THE ROLE OF KININS AND CYTOKINES IN RHEUMATOID ARTHRITIS

by

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Submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF MEDICINE

in the

Department of Experimental and Clinical Pharmacology

Nelson R Mandela School of Medicine

Faculty of Health Sciences

University of Natal

Durban

2001

AUTHOR'S DECLARATION

This study represents original work by the author. It has not been submitted in any other form to the University. Where the 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 and the Rheumatology Unit, Department of Medicine, Nelson R Mandela School of Medicine, Faculty of Health Sciences, University of Natal, Durban, South Africa under the supervision of Professor K. D. Bhoola and Professor G.M. Mody.

SIGNED:_____

B. CASSIM

I certify that the above statement is correct.

SIGNED:_____

PROFESSOR K.D. BHOOLA

DEDICATION

For my mother, Mrs. Fathma Cassim.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS DISSERTATION

1. PUBLICATIONS

Cassim, B., Naidoo, S., Naidoo, Y., Williams, R. & Bhoola, K.D. (1996). Immunolocalisation of the kinin moiety and bradykinin (B2) receptors on synovial fluid neutrophils in rheumatoid arthritis. *Immunopharmacology*. **33**:321-324.

Cassim, B., Naidoo, S., Ramsaroop, R. & Bhoola, K.D. (1997). Immunolocalization of bradykinin receptors on human synovial tissue. *Immunopharmacology*. **36**:121-125.

Williams, R.J., Henderson, L.M., Naidoo, Y., Cassim, B., Elson, C.J. & Bhoola, K.D. (1997). Immunocytochemical analysis of tissue kallikrein and the kinin moiety in rheumatoid synovial fluid neutrophils. *British Journal of Rheumatology*. **36**:420-425.

Bhoola, K., Ramsaroop, R., Plendl, J., Cassim, B., Dlamini, Z. & Naicker, S. (2001). *Biological Chemistry*. **382**:77-89.

Cassim, B., Mody, G. & Bhoola, K. D. (2001). Kallikrein cascade and cytokines in inflamed joints. *Pharmacology & Therapeutics* (accepted for publication).

2. PRESENTATIONS

Cassim B, Naidoo Y, Naidoo S, Williams R, Bhoola K D. Immunolocalisation of the kinin moiety and bradykinin 2 receptors on synovial fluid neutrophils in rheumatoid arthritis. XIVth SARAA Congress, Johannesburg, September 1995.

Cassim B, Naidoo Y, Naidoo S, Williams R, Bhoola KD. Immunolocalisation of the kinin moiety and bradykinin 2 receptors on synovial fluid neutrophils in rheumatoid arthritis. Fourteenth International Symposium on Kinins, Denver, USA. September 1995.

Cassim B, Naidoo Y, Naidoo S, Williams R, Bhoola K.D. Immunolocalisation of the kinin moiety and bradykinin 2 receptors on synovial fluid neutrophils in rheumatoid arthritis. Zeneca Research Day, Durban, October 1995.

Cassim B, Ramsaroop R, Naidoo Y, Bhoola K.D. Immunolocalisation of bradykinin receptors on human synovial tissue. Kinin 96, Durban, October 1996.

Cassim B, Ramsaroop R, Naidoo Y, Bhoola K.D. Immunolocalisation of bradykinin receptors on human synovial tissue. Biennial Conference of South African Rheumatism and Arthritis Association, Durban, March 1997.

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ACKNOWLEDGEMENTS

1. Professors K.D. Bhoola and G.M. Mody for the constant supervision, encouragement and guidance provided during the study.

2. All members of the Kinin Research Team for their assistance, advice and support.

3. Mr. H. Kagoro and Ms E. Gouws for the statistical analysis.

4. Sister N.G. Mkhize for assistance with the sample collection.

5. Mrs D. Pillay, Mrs C Vedalankar, Mrs P Ramhorry and Ms E Nzimande, Department of Medicine for assistance with the manuscript.

6. The University Research Fund (URF) and the Foundation for Research and Development (FRD) for financial support.

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ABBREVIATIONS

AC, adenylate cyclase;
ACE, angiotensin converting enzyme;
ARA, American Rheumatism Association;
ANOVA, analysis of variance;
APP, acute phase protein;
BK, bradykinin;
BSA, bovine serum albumin;
C, Celsius;
C5a, complement chemotactic peptide;
Ca^{2+}, calcium;
CnBr,
COOH, carboxy;
COX, cyclo-oxygenase;
CRP, C-reactive protein;
DAB, diaminobenzidine;
DAG, diacyglycerol;
DAS, disease activity score;
dH_2O, distilled water;
DK, darmkontrahierende substanz or "gut-contracting substance";
DMARDs, disease-modifying anti-rheumatic drugs;
ED4, fourth extracellular domain;
EDTA; ethylenediaminetetraacetic acid;
ELISA, enzyme linked immunosorbant assay;
ESR, erythrocyte sedimentation rate;
f5f, pentafluorophenylalanine;
Factor X1, thromboplastin antecedent factor;
Fc, fusion protein;
FGF, fibroblast growth factor;
FITC, fluorescein isothiocyanate;
G, guanine-nucleotide-binding;
Gc, guanylatecyclase;
gk^{+}, potassium conductance;
GM CSF, granulocyte-macrophage colony stimulating factor;
H&E, haematoxylin and eosin;
h, hour;
5'HT, 5'hydroxytryptamine;
H_2O_2, hydrogen peroxide;
HAQ, health assessment questionnaire;
HCl, hydrochloride;
HEV, high endothelial venule;
HF, Hageman factor;
HFa, activated HF;
HK, high molecular weight kininogen;
H-kininogen, high molecular weight kininogen;
HLA, human leucocytes antigen;
HOE14, D-Arg-(Hyp^3,Thi^5,d-Tic^7,Oic^8)-BK (icatibant);
HUK, human urinary kallikrein;
ICE, interleukin converting enzyme;

