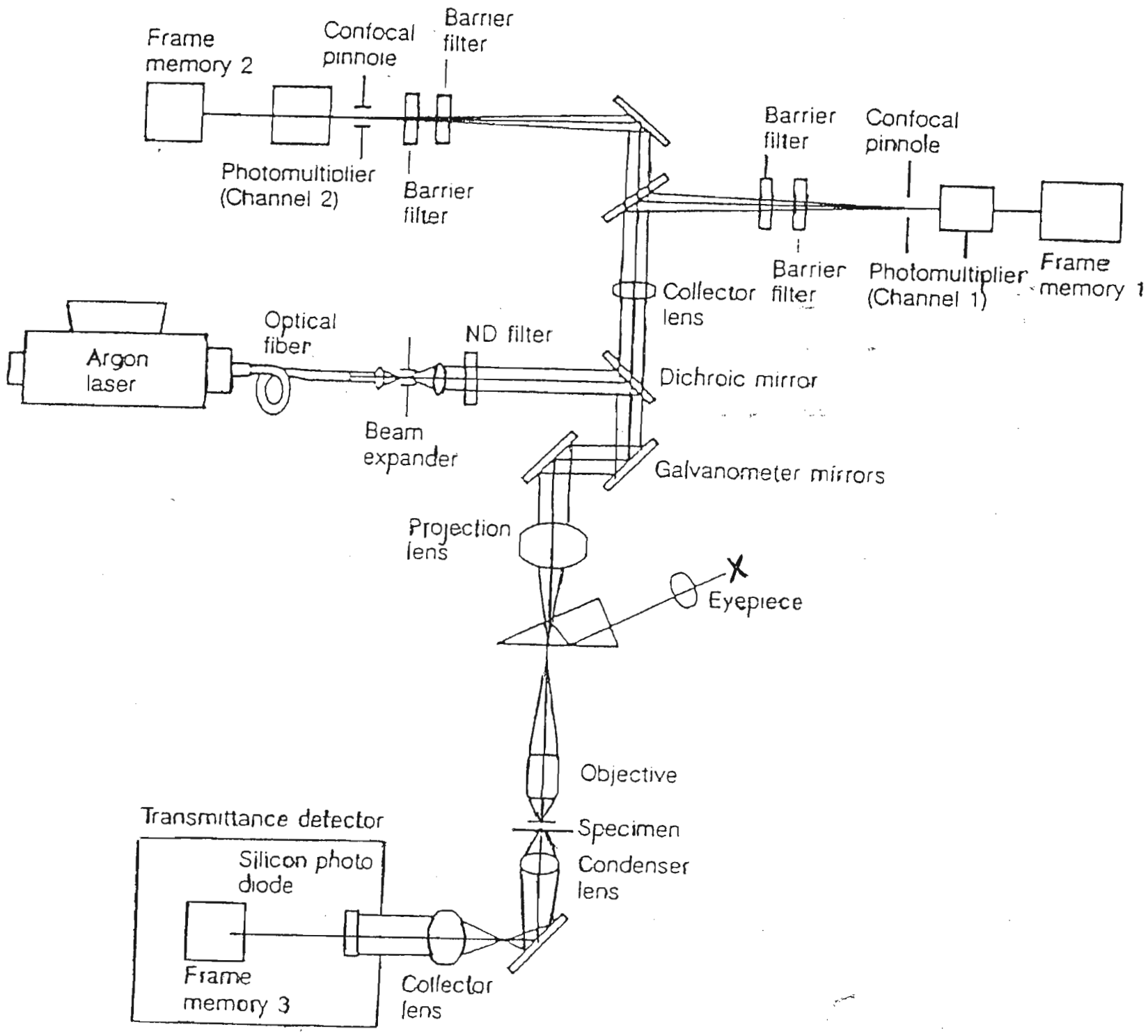


Figure 20: Principles of the laser scanning microscope

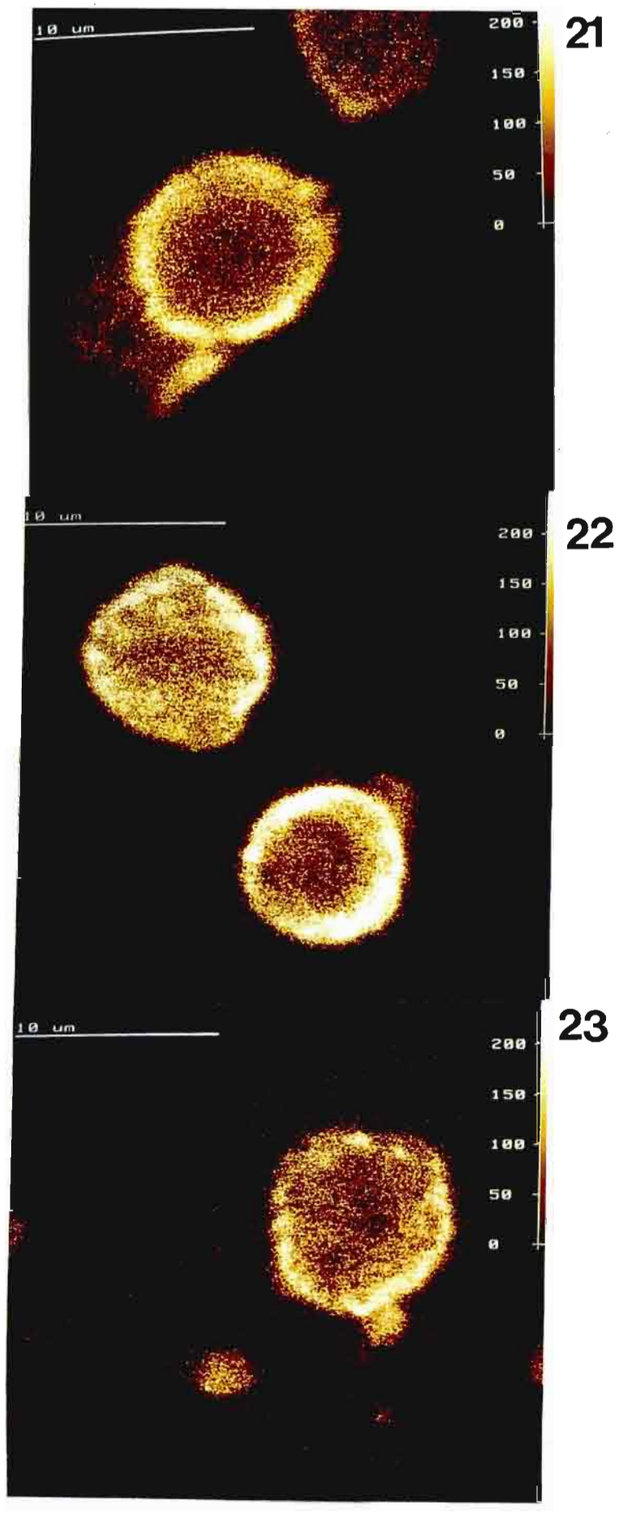


Figure 21: Confocal microscopic images of immunolabelled TK
Figure 22: Immunolabelled ProTK in the human neutrophil
Figure 23: Confocal images of immunolocalised PK

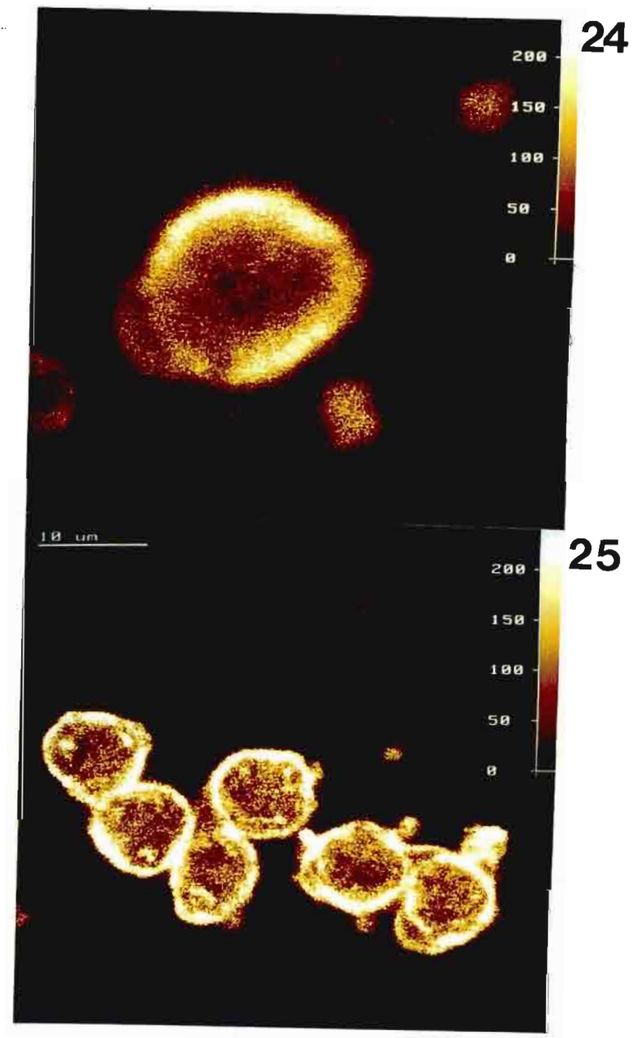


Figure 24: Immunolocalisation of the kininogen molecule on the neutrophil surface
Figure 25: Immunocytochemical localisation of the kinin molecule on the neutrophil surface

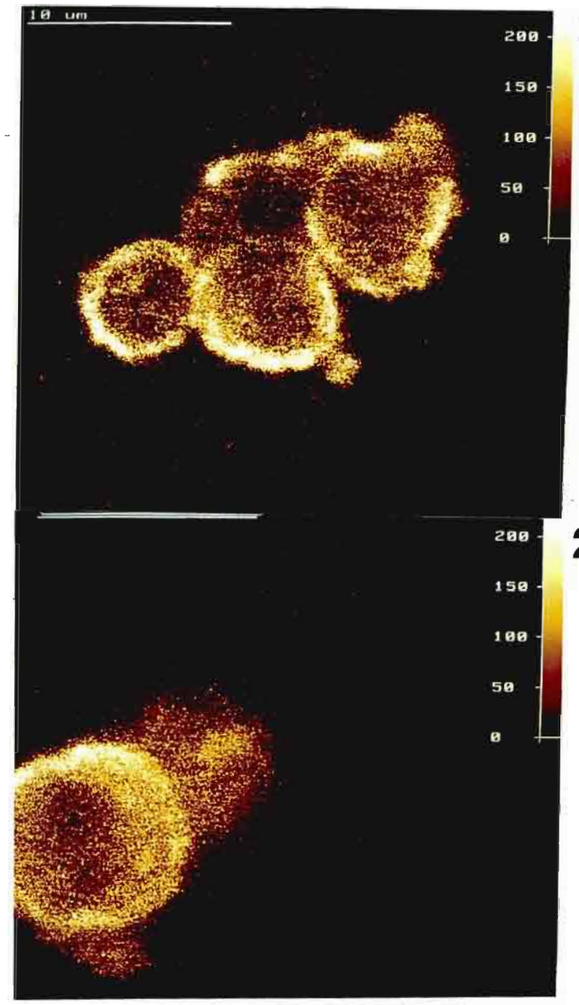


Figure 26: Intense labelling of TK in leukaemic blood cells
Figure 27: Immunolabelling of proTK in blood neutrophils from patients suffering from myeloid leukaema

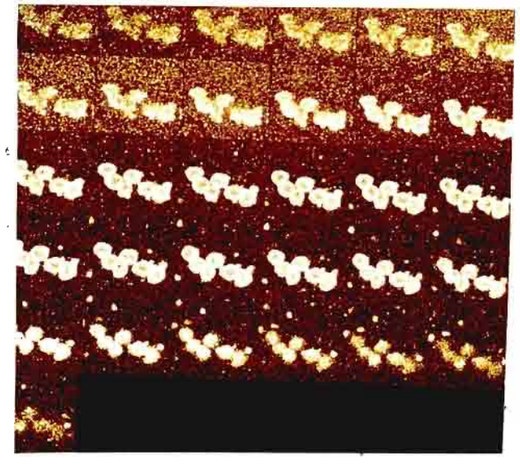


Figure 28: Immunocytochemical localisation of kinin in different optical planes

3.2 *Release of the kinin moiety from kininogen on the external surface of the neutrophil membrane*

3.2.1 **Time-dependence study**

The ability of different enzymes to release the kinin molecule from kininogens located on the neutrophil surface was examined by incubating human neutrophils with both kinin-releasing enzymes and bacterial proteases for different time intervals. Confocal microscopy was used to follow the timed release of kinins from neutrophil-bound kininogens. Neutrophils were incubated with the known kininogenases: TK (fig 29 a-d), PK (fig 30 a-d), trypsin (fig 31 a-d), the bacterial proteases nagarse, and serratiopeptidase. (figs 32, 33 a-d) for 0 (a), 15(b) and 30 min (c). The presence or absence of kinin was confirmed by immunolabelling with the specific SBK1 antibody. The immunolabelling procedure followed FITC labelling and viewing by confocal microscopy. Cells treated with PK, TK and trypsin showed a considerable loss of staining which was completed by 30 min. However, the time-response of the bacterial proteases was considerably longer (observed for a further 2 h), with loss of staining only commencing at 30 min. No labelling was observed in the immunocytochemical control when the antibody was omitted (d).

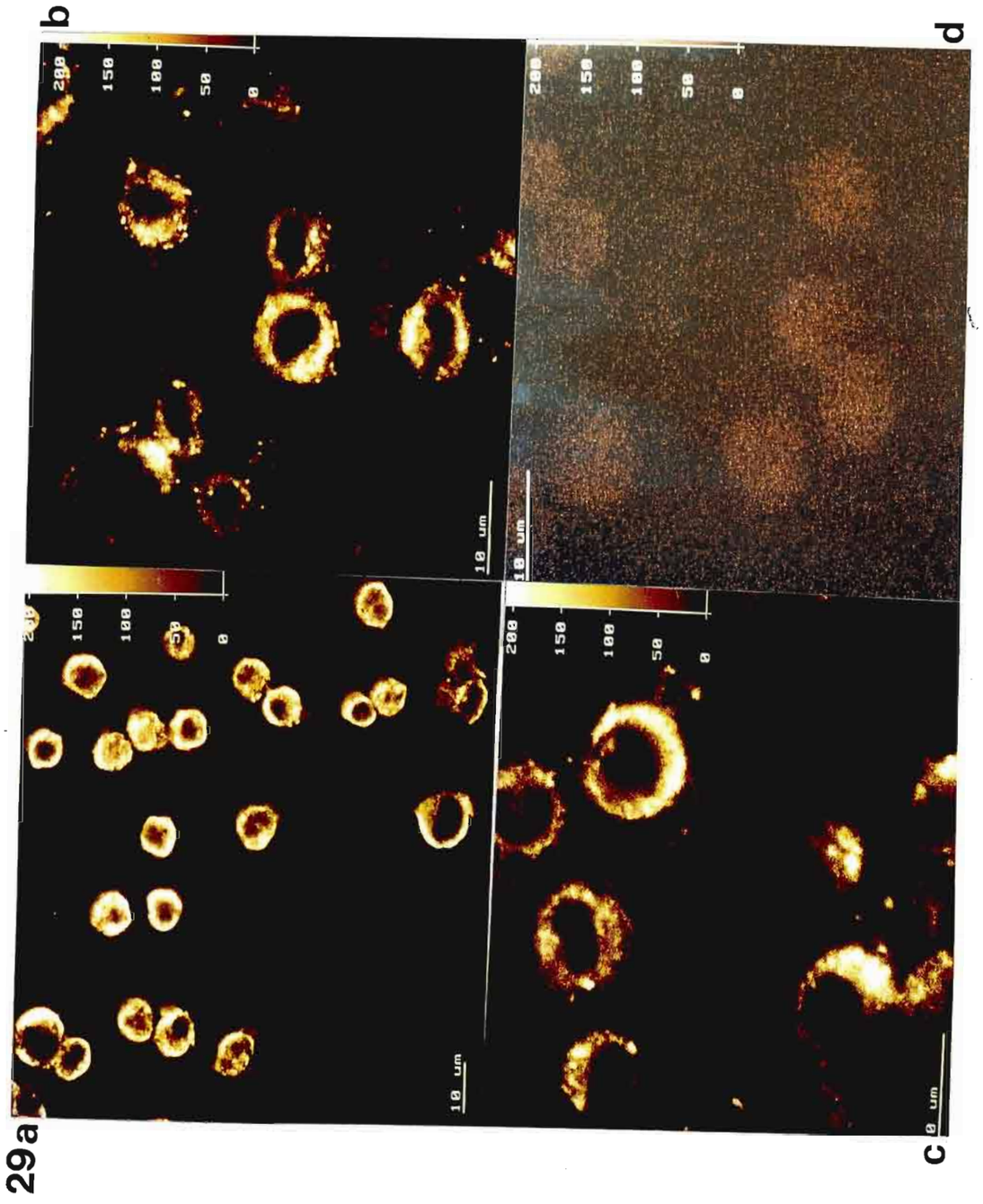


Figure 29: Time-dependent release of kinin after incubation with TK. (a) 0, (b) 15 and (c) 30 min; method control (d)

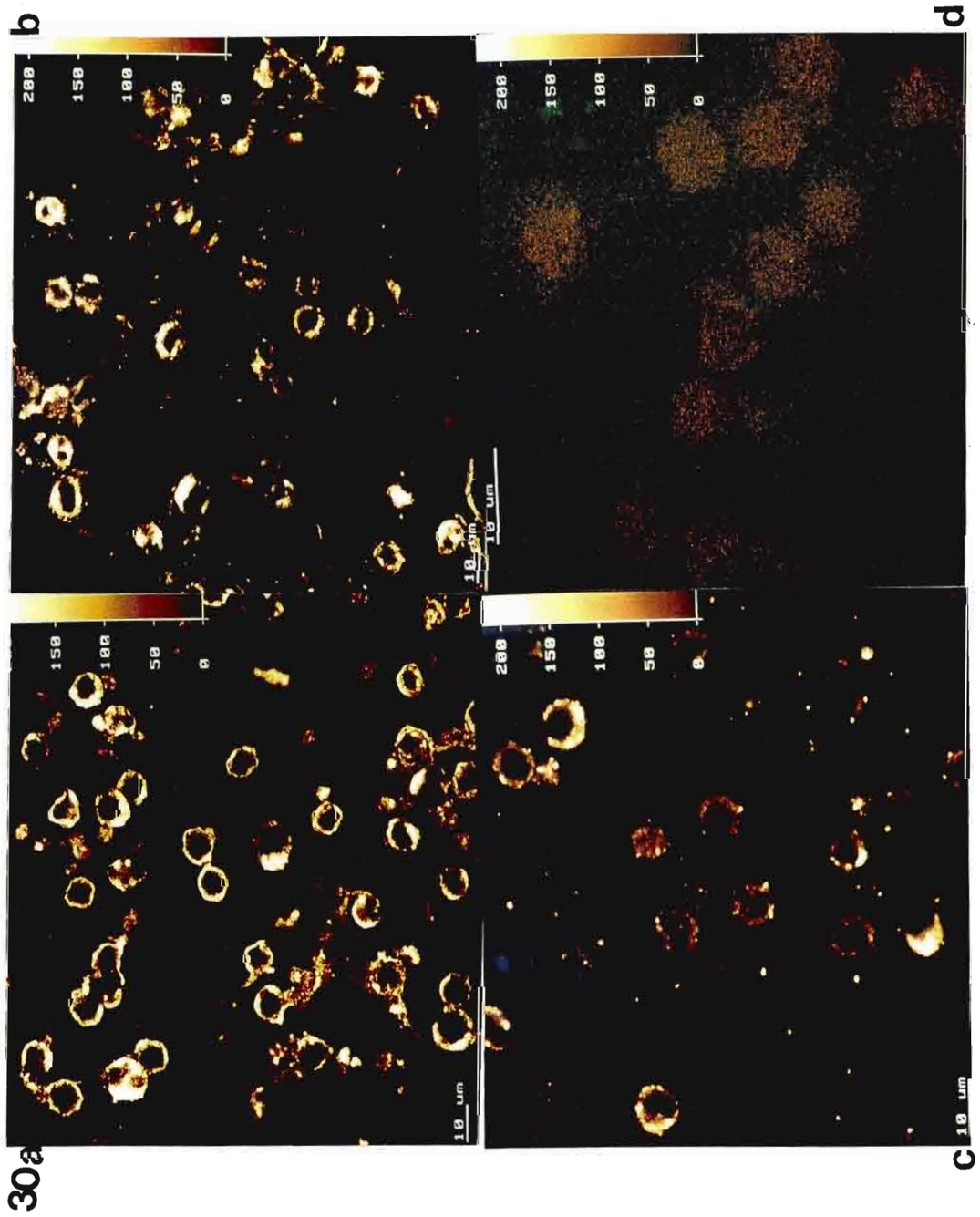
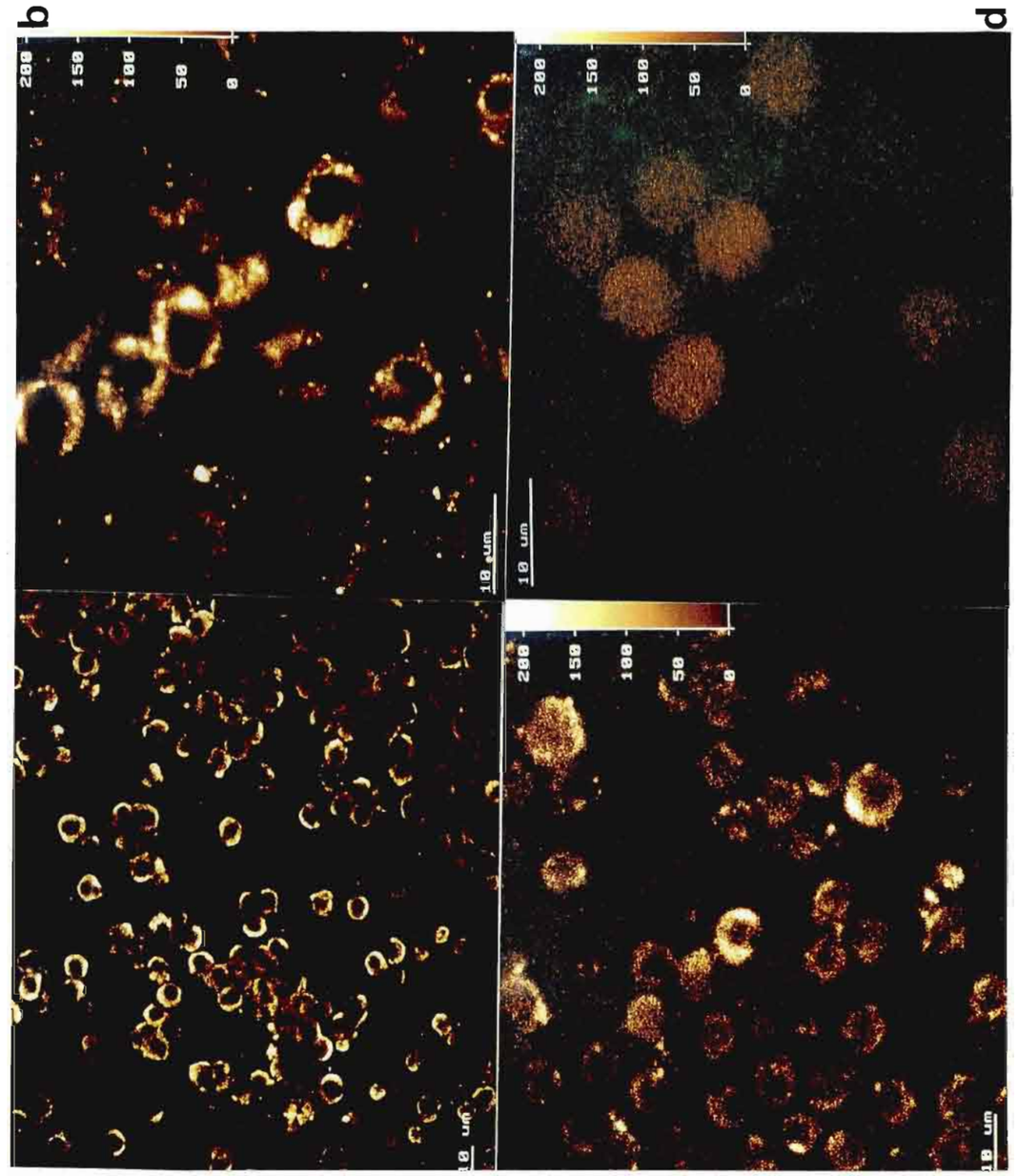


Figure 30: Time-dependent release of kinin after incubation with PK. (a) 0, (b) 15 and (c) 30 min; method control (d)



31a

Figure 31: Time-dependent release of kinin after incubation with trypsin. (a) 0, (b) 15 and (c) 30 min; method control (d)