IcIL-RA, intracellular interleukin 1 receptor antagonist;
ID1, intracellular domain one;
ID2, intracellular domain two;
Ig, immunoglobulin;
Igl, α-2-indanyl-glycine;
IL, interleukin;
IL-1R, interleukin 1 receptor;
IL-1RA, interleukin 1 receptor antagonist;
IL-1RAP, interleukin 1 receptor associated protein;
IP(3), inositol trisphosphate;
K11-ACE, kininase II-angiotensin converting enzyme;
K11-KEP, kininase II neutral endopeptidases;
K1-CPM, kininase caboxypeptidase M;
K1-CPN, kininase 1 carboxypeptidase N;
kb, kilobase;
kDa, kilodalton
KK, kallikrein-kinin
KKS, keratoconjunctivitis sicca;
KLK, tissue kallikrein multigene family;
KLK1, tissue kallikrein gene;
KLK2, human glandular kallikrein gene;
KLK3, prostate specific antigen gene;
LK, low molecular weight kininogen;
L-kininogen, low molecular weight kininogen;
LPS, lipopolysaccharide;
LTB4, leucotriene B4;
M,molar;
µl, microlitre;
MCP, metacarpophalangeal;
mg, milligram;
$MgCl_2$, magnesium chloride;
MHC, major histocompatibility complex;
min, minute;
ml, millilitre;
mM, millimole;
mm, millimetre
MMPS, metalloproteinases;
mRNA, messenger ribonucleic acid;
MTX, methotrexate;
NaCl, sodium chloride;
NADPH, nicotinamide adenine dinucleotide phosphate(reduced form);
NESI, normal epithelial cell-specific 1 gene;
N-fMLP, formyl-methionyl-leucyl-phenyl-alanine;
Ng/ml, nannogram per millilitre;
NH_2, amino;
NK, neurokinin;
nm, nanometre;
NSAIDs, non-steroidal anti-inflammatory drugs;
OA, osteoarthritis;
Oic, octrahydroindole-2-carboxylic acid;

PAP, peroxidase-anti-peroxidase;
PAF, platelet activating factor;
PBS, phosphate buffered saline;
PDGF, platelet derived growth factor;
PG, prostaglandin;
ρg, picagram
PI, phosphatidyl inositol;
PIP, proximal interphalangeal;
PK, plasma kallikrein;
PLA_2, phospholipase A_2;
PMN, polymorphonuclear;
pNA, para-nitroaniline;
pNPP, p-nitrophenyl phosphate;
PPK, plasma pre-kallikrein;
PPT, pre-protachy-kinin;
RA, rheumatoid arthritis;
RF, rheumatoid factor;
ROI, regions of interest;
RT, room temperature;
rTK, recombinant tissue kallikrein;
s, soluble;
SAA, serum amyloid A;
SBTI, soya bean trypsin inhibitor;
SEM, standard error of mean;
SF, synovial fluid;
SPDP, succimidyl-3(2-pyridyldithio)propionate;
Tic, D tetrahydroisoquinolone-3-carboxylic acid;
TGF, transforming growth factor;
TIMP, tissue inhibitors of metalloproteinases;
TK, tissue kallikrein;
TLSP, trypsin-like serine protease;
TNF, tumor necrosis factor;
TNF-R, tumor necrosis factor receptor;
u, unit;
v/v, volume for volume;
Zn, zinc

ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by inflammatory synovitis. The histopathological features include synovial hyperplasia, an inflammatory cell infiltration, angiogenesis and an inflammatory exudate into the synovial joint with progression to bone and joint destruction. While the exact aetiology of RA is unknown, a number of inflammatory cells and mediators have been implicated in the pathogenesis. Kinins are vasoactive peptides that have the capacity to induce the cardinal features of inflammation and considerable evidence exists for a role for the kallikrein-kinin cascade in inflammatory arthritis. The proinflammatory cytokines are also important mediators in rheumatoid arthritis and there is evidence for a functional relationship between the kallikrein-kinin and cytokine cascades in rheumatoid arthritis.

Methods: Following approval from the Ethics Committee of the University of Natal, synovial tissue samples were obtained at arthroscopy from patients with RA and at autopsy (for controls). The tissue samples were processed for light microscopy and immunostained by the immunoperoxidase method to detect tissue kallikrein and the kinin B1 and B2 receptors. The intensity of the immunostaining was quantified by image analysis.

Blood and synovial fluid samples were obtained from patients with RA and blood from age and sex matched healthy volunteers. The RA patients were assessed clinically to determine the degree of disease activity and the presence or concomitant diseases. Disease activity was determined by the duration of morning stiffness, the twenty eight tender and swollen joint counts, pain on a visual analogue scale, patient's and physician's global assessment of disease activity (Likert scale), a local activity index, the modified Health Assessment Questionnaire (HAQ), disease activity score (DAS) and the erythrocyte sedimentation rate (ESR) and C reactive protein (CRP).