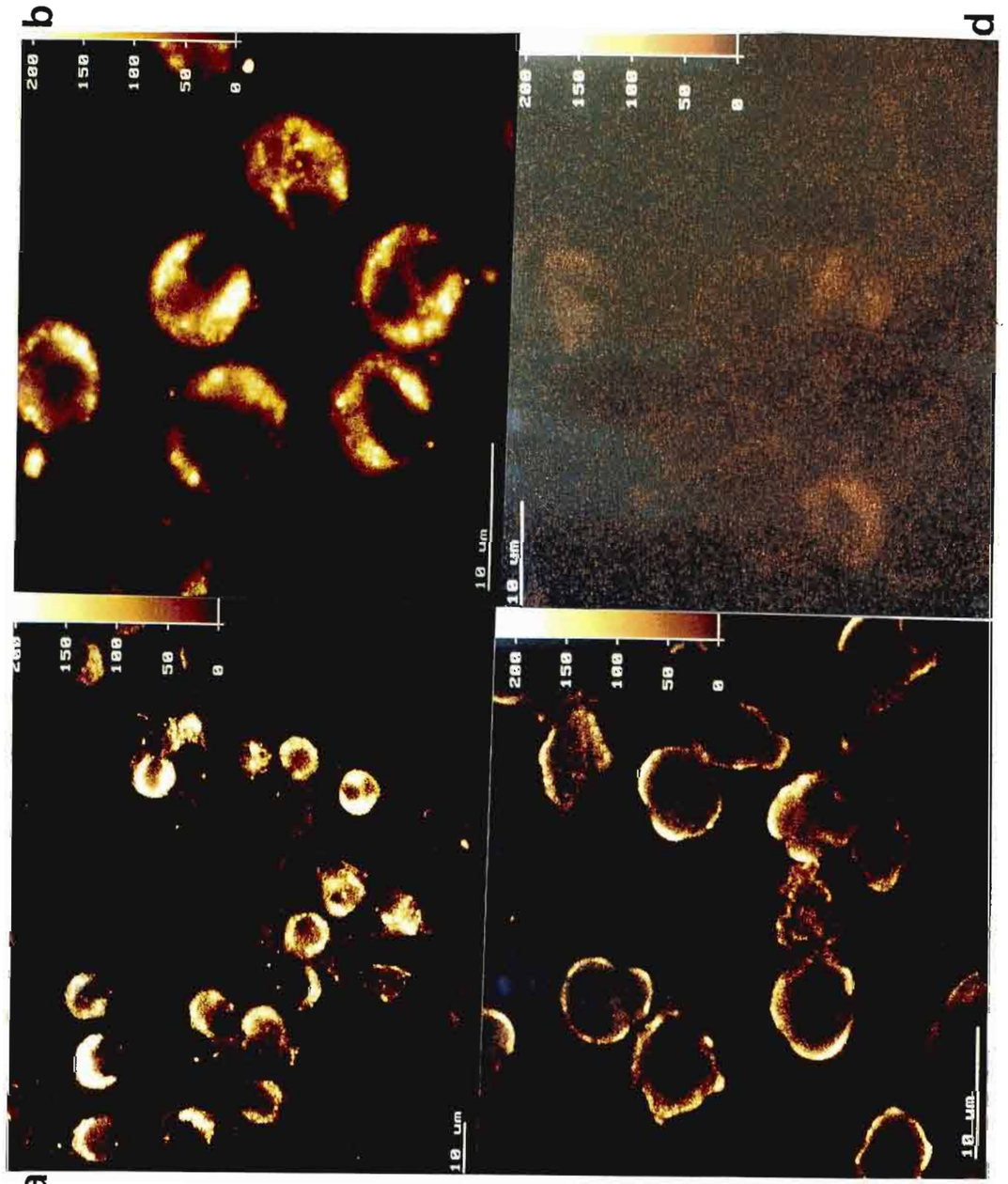


Figure 32: Time-dependent release of kinin after incubation with nagarse. (a) 0, (b) 15 and (c) 30 min; method control (d)

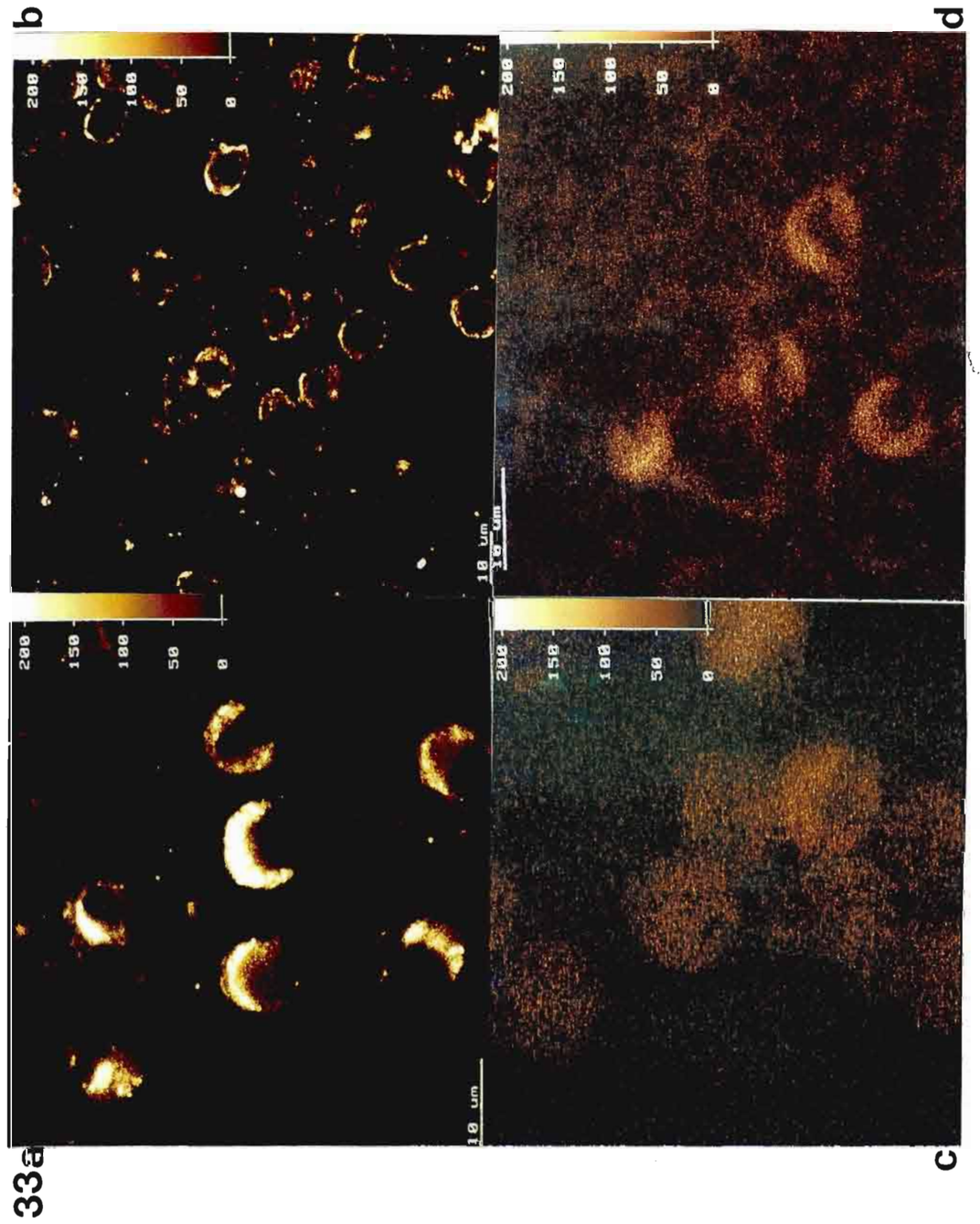


Figure 33: Time-dependent release of kinin after incubation with serratiopeptidase. (a) 0, (b) 15 and (c) 30 min; method control (d)

3.2.2 **Dose-response study**

In order to determine the potency of the different doses of the various enzymes used for the release of kinin from the neutrophil surface, neutrophils were incubated with 20, 100 and 500 ng/ml of the enzyme. After 20 min, the kinin antibody SBK1 was used in the labelling procedure. It was observed that for TK and PK, an increase in the concentration of enzyme used resulted in a rapid drop in staining (figs 34 a-d, 35 a-d, respectively). However, with trypsin and the bacterial proteases, this decrease in staining was gradual (figs 36 a-d, 37 a-d, 38 a-d). There was no labelling in the immunocytochemical controls when the antibody was omitted (d).

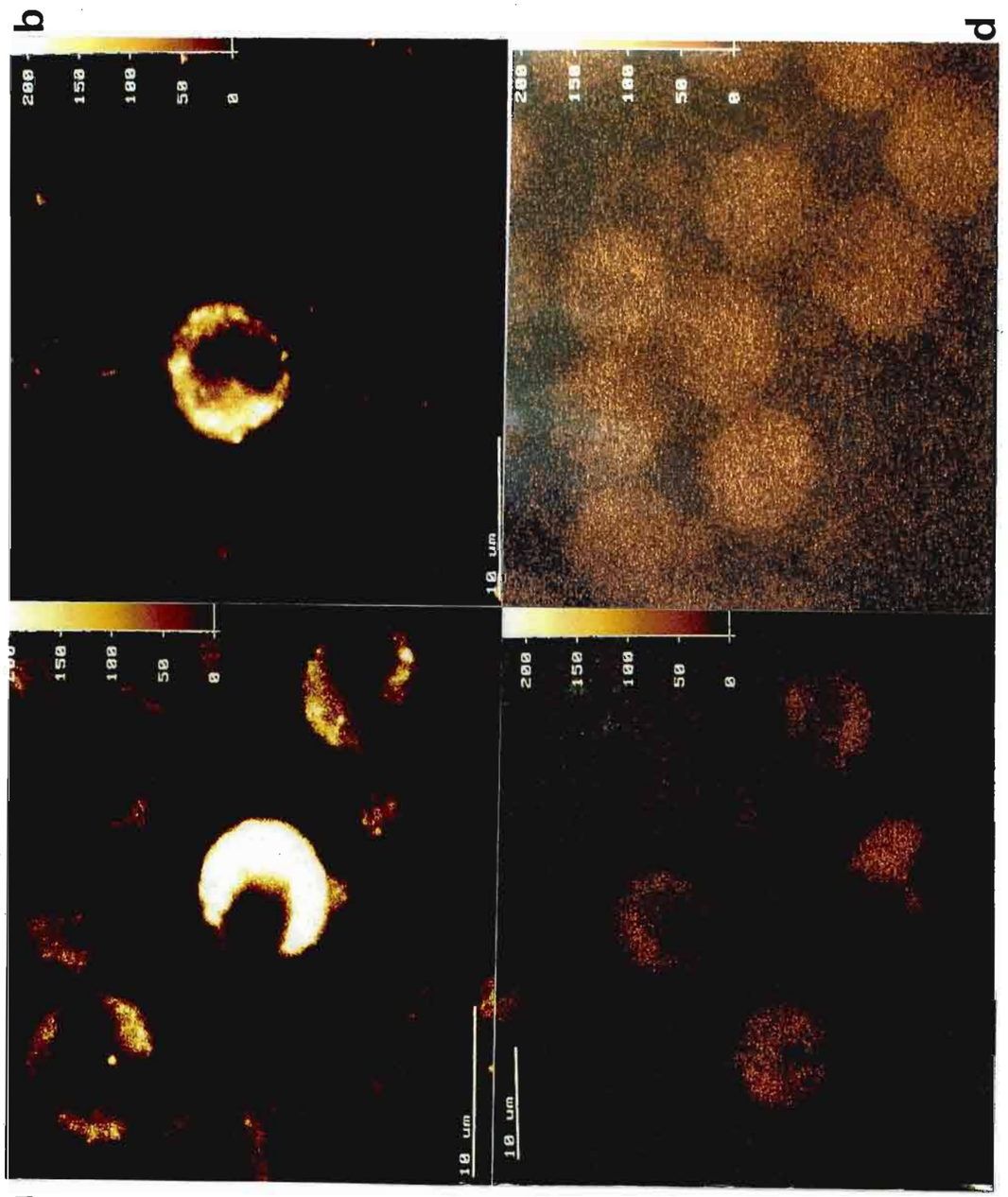


Figure 34: Release of kinin from the neutrophil surface after incubation of the neutrophils with different doses of TK.
(a) 20, (b) 100 and (c) 500 ng/ml; method control (d)