In the synovial fluid (SF) samples, the functional activity of tissue kallikrein (TK) was demonstrated using an amidolytic assay and the total amount of TK was measured in a sandwich enzyme linked immunosorbent assay (ELISA). The Pearson's correlation co-efficient was used to correlate the TK levels with measures of disease activity. Further, basal and generated kinins were measured in the SF by competitive ELISA, and the levels correlated with measures of disease activity.

In the cytokine study, interleukin 1β (IL 1β) and tumour necrosis factor β (TNFβ) were measured in the synovial fluid samples by ELISA, and the relationship between the cytokine levels and disease activity as well as TK, basal and generated kinins determined.

Neutrophils were isolated from the blood and synovial fluid samples from the rheumatoid arthritis patients and from the blood samples from healthy volunteers. The circulating and synovial fluid neutrophils were immunostained to detect tissue kallkrein, the kinin moiety in the kininogen molecule and kinin B1 and B2 receptors, and the immunofluorescence visualized by confocal microscopy. The images were digitally analysed using the Analysis 2.1 Pro system. The Kruskal Wallis and one-way ANOVA tests were used to compare the mean intensity of immunostaining in the control neutrophils with that present on the circulating and SF neutrophils harvested from RA patients. The intensity of labeling for these antigens was correlated with measures of disease activity.

Results:

1. Synovial tissue samples: Labeling for tissue kallikrein was observed in the synovial lining and endothelial cells in control and rheumatoid tissue. There was a significant increase in the intensity of TK labeling in the endothelial cells of the rheumatoid tissue ($p < 0.05$). The kinin B1 and B2 receptor were visualized in the synovial lining cells, endothelial cells and the subintimal fibroblasts and macrophages in the control

and rheumatoid synovial samples, with a significant increase in B1 receptor labeling in the synovial lining cells in rheumatoid synovial tissue ($p < 0.01$).

2. Tissue kallikrein activity and the total TK concentration was measured in the synovial fluid obtained from 20 patients with RA. There was no direct correlation between the between the enzymic and antigenic tissue kallikrein. There was a significant negative correlation between the enzymic TK and the twenty eight swollen joint count ($r = -0.464$; $p < 0.05$).

3. There was a significant negative correlation between the basal kinin and generated kinin levels ($r = -0.454$; $p < 0.05$). In addition, there was a negative correlation between the basal kinin levels and the CRP ($r = -0.537$; $p < 0.05$) and the disease activity score ($r = -0.458$; $p < 0.05$). In contrast, there was a positive correlation between the generated kinin levels and the twenty-eight tender and swollen joint counts ($r = 0.536$; $p < 0.05$ and $r = 0.509$; $p < 0.05$ respectively), the ESR ($r = 0.598$; $p < 0.01$), CRP ($r = 0.725$; $p < 0.01$) and the disease activity score ($r = 0.676$; $p < 0.01$).

There was a significant correlation between the SF levels of IL 1β and pain ($r = 0.462$; $p < 0.05$), physician's global assessment of disease activity ($r = 0.549$; $p < 0.05$), 28 tender joint count ($r = 0.472$; $p < 0.05$) and CRP ($r = 0.530$; $p < 0.05$). Although there appeared to be a correlation between the IL 1β and disease activity score, this was not significant ($r = 0.412$; $p = 0.07$). In addition, the levels of synovial fluid TNF α correlated with the 28 tender joint ($r = 0.458$; $p < 0.05$) count and CRP ($r = 0.653$; $p < 0.01$).

4. There appeared to be a trend towards a negative correlation between the SF amidase TK levels and IL 1β, however this was not significant. While there was no direct relationship between the SF levels of IL 1β and the generated kinins, there was a positive correlation between low to moderate levels of IL 1β and the generated kinins ($r = 0.51$,

$p < 0.05$). In contrast, there was a negative correlation with higher levels of IL 1β ($r = -0.5$, $p < 0.05$).

5. Immunoreactive TK, kinin moiety and the B1 and B2 receptors were visualized on the circulating neutrophils from the healthy volunteers and the circulating and SF neutrophils from the RA patients. There was no statistically significant difference in the mean intensity of TK labeling in the circulating neutrophils from healthy volunteers (n=8) and the circulating and synovial fluid neutrophils of the RA patients n=8). However, when the intensity of labeling of the SF neutrophils (n=80) from the RA patients was compared to the circulating neutrophils (n=80) of healthy volunteers, there was a significant loss of TK labeling in the SF neutrophils of the RA patients (1-Way ANOVA, $p < 0.01$). In the RA patients, there was a loss of the kinin moiety from both the SF and circulating neutrophils compared to controls (Kruskal-Wallis: $p < 0.05$ and < 0.01 respectively). Although there was a clear increase in the intensity of labeling of the kinin B1 receptor on the SF neutrophils from RA patients (n=8), when compared to the circulating neutrophils from healthy volunteers (n=8), the mean values did not reach significance (Kruskal Wallis; $p > 0.05$). However, a significant increase in B1 receptor labeling was observed on both the circulating and SF neutrophils of the RA patients (n=80) when compared to circulating neutrophils of the healthy volunteers (n=80) (1 Way ANOVA, $p < 0.01$ and < 0.05 respectively). In addition, there was a positive correlation between the immunoreactivity for the B1 kinin receptor on the circulating neutrophils from the RA patients and the local activity index ($r = 0.783$; $p < 0.05$). Although there was a clear increase of the kinin B2 receptor on the circulating and SF neutrophils from the RA patients compared to circulating neutrophils from healthy volunteers, this was only significant for the circulating neutrophils from the RA patients (Kruskal-Wallis, $p = 0.05$). There was no correlation between the

intensity of labeling of TK, the kinin moiety and the B2 receptor and measures of disease activity.