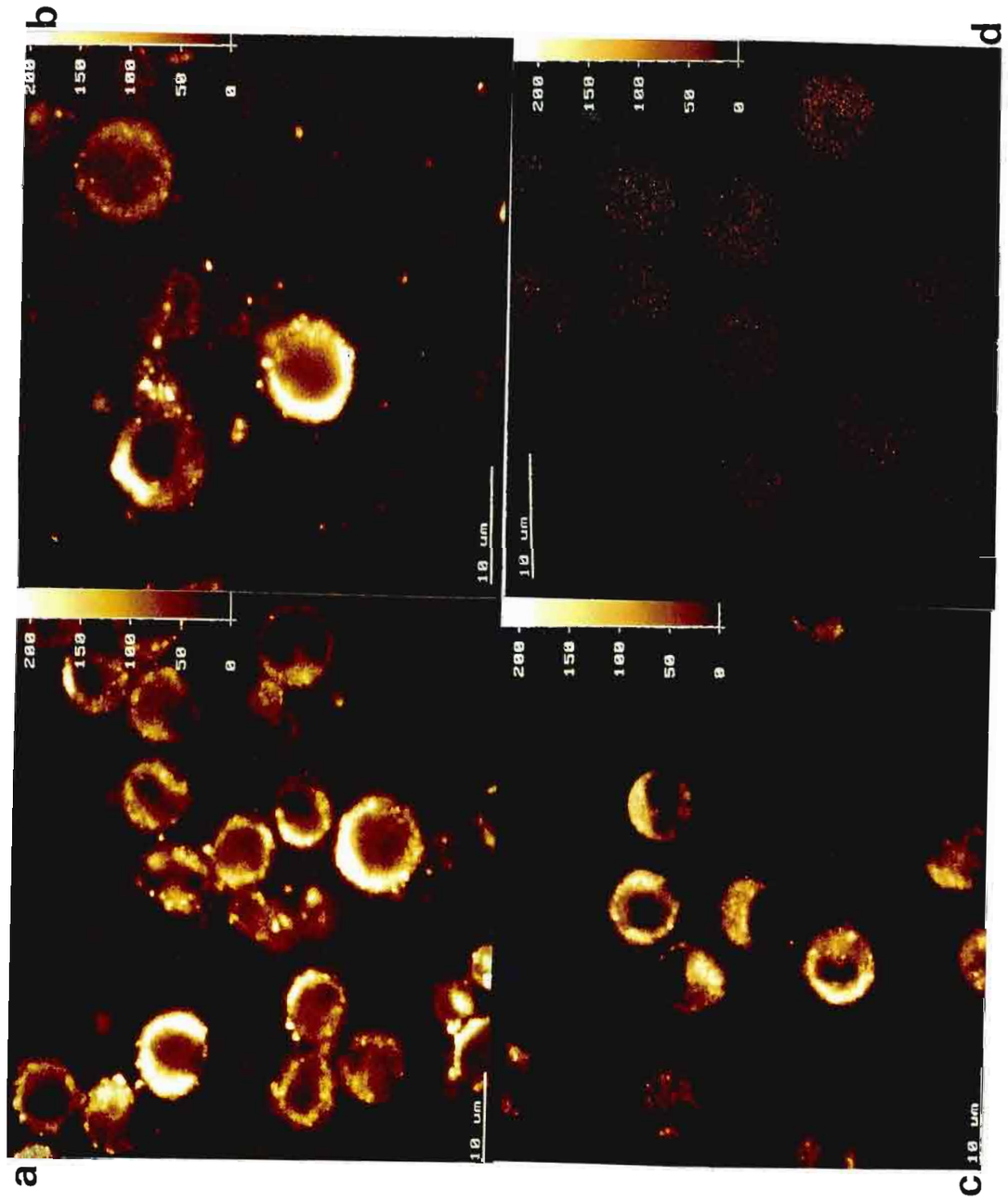


Figure 35: Release of kinin from the neutrophil surface after incubation of the neutrophils with different doses of PK.
(a) 20, (b) 100 and (c) 500 ng/ml; method control (d)

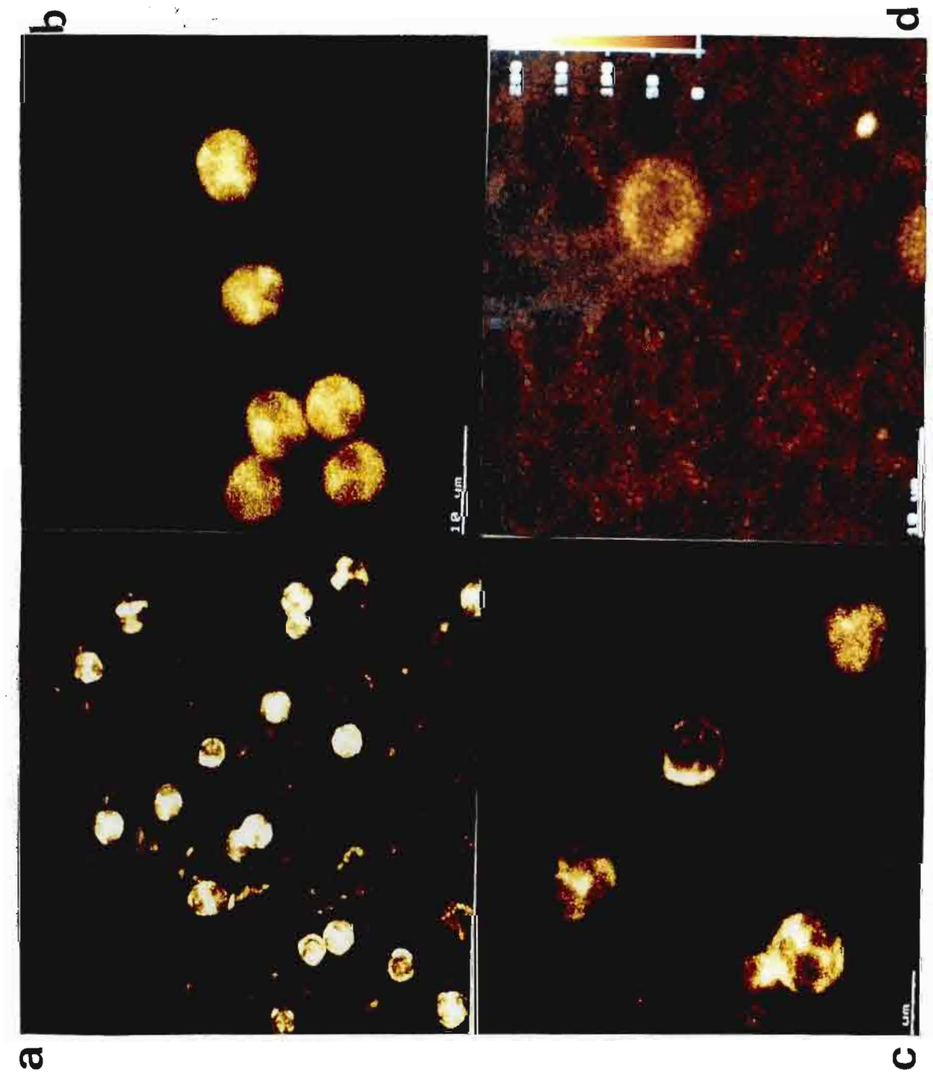


Figure 36: Release of kinin from the neutrophil surface after incubation of the neutrophils with different doses of trypsin.
(a) 20, (b) 100 and (c) 500 ng/ml; method control (d)

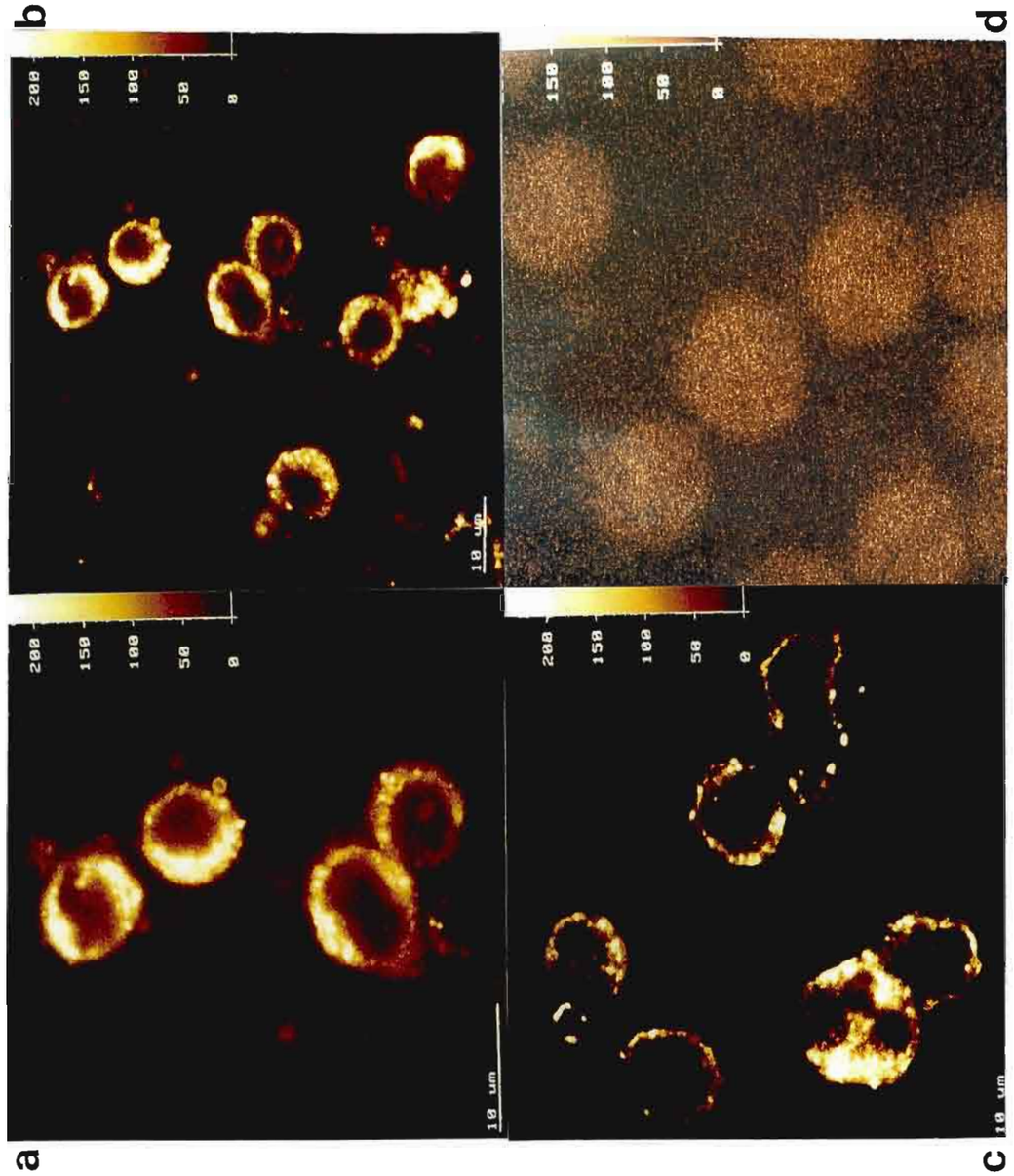


Figure 37: Release of kinin from the neutrophil surface after incubation of the neutrophils with different doses of nagarse.
(a) 20, (b) 100 and (c) 500 ng/ml; method control (d)