Discussion and conclusions

1. This study provides the first evidence for the localization of TK and the kinin receptors in control and rheumatoid synovial tissue using antibodies specific for each protein and standard immunolabelling techniques. Synovial fibroblasts, macrophages and endothelial cells through the release of enzymes and cytokines have the ability to mediate the inflammatory changes and cartilage and bone destruction in RA. The presence of TK and the kinin receptors in these cells therefore provides evidence for a pathogenetic role for the kallikrein-kinin cascade in RA.

2. In addition, in RA there is an exudation of fluid into the joint space. Tissue kallikrein has been previously reported in the synovial fluid obtained from RA patients, however the correlation of TK levels and disease activity has not been previously studied. The negative correlation between enzymic TK and the twenty-eight swollen joint count, an indicator of disease activity suggests that there is a consumption of TK in inflammation, presumably due to increased kininogenase activity. Similarly, the kinin generating capacity of synovial fluid obtained from RA patients has been previously reported, however, this is the first study demonstrating a link between kinin generating capacity and validated markers of disease activity. The kinin generating capacity is a complex and dynamic cascade involving the bioregulation of all the components of the kallikrein-kinin system and is therefore more likely to accurately define the role of kinins in inflammatory arthritis than are individual components of the kallikrein-kinin cascade. Measurement of tissue kallikrein and basal kinins is affected by the presence of natural inhibitors and their short half-life in biological fluids. In addition to the synovial fluid study a decrease in the urinary TK

activity and an increase in urine kinin generated kinins was demonstrated, suggesting that there is a systemic activation of the kallikrein kinin cascade in RA.

3. Although there is evidence for an interaction between the kallikrein-kinin and cytokine cascades in inflammation, in this study a direct correlation between the levels of interleukin 1β and tumour necrosis factor β in the synovial fluid and TK and generated kinin levels was not found. This may be due to the wide variations in the levels of cytokines, the presence of inhibitors and anti-inflammatory cytokines, or a complex and dynamic relationship between the two cascades. However, the correlation of both generated kinins and interleukin 1β and tumour necrosis factor β with disease activity provides circumstantial evidence for a synergistic role for these mediators in inflammatory arthritis.

4. In the neutrophil study, loss of immunoreactive tissue kallkrein and kinin moiety from the neutrophils obtained from RA patients was demonstrated. This finding supports the hypothesis that kinins are released from the neutrophils by the enzymatic action of tissue kallkrein and suggests that the kallkrein-kinin system is activated both locally and systemically in patients with RA. Further, there was upregulation of the both the kinin B1 and B2 receptors on the neutrophils from the RA patients. While the B2 receptor is thought mediate most of the actions of kinins, the correlation of the intensity of B1 receptors and the local activity index implies that the B1 receptor may be important in inflammation.

5. These findings provide convincing evidence for the role of the kallikrein-kinin cascade in the pathogenesis of inflammation in RA. Further development of kinin receptor antagonists may provide a novel therapeutic modality.

CHAPTER 1

INTRODUCTION

1.1 THE SYNOVIAL JOINT

Diarthrodial or synovial joints are lined by a soft connective tissue lining or synovium composed of a matrix of numerous microfibrils (Edwards, 1987), and abundant proteoglycan aggregates within which lie the synovial cells. Normal or healthy synovium (Fig.1.1) consists of a superficial cellular layer called the lining layer or intima, approximately 20 mm and a deeper subintimal layer of compact tissue carrying a rich vascular plexus (Wilkinson and Edwards, 1989). The intimal cells are of two types; one resembling the macrophage (Type A) and the other fibroblast like (Type B) which produces hyaluronan (Barland *et al.* 1962). Within the joint spaces is a small amount of synovial fluid that is rich in hyaluronan. The contact surfaces of adjoining bone are covered by hyaline cartilage, which is mainly comprised of water and proteoglycan aggregates restrained in an arching framework of type 11 collagen fibres. The proteoglycan is made up of chrondroitin sulfphate and keratin sulphates.

In an inflamed joint, early changes include endothelial cell damage, synovial oedema, fibrin deposition, polymorphonuclear (PMN) leucocyte invasion and mild lining cell hyperplasia (Schumacher and Kitridou, 1972). This is followed by further synovial hyperplasia, infiltration of the sublining layer with mononuclear cells, T and B lymphocytes, macrophages and plasma cells, overgrowth of a fibrovascular granulation tissue known as the pannus and striking proliferation of blood vessels or angiogenesis (Fig.1.2). The markedly hyperplastic pannus becomes locally invasive at the synovial interface with cartilage and bone with subsequent cartilage and bone destruction. In addition, there is an increase in synovial fluid due to exudation and increased vascular permeability. The synovial fluid is abundant with neutrophils ranging from <1000 to >100 000/mm^3. These pathological changes result from the activation and complex interaction

of a number of different cells and inflammatory mediators. The aetiology, degree of synovitis and joint damage varies in the different arthritides.

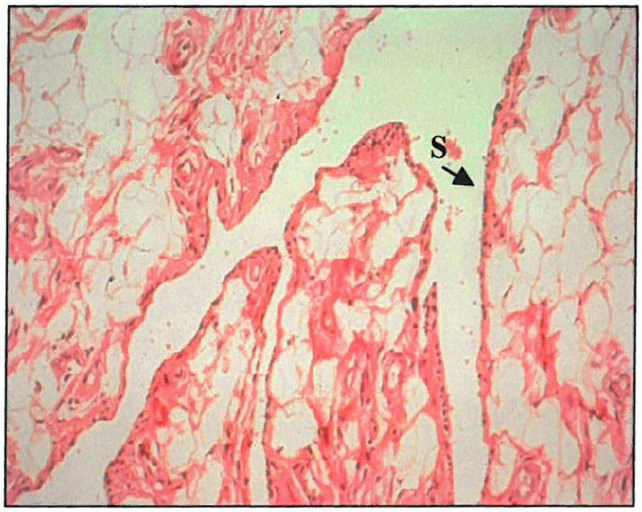


Figure 1.1 Normal synovium
Photomicrograph of normal synovial tissue showing a single layer of synovial lining cells (S).

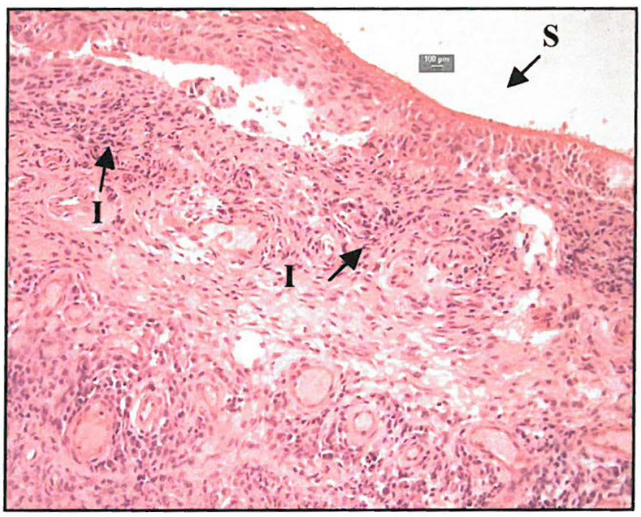


Figure 1.2 Inflamed synovium
Photomicrograph of synovial tissue from a patient with rheumatoid arthritis showing hypertrophy of the synovial layer (S) and a dense inflammatory cell infiltrate in the subintimal layer (I).

1.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an immune-mediated systemic disease characterized by chronic widespread synovitis resulting in bone and joint damage. It affects about 1% of the world's population and is more common in women (Lawrence, 1961). Initially considered benign, RA is now recognized as a severe progressive disease with increased morbidity and mortality (Pincus and Callahan, 1989).

1.2.1 Clinical presentation

The onset of RA is generally insidious with systemic features such as morning stiffness, fatigue and pain followed by joint inflammation. The typical presentation is that of a symmetrical polyarthritis. The most commonly involved joints include the wrists, metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints. However, the large joints are also affected. In active disease, the joints are tender and swollen joints with or without effusions. The synovial fluid consists of a large number of neutrophils. Persistent inflammation leads to progressive damage of joints with the development of deformities and bone and cartilage destruction. In addition, a number of extra-articular features may occur. These include nodules, vasculitis, keratoconjunctivitis sicca (KKS), Sjogren's syndrome and serositis. Diagnostic criteria have been established by the American Rheumatism Association (ARA) (Arnett *et al.* 1988) (Table 1.1).

The prognosis of RA is determined by the activity and severity of the disease (Wolfe, 1997). Disease activity refers to the extent of current inflammation and can be measured by clinical and laboratory criteria. Clinical measures include duration of morning stiffness, extent of joint tenderness and swelling, intensity of pain measured on a visual analogue scale, global measures of disease activity using either a categorical or visual analogue scale

and functional assessment. Severity of the disease is usually measured by outcome measures such as death, disability and radiological progression.

Table 1.1 The 1987 revised criteria for the classification of rheumatoid arthritis*

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joints	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joints
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in (2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on postero-anterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

*For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks.

1.2.2 Diagnostic features

Rheumatoid factors (RF) are antibodies directed against the Fc fragment of immunoglobulin G (IgG). While not specific for the disease, RF is present in 75% of patients with RA, and a positive RF has been shown to predict prognosis and the later development of radiological damage (Richardson and Emery, 1996).

Acute phase response

The proinflammatory cytokines, interleukin 1, 6 (IL-1, IL-6) and tumor necrosis factor (TNF), stimulate the hepatic synthesis of acute phase proteins, namely, C-reactive protein (CRP), fibrinogen, haptoglobin and serum amyloid A (SAA). Measurement of the acute phase proteins can be used to quantify and monitor disease activity. The two most commonly used tests are the CRP and erythrocyte sedimentation rate (ESR).

The CRP is generally accepted as the most accurate measure of the acute phase response. It does not vary with age and sex, and in healthy individuals, basal concentrations are very low. In response to active inflammation, there is a rapid and early rise up to 1000 fold. The ESR is an indirect measure of the acute phase response. It measures the rate of sedimentation of erythrocytes in a vertical tube over a standard period of 1 hour. The ESR is determined by the negative electrical charge on the erythrocyte surface, which prevents aggregation. Certain plasma proteins for example fibrinogen decrease the negative charge and therefore increase the sedimentation rate. The CRP and ESR are both surrogate markers for disease activity, and assist in the monitoring of therapy. Persistent acute phase response is associated with an increased rate of radiological progression.