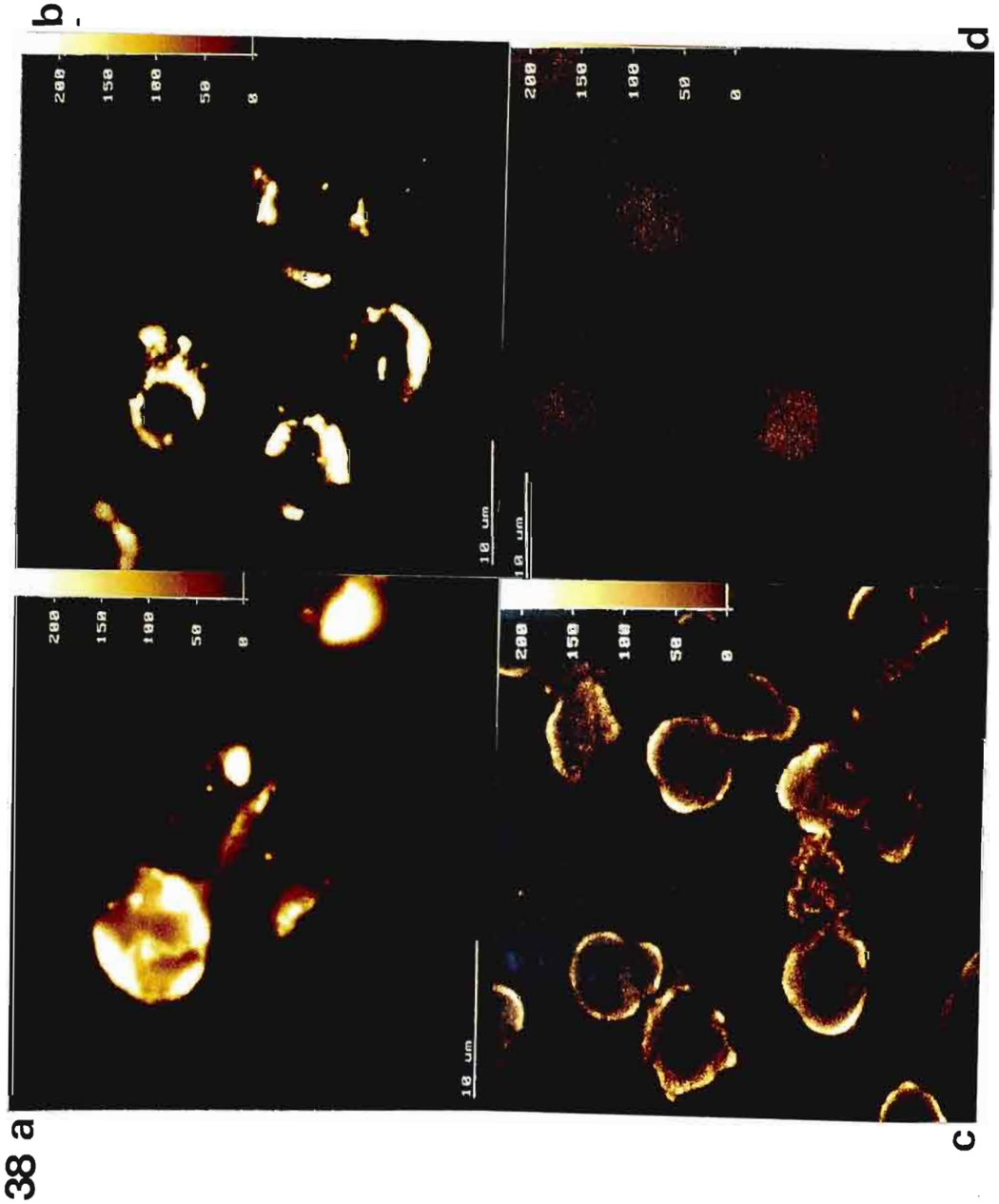


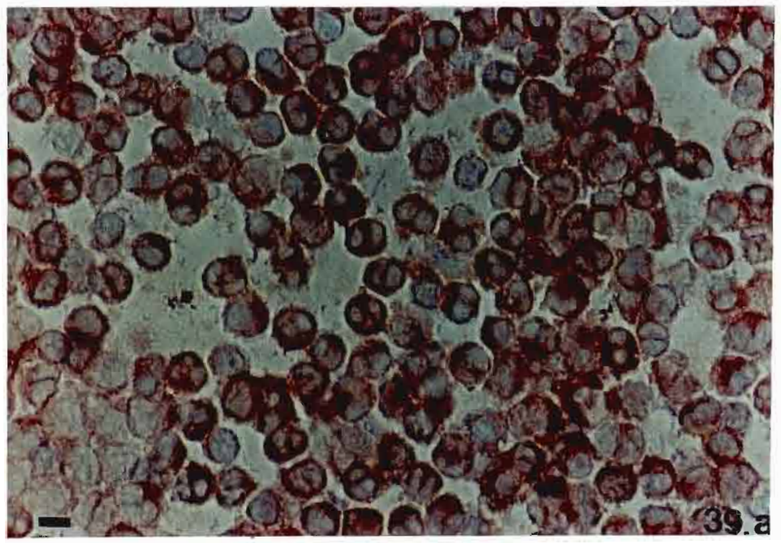
Figure 38: Release of kinin from the neutrophil surface after incubation of the neutrophils with different doses of serratiopeptidase.
(a) 20, (b) 100 and (c) 500 ng/ml; method control (d)

3.3. *Infections*

Discovery of kininogen together with the localisation of PK on domain 6 and the intracellular localisation of tissue kallikrein has led to the novel view that during degranulation of neutrophils, under conditions in which the proTK and preTK could be activated, could provide a mechanism for release of kinin from the surface of neutrophils. In sepsis, a major manifestation is hypotension, a cardinal property of kinins. Therefore, the question posed was, whether or not kinin could be released from neutrophils by the kallikreins themselves, and/or by bacterial proteases? In the first instance, therefore, the time- and dose- dependent experiments were designed. Once the possibility of the release of kinin by the kininogenases (tissue and PKs, trypsin) and bacterial proteases was established, experiments were undertaken to determine whether kinin could be released from circulating neutrophils of patients with bacterial infections.

3.3.1. **Tuberculosis meningitis and pneumonia**

In ten patients with tuberculosis meningitis, immunolabelling (with AEC for light microscopy) indicated a distinct loss of the kinin moiety on the neutrophil surface (fig 39 b compared to the normal fig 39 a). A loss of the reaction product observed when neutrophils from 10 pneumonia patients were labelled with SBK1 (fig 40 a-normal, fig 40 b-loss of kinin) indicated a loss of kinin from the kininogen molecule but this loss of the kinin molecule was not profound. There was no labelling for the antigen when the primary antibody was omitted (fig 41).



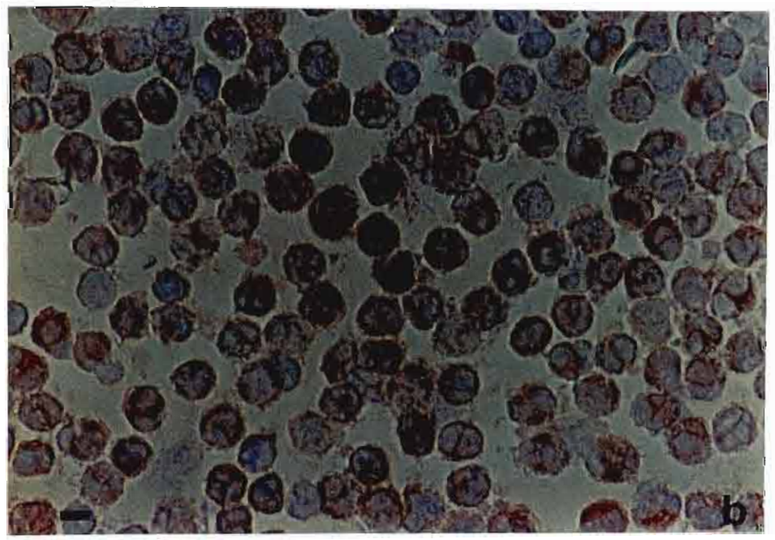
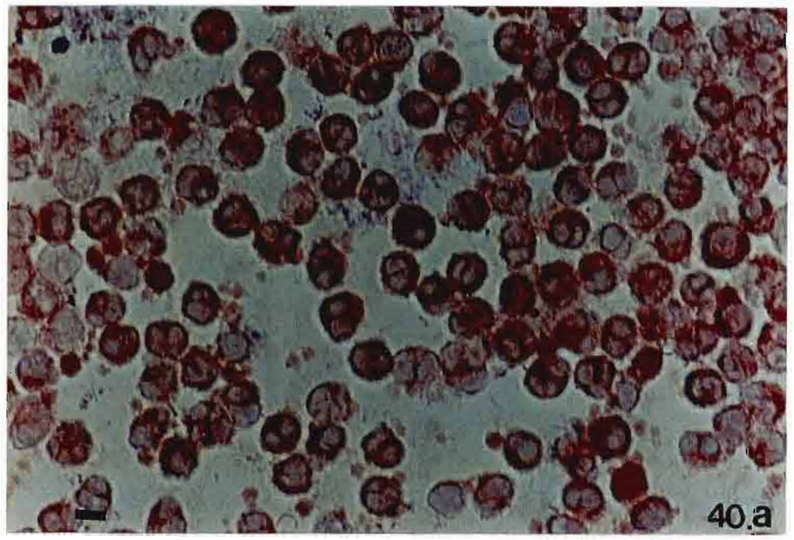


Figure 39: Immunolocalisation of the kinin moiety of kininogen molecule on the surface of neutrophils

(a) Neutrophils from normal healthy volunteers

(b) Neutrophils from patients suffering from tuberculosis meningitis

(Bar = 10 μ)



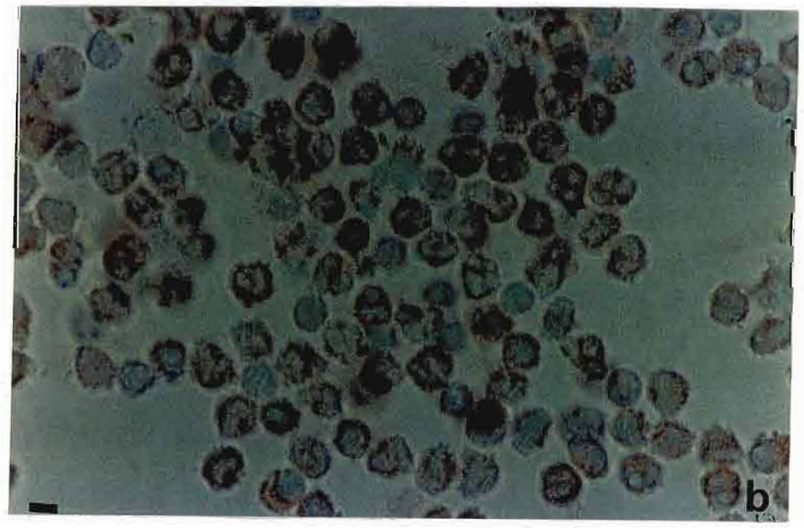


Figure 40: Immunolocalisation of the kinin moiety of the kininogen molecule on the surface of neutrophils
(a) Neutrophils from healthy volunteers
(b) Neutrophils from patients suffering from pneumonia
(Bar = 10 μ)

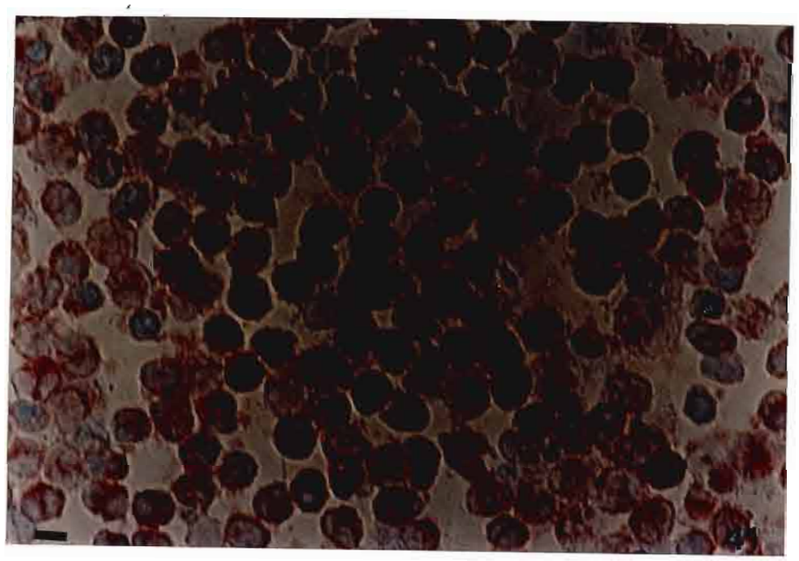


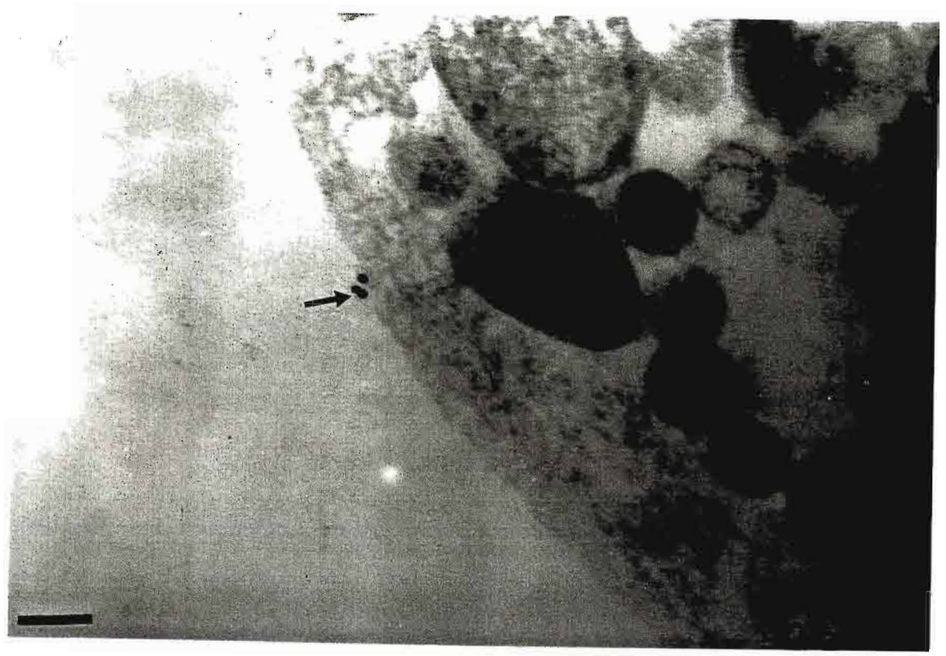
Figure 41: Control labelling for the kinin moiety of kininogen molecule on the surface of neutrophils.
The control consisted of omission of the primary antibody
(Bar = 10 μ)

The cells were subjected also to electron microscopy immunolabelling procedures as previously described. Eight out of 10 pneumonia patients showed a distinct loss of kinin on the surface of the neutrophil (fig 42 a -normal, fig 42 b-loss of kinin) confirming the light microscopy results.

As already observed with electron and light microscopy, there was a distinct loss of the kinin moiety on the surface of circulating neutrophils of pneumonia patients as illustrated by confocal microscopy (fig 43 a-normal, fig 43 b-loss of kinin).

3.5. *Clinical disorders*

An inflammatory response is made up of many stages, the first of which is changes in vascular calibre and tone and an increase in vascular permeability that results in the formation of protein-rich exudates. Neutrophils are attracted to the site of inflammation by chemotactic factors formed in the very early stage of inflammation. They initiate migration by causing neutrophils to marginate and adhere to the endothelium of capillaries (Ryan et al., 1977; Hess et al., 1994). Neutrophils then form endothelial gaps and migrate into the interstitial tissue spaces via these gaps. The mechanism by which neutrophils form endothelial gaps and migrate through them is unknown. However, the immunolocalisation of TK, HK and LK and PK on the human neutrophil is significant, and suggests a possible role in the diapedesis of these cells between capillary cells (fig 44). As a typical example of an inflammatory disorder, rheumatoid arthritis patients were chosen to determine whether kinin could be released from circulating neutrophils.