Radiology

In the early stages of RA, soft tissue swelling may be the only radiological finding. However, persistent inflammation results in joint and cartilage damage. The ARA has designated 4 anatomical stages to describe the physical deterioration of the joints. In stage I, there is osteoporosis, but the joint line is intact. Stage II is characterized by slight diminution of the joint line and early subchondral erosions, while obvious cartilage and bone lesions are present in stage III and gross destruction in stage V.

1.2.3 Pathogenesis

The exact aetiology remains unknown however; it is believed that an unknown antigen triggers an exaggerated immune response in a genetically susceptible individual. The inflammatory process in RA is multifactorial and may result from a number of different mechanisms (Fig. 1.3):

- antibody mediated complement activation and cytotoxic cellular injury.
- immune complex deposition with activation of the complement system and generation of pro-inflammatory mediators
- T cell mediated either through direct cytotoxic or via the elaboration of cytokines.

1.2.3.1 Genetic factors

A higher incidence of RA among families of patients with RA (Spector, 1990), and a concordance frequency of 30% in monozygotic twins compared to 5% in dizygotic twins (Lipsky, 1991) support a genetic predisposition for RA. A significant association between RA and the major histocompatibility complex (MHC), human leucoytes antigen (HLA), has been reported. A higher prevalence of HLA DR4 and DR1 has been reported in various populations. The predisposition to the disease is conferred by a 'shared epitope'

present in the third hypervariable region of the DRB1 gene. This epitope is encoded by DR4 and non-DR4 alleles, namely DR1, DR10 and DRw6. In addition, the epitope is also associated with more severe disease. However, only 20–30% of the cause of RA can be attributed to genetic factors.