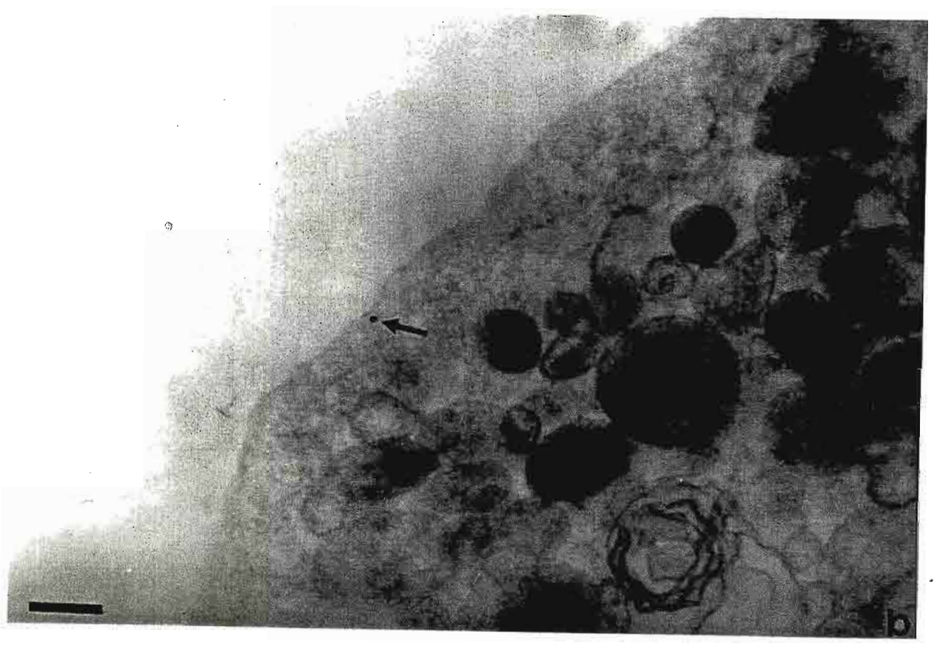
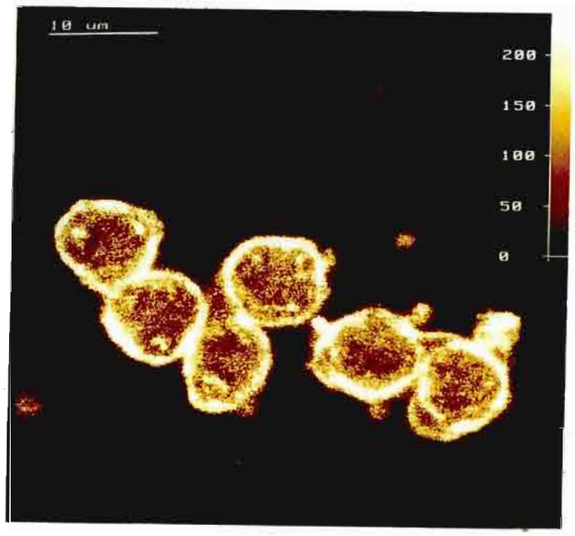


Figure 42: Electron micrograph of the neutrophil showing kinin on the surface of neutrophils
(a) Neutrophils from normal, healthy volunteers
(b) Neutrophils from pneumonia patients
(Bar = 100 nm)



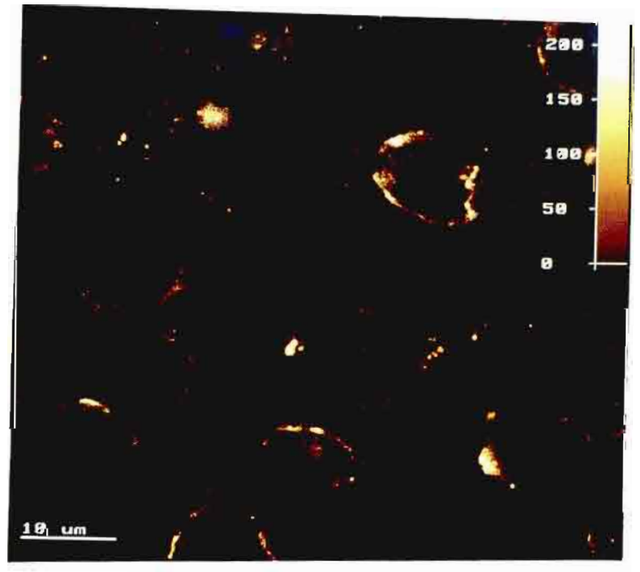


Figure 43: Confocal microscopy images of kinin on the surface of neutrophils
(a) Neutrophils from normal, healthy volunteers
(b) Neutrophils from pneumonia patients

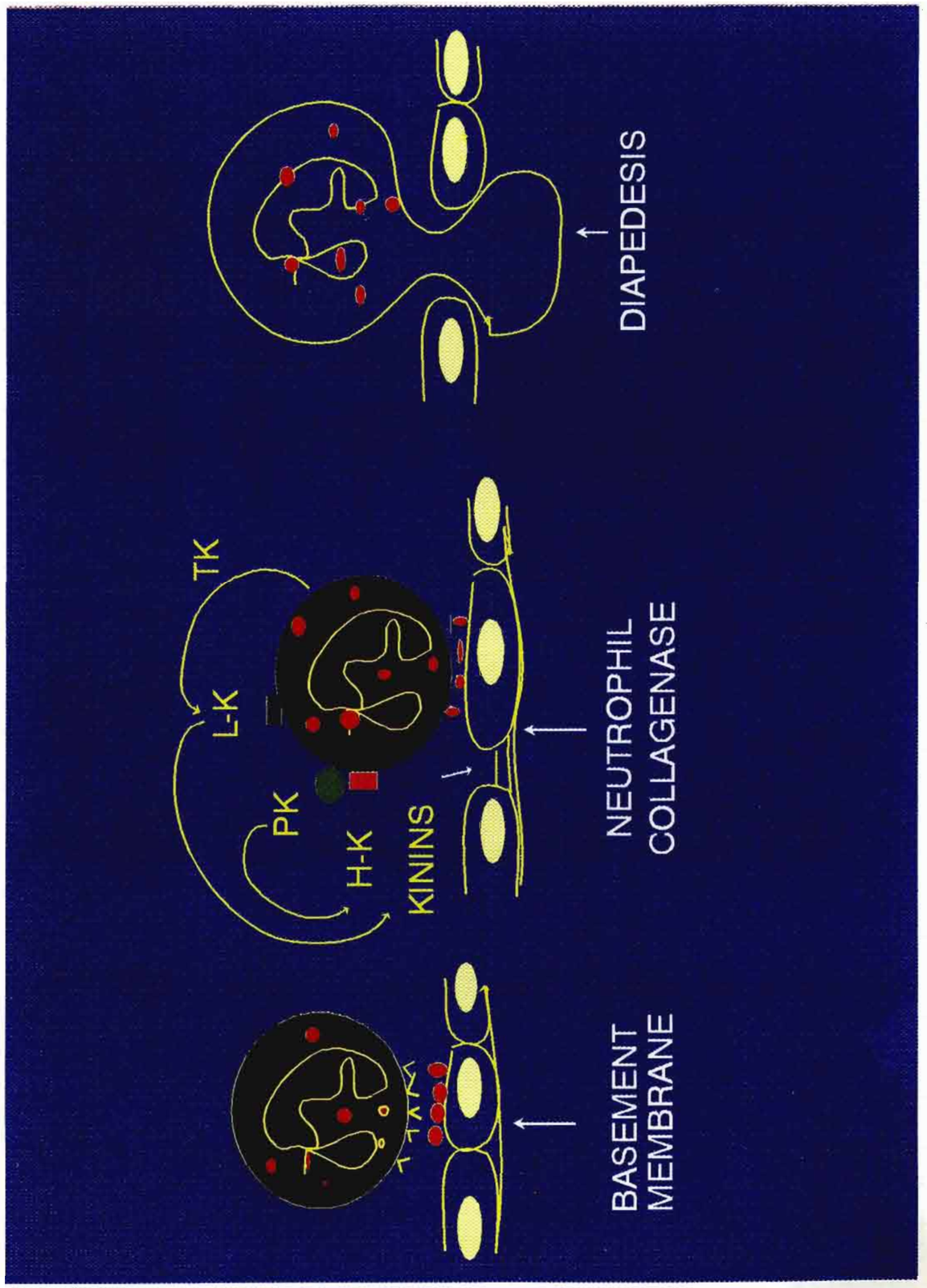


Figure 44: Diapedesis of neutrophils and migration through capillary cells

Rahman et al. (1994) reported that pain and swelling in inflammatory joint disease may be mediated by kinins formed by the enzymatic action of kallikreins. Furthermore, the hypothesis that kinins generated by the neutrophil-borne contact system might act in an autocrine manner is consistent with the finding that activated PK causes the release of elastase from neutrophils. Henderson et al. (1994) concluded that attachment to HK is a major requirement for the observed effect of PK on neutrophils to release kinins. The released kinins could regulate neutrophil function by activation of autocrine receptors. The question of whether or not the released kinin could affect kinin receptors on the neutrophil was examined by immunolocalisation of the B_2 receptors on the neutrophil surface by confocal microscopy. It was observed that there was an increased signal for B_2 receptors only on synovial fluid neutrophils (Fig 45), but not on those in the circulation (Fig 46). There appeared to be no upregulation or induction of the B_1 receptor in the neutrophil isolated from rheumatoid arthritis patients.

A credible aim therefore was to determine whether kinin could be released from the circulating neutrophils of patients with rheumatoid arthritis. Structural detail of the neutrophil was demonstrated on phase contrast microscopy (fig 47). The image processing programme of the confocal miscroscope was used to record the fluorescence emitted from the FITC labelled secondary antibody linked to the primary antibody with pseudocolour gradients. In all of the 25 RA patients, there was a distinct loss of the kinin moiety from the circulating (Fig 48) and synovial fluid neutrophils (Fig 49) as compared to normal neutrophils (refer to fig 25).

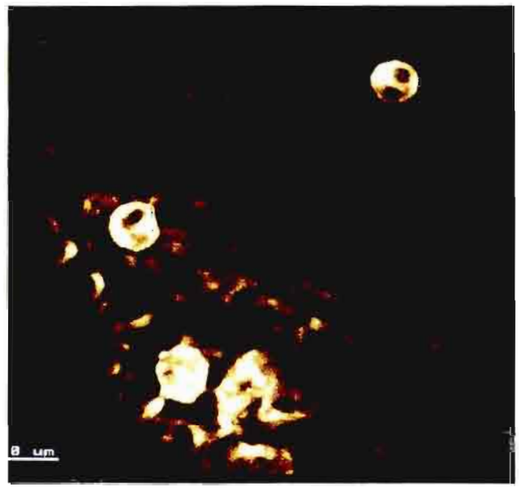
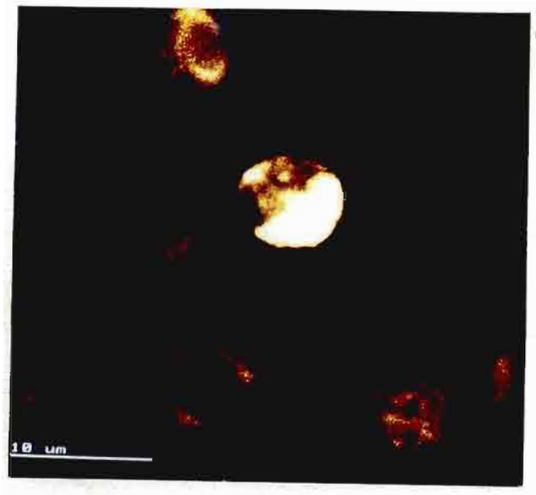


Figure 45: Synovial fluid neutrophils from rheumatoid arthritis patients immunolabelled for B_2 receptors



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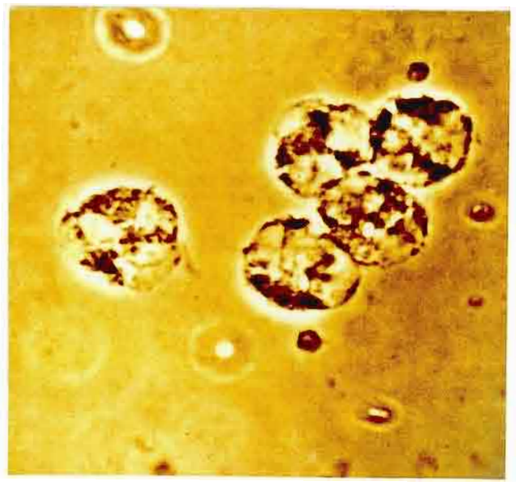