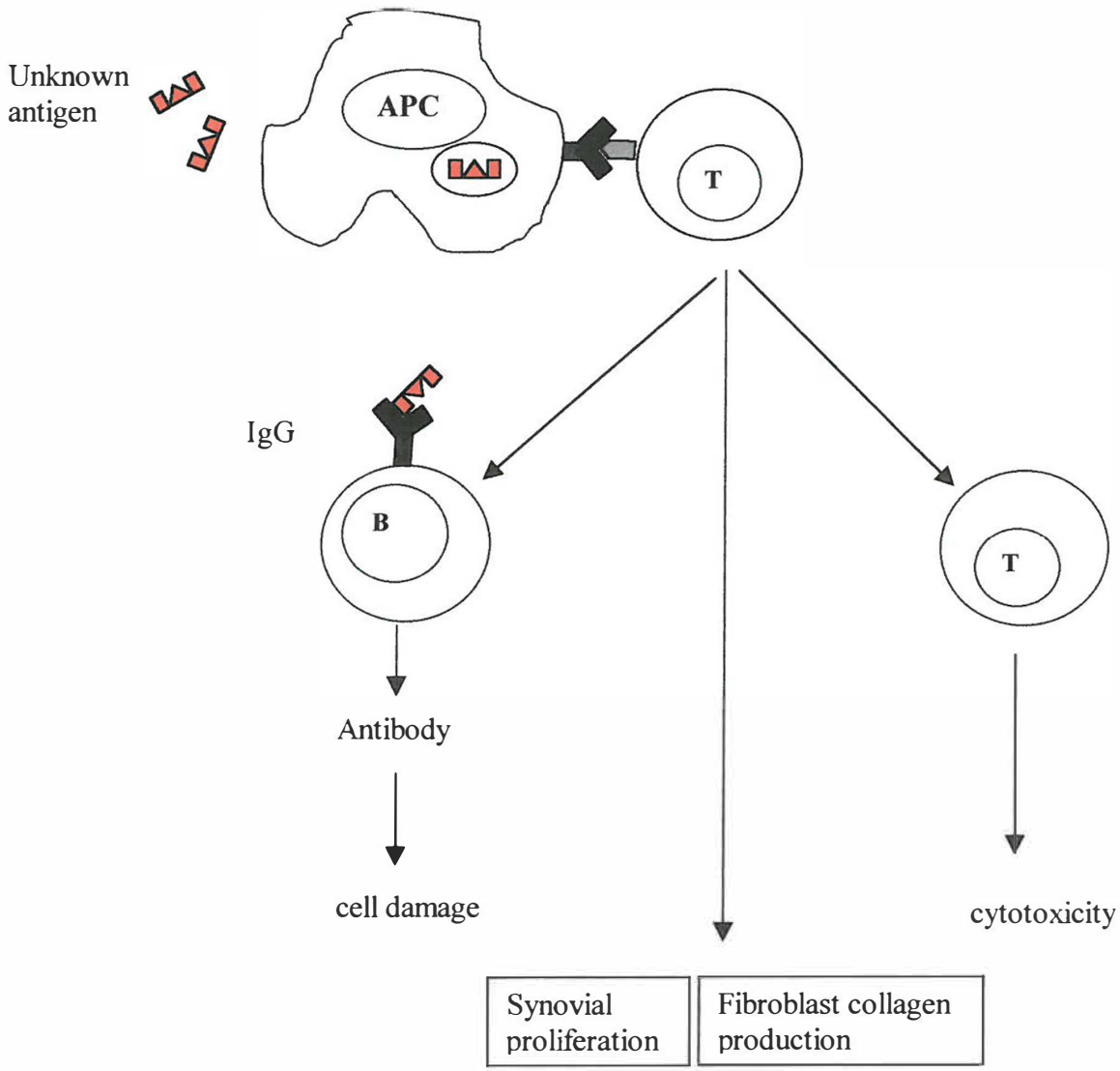


Figure 1.3 Pathogenesis of rheumatoid arthritis

1.2.3.2 Primary mediators of synovitis

1.2.3.2.1 The kallikrein -kinin cascade

The *"contact system"* consists of a group of four proteins which circulate in plasma in inactive forms, and are activated to provide a host defence system (Fig. 1.4). It consists of Hageman factor (HF) or coagulation factor X11, prekallikrein, high molecular weight kininogen (H-kininogen) and coagulation factor X1 (plasma thromboplastin antecedent). Most of the plasma kallikrein exists as a proenzyme complexed to H-kininogen. The contact system is activated by contact with negatively charged surfaces found in a variety of substances, including monosodium urate and calcium dihydrate crystals, collagen, vascular basement membranes, glycosaminoglycans and immune complexes. Activation is initiated by the binding of HF to negatively charged surfaces with cleavage of HF to form activated HF (HFa). HFa then cleaves the prekallikrein-H-kininogen complex to release the active enzyme, plasma kallikrein, which has the ability to cleave bradykinin from H-kininogen. Bradykinin is a potent mediator of inflammation because of its ability to cause vasodilatation, and increased vascular permeability. In addition, it produces pain, causes leucocyte margination in blood vessels and has the ability to release cytokines from monocytes. Closely related to the kinin system are the clotting and fibrinolytic systems. Plasmin, a peptide cleaved from fibrin, is capable of activating HF and cleaving the C3 component of complement. In addition, plasmin possesses chemotactic activity and increases vascular permeability.

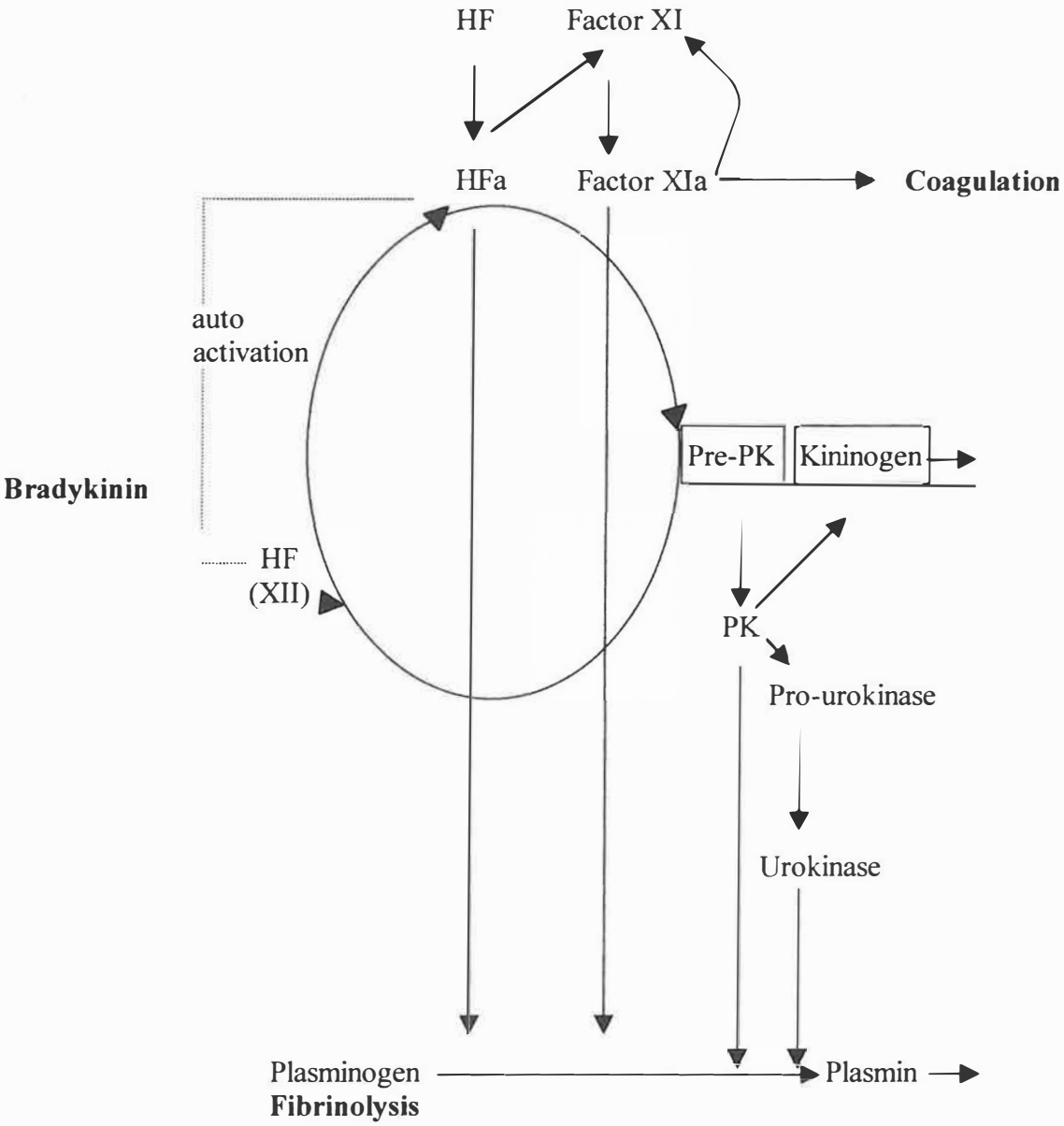


Figure 1.4 Contact activation cascade

(HF, hageman factor; Hfa, activated Hageman factor; PK, plasma kallikrein).