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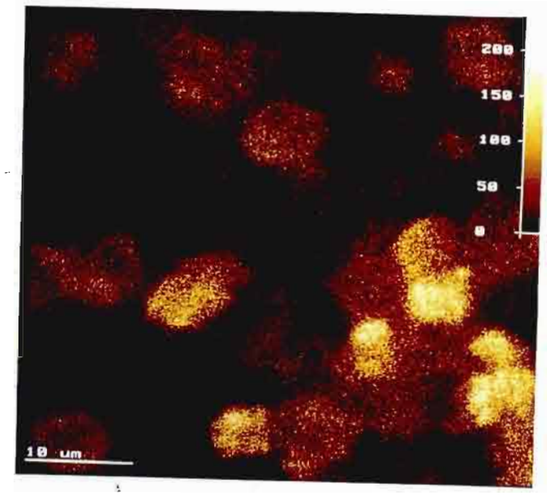
Figure 46: Circulating neutrophils from rheumatoid arthritis patients immunolabelled for B_2 receptors

Figure 47: Phase contrast microscopy showing structural detail of neutrophil



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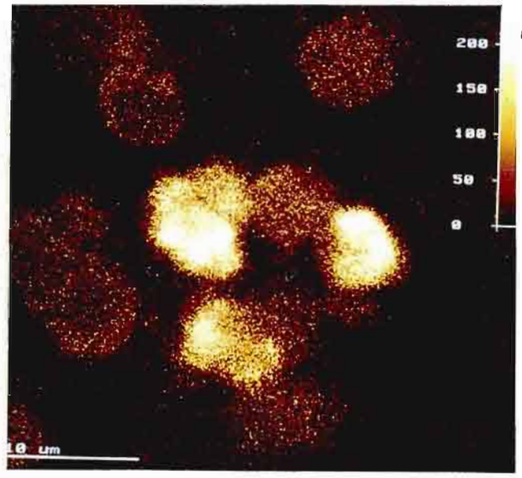

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Figure 48: Circulating neutrophils from rheumatoid arthritis patients immunolabelled for immunoreactive kinin

Figure 49: Synovial fluid neutrophils from rheumatoid arthritis patients immunolabelled for immunoreactive kinin

CHAPTER 4

DISCUSSION

In studies by Henderson et al. (1994), the Hageman factor XII-kallikrein contact-phase assembly was visualised on the external surface of the neutrophil cell membrane. A first step in the present study therefore, was to confirm these novel findings. Because of the binding of the HK-plasma pre-kallikrein complex on the neutrophil membrane (Henderson et al., 1992), activation of PK may result in the formation of bradykinin. The sequence of biochemical events of such a phenomenon involves the formation of the kinins from HK or LK on the surface of the neutrophil, either by the FXII-mediated activation of PK or by the release of TK in its active form. The spatial configuration of the contact-phase proteins therefore, uniquely places the neutrophil to participate in inflammatory disorders and in infections.

The intracellular identification of tissue prokallikrein in neutrophils and myeloid stem cells strongly suggested the *in situ* synthesis of tissue kallikrein (TK) in granulocytes. The appearance of maximal labelling as a ring on the surface of the cell indicated that the kinin moiety is associated with the surface membrane of the cell.

4.1. Profiles of imaging techniques to visualise tissue kallikrein and the kinin moiety on the external surface of the neutrophil membrane

In immunocytochemistry one detection marker widely used is the peroxidase-anti-peroxidase (PAP) complex. The PAP rings consist of 3 molecules of horseradish peroxidase and two antibodies (fig 5). PAP will react only with the free binding site of the specific anti-immunoglobulin in the link serum. For this reason it provides a highly specific method of

labelling. This is also a highly sensitive method of labelling because of the amplifying effect of the enzymatic activity. A few molecules of protein at a site can therefore generate a much larger amount of reaction product upon incubation. Results of the PAP labelling experiments clearly indicated that the kinin moiety of the kininogen molecule was released from the neutrophil surface in patients with infections. Immunogold studies were undertaken to confirm at the ultrastructural level, the presence or absence of the kinin moiety from the surface membrane of these neutrophils.

In addition to the initial light microscopy experiments with AEC marker that illustrated a release of the kinin molecule from kininogen, using electron and confocal methods of labelling and visualisation, it was also observed that there was a loss of the kinin moiety from the kininogen on the external surface of neutrophils harvested from patients with infections.

With a laser scanning confocal microscope it is possible for emitted light from one very small area only to be detected, by allowing the light to pass through a very small aperture. Therefore, no scattered light from other planes of the tissue that may obscure the final image, is detected. A representative image of any focal plane is obtained by scanning, or combining recording data from illuminated areas in a grid format. Optical sectioning is possible by viewing consecutive focal planes of the specimen. Therefore, the need for physical sectioning of a specimen is eliminated.

The advantage of this method of visualisation is that it is also possible to view live cells and furthermore, a transmitted light source and detector makes phase contrast visualisation

possible. A phase contrast image may be obtained from an unstained specimen. With two channels available, separate images of double labelled samples may be obtained. Images may be stored on the hard drive, stiffies or on a WORM (write once read many) drive. It is also possible, for record purposes, to photograph images off a flat, non-reflective screen. Specific advantages of the software package are that magnification bars calculated by the computer, as well as an intensity bar, indicating the intensity of fluorescence, may be added to the recorded image. Morphometric analysis, as well as 3D reconstruction of a thick specimen, are just two of the complex calculations feasible. A definite advantage is the ability to obtain a vertically scanned image from a specimen. This mode of imaging will allow the observation of the interaction between bacteria and cells in tissue culture.

The confocal images of fixed, permeabilised neutrophils provided multi-dimensional constructs of the neutrophil, and the intensity of fluorescence reflected the relative amounts of the kinin molecule detected on neutrophils harvested from both healthy blood as well as from disease states.

4.2. Release of kinin

The coupling of kininogen to appropriate receptors on the surface of the neutrophil may serve several functions, viz: (1) surface bound kininogens on platelets, together with prekallikrein and factor XI could locally trigger the endogenous coagulation cascade (fig 50), or (2) in the case of neutrophils, proteolytic processing of surface bound LK and HK by the kallikreins could form kinins. The locally released kinin may enhance the passage of the neutrophils into the extracellular space by causing the endothelial cells to retract, and

thereby opening the junctions between the cells (Gabbiani et al., 1970; Oyvin et al., 1970; Majno et al., 1972). This mechanism would promote the transudation of plasma constituents by controlling vascular permeability, and also the passage of circulating neutrophils (diapedesis) into the interstitial tissue space surrounding the site of injury or inflammation (Wright et al., 1979).

4.3. Role in infections and sepsis

It was observed that the inflammatory response to a number of stimuli, including infection, severe trauma, and burns appears to be similar, independent of the initiating cause. This response is characterised by fever, rapid heart rate, rapid respiratory rate and leucocytosis. In more serious forms, these signs and symptoms may progress to hypotension, shock, organ dysfunction and/or failure and death. This state has been defined as sepsis and in the more severe states as septic shock, when associated with infection. Loss in the amount of the kinin moiety residing in the kininogen molecules on the surface of the neutrophil has been demonstrated in sepsis patients, and therefore, may be a manifestation of sepsis (Naidoo et al., 1994b). This *in vitro* study on infectious states supports the view that circulating bacterial proteases, either directly or by complex cascade mechanisms, may release kinins from the surface of neutrophils - a finding of considerable clinical importance. Patients presenting with pneumonia also showed a distinct loss of the kinin moiety on the surface of isolated neutrophils (Naidoo et al., 1996a).

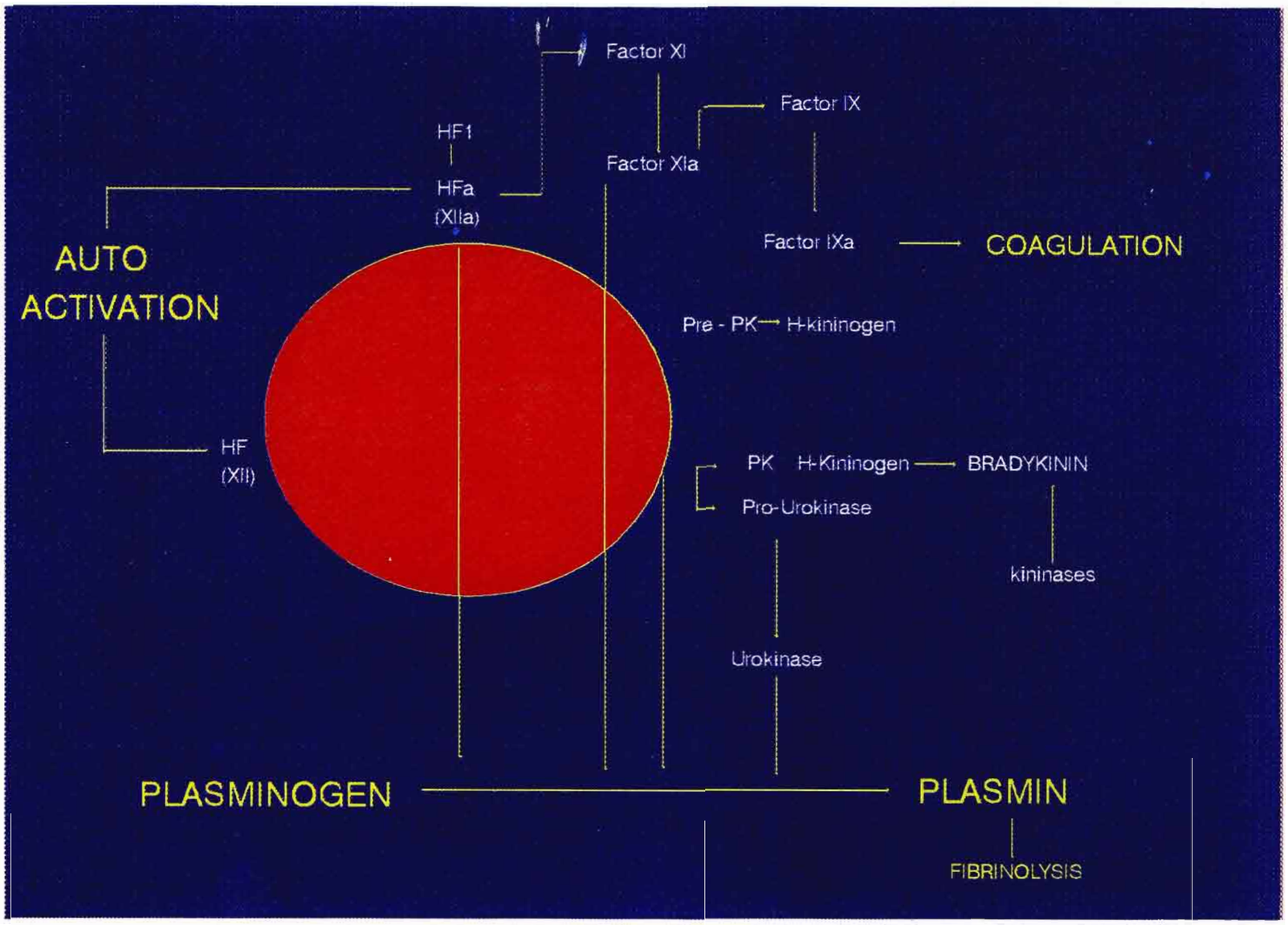

Figure 50: Contact phase assembly on the surface of the neutrophil cell membrane

4.4. *Neutrophil chemotaxis and role in inflammatory disorders*

Complex mechanisms, which come into play at the receptors on the cell surface, control cellular interactions and these include adhesion molecules, selectins, integrins and the superfamily of G-proteins. Furthermore, the soluble cell mediators, cytokines, and components of the extracellular matrix, such as collagen also play a pivotal role in cellular interactions. Disturbance of any one of these systems may evoke pathological disorders. In the development of inflammation, adhesion molecules play an essential role in the movement of cells to the inflammatory sites. After they have marginated, the activated cells migrate by diapedesis towards the site of inflammation, by following of chemotactic signals. The adhesion between the cells, alone, is insufficient to induce their migration. The adherence phenomena depend on a process that is strictly controlled by cytokines and enable the intervention of cell-cell reactions and cell-protein recognition of the extra-cellular matrix. Cytokines play a key role in the control of the expression and avidity of membrane receptors for ligands.

The most important chemotactic factors which attract neutrophils and cause them to marginate along the endothelium of capillaries are complement C5a and interleukin-1 (IL1). Thereafter, the neutrophils undergo diapedesis through the endothelial cell gaps. *In vivo* studies suggest that the endothelial cells undergo certain changes during injury and make themselves "sticky" for the circulating neutrophils which are the most mobile inflammatory cells. Regulation by IL-2 and tumour necrosis factor (TNF) of the de novo synthesis of adhesion molecules showed an association with the adhesion process in experiments with human umbilical vein endothelial cells (Henderson et al., 1994; Melmon et al., 1967;

Kaplan et al., 1989).

The significance and functional importance of the components of the kinin system in inflammatory disorders continues to unfold with molecular mapping, and by advances in the geneology, gene characterisation and cloning of the kinin receptors. A powerful tool for elucidating the physiopathological actions of kinins has been established by the discovery of specific, competitive antagonists of kinin receptors. The clinical manifestations of systemic inflammatory response syndromes associated with infection, severe trauma, burns and pancreatitis appear to be similar, and attributable, at least in part to kinins.

Though much knowledge has accumulated on the contact phase activation process, the nature of the physiological contact surface has remained elusive. Henderson and colleagues (1994) suggested a novel mechanism for the formation of kinins from kininogens on the surface of neutrophils. They suggested that kinins are formed on the surface of the neutrophil either by the activation of PPK (causing the release of bradykinin from HK by activated PPK), and/or the release of active TK. It was also proposed that released kinin initiates margination, orientates and evokes the passage of the neutrophil into the extracellular space by causing the endothelial cells to retract (Henderson et al., 1994).

Fluid accumulation in the joints of patients with rheumatoid arthritis, an immune driven disorder involving the release of cytokines and the activation of B and T lymphocytes, resembles that of inflamed tissue. Enzyme systems identified in synovial fluids include plasma transudates and degranulating neutrophils, and cellular damage occurs from the enzymatic properties of the kallikreins. Many neutrophils invade rheumatoid synovial

vessels, migrate to the luminal surface of the synovial membrane and accumulate in large numbers in the synovial fluid (Dularey et al., 1990). Therefore, TK probably reaches the synovial fluid from both neutrophils and plasma exudates. Secretion from even a small number of neutrophils in rheumatoid arthritis synovial fluid could result in the activation of the enzyme and the subsequent formation of kinins during episodes of acute inflammation within the joints.

Most of the PK in synovial fluid exists as a proenzyme complexed to HK. Conversion of plasma prekallikrein to its active form may be triggered through activation of HK by tissue matrix components such as proteoglycans, urate crystals or pyrophosphates (Cassim et al., 1995). PK is also believed to cause significant conversion of latent collagenase to its active form *in vitro* (Dularey et al., 1990) and this could be an important property of PK in the joint space. This activated collagenase could be responsible for the extensive cartilage and bone destruction seen in patients with rheumatoid arthritis, in which neutrophils invade the synovium and migrate in large numbers to the synovial fluid. In particular, the synovial membrane and the joint space are infiltrated with numerous neutrophils, which carry in and on their surface the kinin and fibrin generating proteolytic enzymes and cascade proteins (Kaplan et al., 1989).

4.5. Kinin receptors and nitric oxide formation

Most of the well characterised functional actions of kinins appear to be mediated by activation of the B_2 receptor. Ongoing studies on neutrophils isolated from whole blood of patients with rheumatoid arthritis as well as from synovial fluid indicate that the density of the B_2 receptor is increased on neutrophils isolated from the circulation while neutrophils isolated from synovial fluid show a loss of the B_2 receptor (Cassim et al., 1995). However, there appears to be no change in the regulation of the B_1 receptors on neutrophils isolated from patients with different disease states, compared to those from normal, healthy volunteers.

Kinin receptors have a seven transmembrane loop configuration and are linked to G-proteins. One signal transduction event that kinins generate is the formation of nitric oxide. An important source of nitric oxide could be the C terminal arginine which is released following the conversion of bradykinin or kallidin into the appropriate des Arg^9 kinin molecule (fig 51). Nitric oxide (NO), is a unique biological mediator that has been implicated in a variety of physiological and pathophysiological conditions (Rodeberg et al., 1995). It has been found to be an important regulator of vascular tone and may be the mediator of the hemodynamic changes in sepsis.

Since the only physiological nitrogen donor for NO synthesis is arginine, metabolism of this amino acid may play a role in the regulation of NO synthesis during sepsis. Regardless of its origin, endogenous NO is produced through the conversion of L-arginine to L-citrulline by NO synthase (NOS) (Berdeaux, 1993). Several isoforms of the enzyme have

been isolated, purified and cloned. NOS-type 1 (isolated from brain) and type III (isolated from endothelial cells) are termed "constitutive NOS" and produce minute amounts of NO which elicits physiological responses. NOS-types II and IV are the "inducible NOS" since their activation is only promoted under physiological conditions where macrophages exert cytotoxic effects in response to cytokines. Unlike NOS-types I and III, activation of NOS-type II in these cells induces the formation of sufficiently large amounts of NO which act as a defence mechanism of the immune system. Recent studies have identified the induction of NO synthesis in many cell types as part of the host response to sepsis and inflammation (Morris and Billiar, 1994), with both detrimental and beneficial effects that depend on amount, duration and anatomic site of NO synthesis.

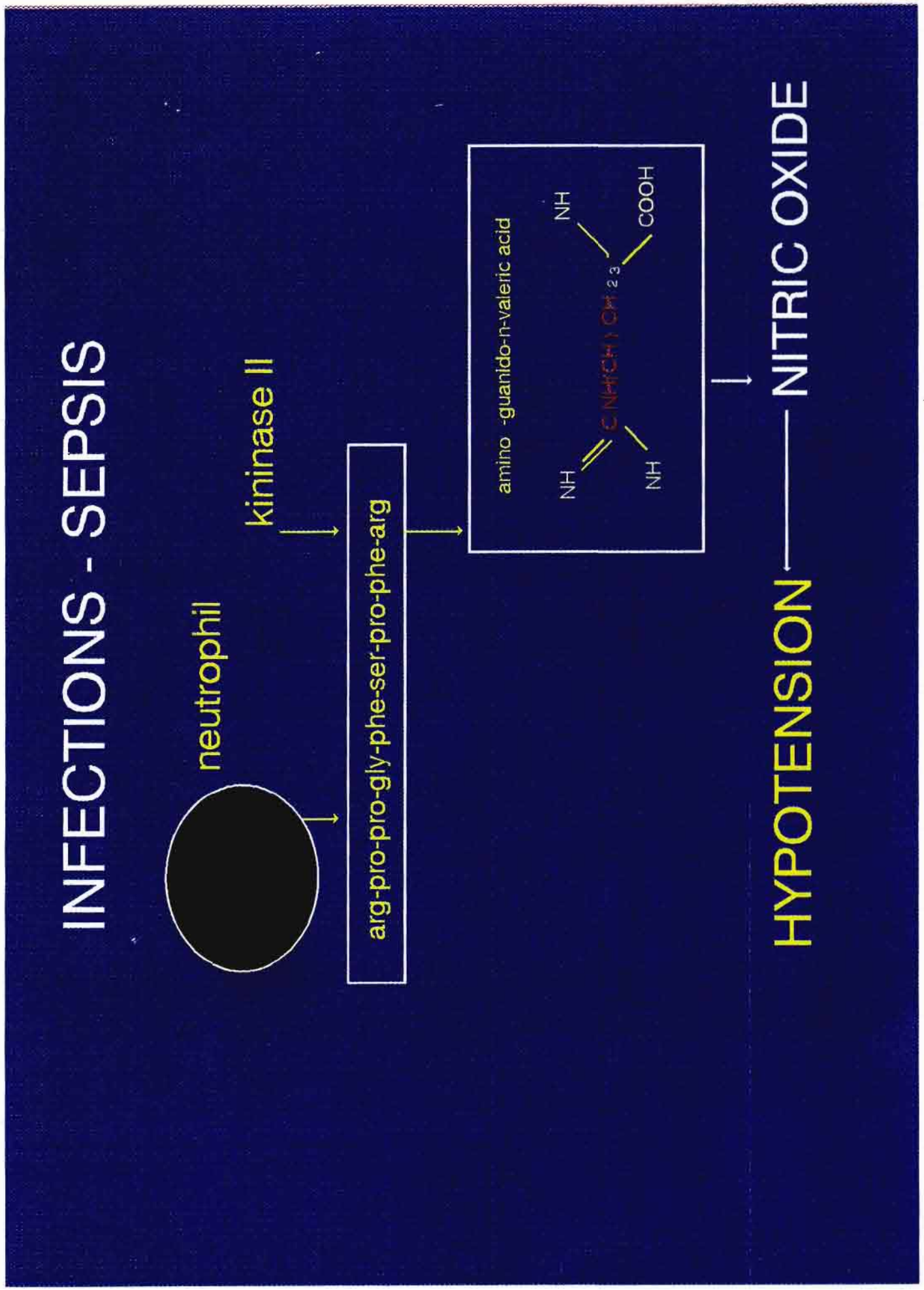

Figure 51: Role of kinin in nitric oxide formation

4.6 Conclusions

4.6.1 This study supports the hypothesis that all of the essential molecular components of the kallikrein-kinin system may be located in or on the surface of the neutrophil.

4.6.2 The time-dependence and dose-response kinin release experiments indicate that the kinin moiety on the neutrophil surface is readily accessible for the enzymatic action of kallikreins, and a major loss of kinin apparently occurs upon exposure of the cells to active kallikreins and bacterial proteases.

4.6.3 Experiments demonstrated an apparent loss of the kinin moiety from the kininogens bound to the external surface of neutrophils in infections and inflammatory disorders such as rheumatoid arthritis.

4.6.4 There appears to be no induction of the B_1 and B_2 receptors in neutrophils isolated from normal, healthy blood or blood from patients suffering from rheumatoid arthritis. However, there is an upregulation of kinin B_2 receptors in neutrophils from synovial fluid isolated from rheumatoid arthritis patients.

4.7 Future experiments

4.7.1 A question that arises from this study is how the novel finding of a loss of the neutrophil kinin moiety can be used to investigate new strategies for the treatment in infectious states. With the development of appropriate kininogenase and kinin peptide antagonists, this prove useful in drug therapy.

4.7.2 Since most of the functions of kinins appears to be mediated by the action of kinin receptors, future experiments are necessary to determine the conditions under which there is upregulation or downregulation of these receptors. Experiments involving the induction of the neutrophil B_1 receptor in sepsis would be significant to this end.

4.7.3 Another important direction is comparative analysis of the signal transduction system regulated by the activation of the B_1 and B_2 kinin receptors.

4.7.4 The role of NO in neutrophil adhesion should be investigated to elucidate whether NO regulates neutrophil-endothelial cell interactions, and hence the modulation of the inflammatory response.

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APPENDICES

Appendix 1

Data sheet of all patients

Patient name	
Age	
Sex	
Pharmacology reference number	
Diagnosis	
Date of sample collection	

For all patients, circulating neutrophils were isolated for immunocytochemistry.

Synovial fluid was also isolated from rheumatoid arthritis patients.

Appendix 2

Profiles of tuberculosis meningitis patients

Patients	Age	Sex	Reference no.	Date of blood collection
1	42	M	P22	13/07/93
2	38	M	P27	15/07/93
3	45	M	P28	15/07/93
4	35	M	P35	22/07/93
5	40	M	P52	03/08/93
6	49	F	P70	19/01/94
7	42	M	P73	10/01/94
8	40	M	P99	09/02/94
9	30	M	P130	25/10/94
10	34	M	P135	21/11/94
11	44	M	P136	21/11/94

Appendix 3

Profiles of pneumonia patients

Patients	Age	Sex	Reference no.	Date of blood collection
1	45	M	P120	05/10/94
2	21	M	P121	05/10/94
3	35	M	P122	05/10/94
4	42	M	P125	05/10/94
5	32	M	P126	15/10/94
6	26	M	P127	15/10/94
7	30	F	P128	19/10/94
8	29	M	P129	19/10/94
9	40	M	P137	22/10/94
10	47	F	P138	22/10/94

Appendix 4

Profiles of myeloid leukaemia patients

Patients	Age	Sex	Reference no.	Date of blood collection
1	60	M	P183	28/06/95
2	18	M	P184	28/06/95
3	12	M	P185	28/06/95
4	14	M	P186	27/06/95
5	60	M	P188	03/07/95
6	43	F	P201	11/10/95
7	38	M	P202	11/10/95
8	47	M	P203	11/10/95
9	58	M	P204	11/10/95
10	50	M	P231	26/01/96

Appendix 5

Profiles of rheumatoid athritis patients

Patients	Age	Sex	Reference no.	Date of sample collection
1	40	F	P134	18/11/94
2	28	M	P124	05/10/94
3	64	F	P154	02/03/95
4	69	M	P156	09/03/95
5	45	F	P157	09/03/95
6	61	F	P158	09/03/95
7	31	F	P160	16/03/95
8	50	F	P159	16/03/95
9	60	F	P161	16/03/95
10	49	M	P162	16/03/95
11	61	F	P164	23/03/95
12	50	F	P165	23/03/95
13	47	F	P166	23/03/95
14	62	F	P167	23/03/95
15	39	F	P169	30/03/95
16	34	F	P170	30/03/95
17	55	F	P172	06/04/95
18	37	F	P173	06/04/95
19	54	F	P174	06/04/95
20	54	F	P178	20/04/95
21	52	F	P179	20/04/95
22	24	F	P180	20/04/95
23	50	F	P181	16/05/95
24	48	F	P149	23/02/95
25	47	F	P150	23/02/95