Overview

Kinins are potent vasoactive peptides that are thought to be responsible for the cardinal signs of inflammation as well as a number of other biological activities. The kinins, bradykinin and lys-bradykinin (kallidin) are cleaved from endogenous substrates called kininogens by the serine proteases, kallikreins. Two types of kallikreins, tissue and plasma, have been identified. Tissue kallikrein (TK) releases lys-bradykinin from L-kininogen (low molecular weight kininogen or LK) and plasma kallikrein (PK) releases bradykinin from H-kininogen (high molecular weight kininogen or HK) (Fig. 1.5).

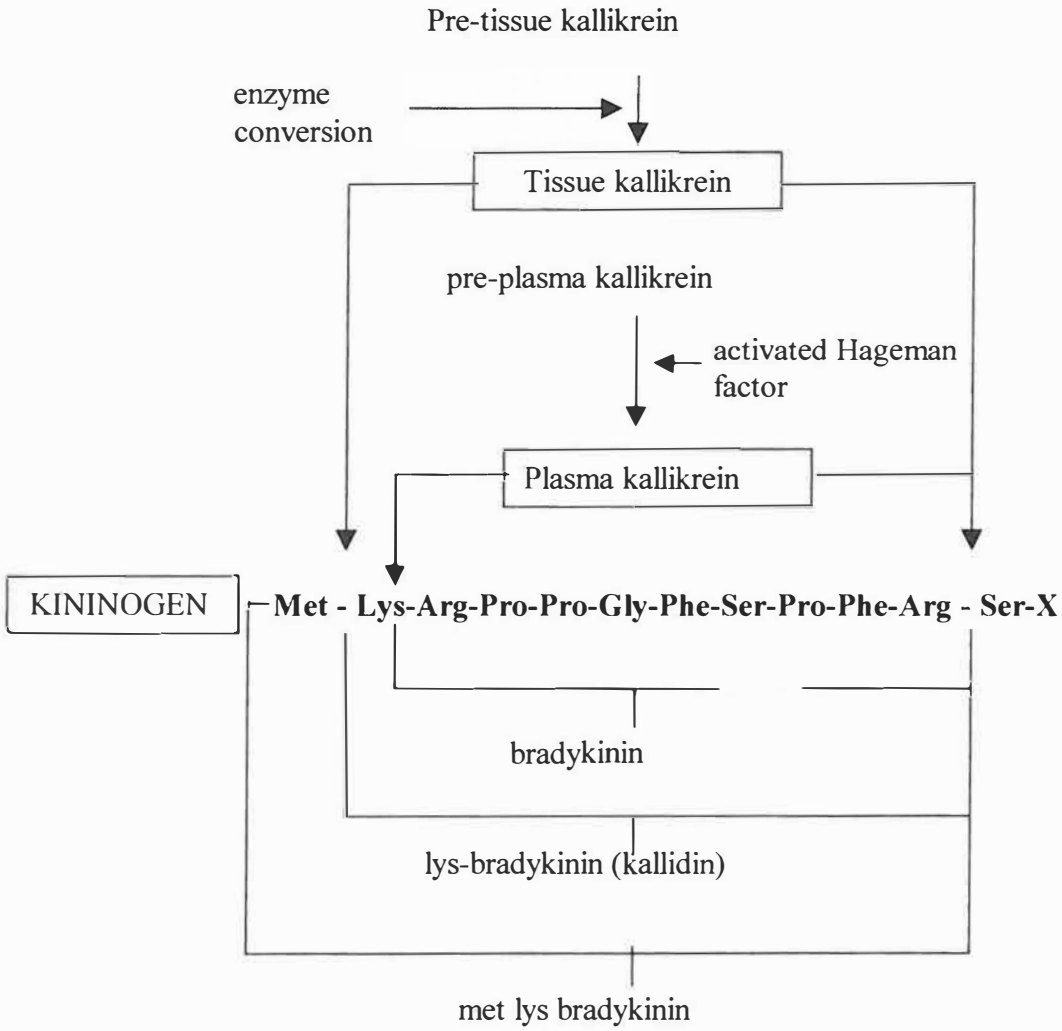


Figure 1.5 Cleavage sites on kininogen molecule for the release of kinins

Although the half-life of kinins in the circulation is considered to be very short (less than 30 seconds), paracrine formation of kinins in extracellular spaces is known to have a longer half-life. Kinins once formed, are degraded by kininases. The actions of kinins are mediated via G-protein linked kinin receptors, with subsequent activation of a series of second messengers by signal induction. A number of receptor antagonists have been developed for clinical use.

Historical background

In 1909, Abelous and Bardier, reported the presence of a hypotensive substance in normal human urine (Abelous and Bardier, 1909) that they called urohypotensin. This was subsequently confirmed by Frey (1926). The compound was later isolated and demonstrated in blood, pancreas and the salivary glands (Frey *et al.* 1932, 1950). Since it was assumed that this substance originated from the pancreas (kallikreas in Greek), they named it kallikrein. Later, it was demonstrated that when urinary kallikrein was incubated with serum it enzymatically released a smooth muscle contracting substance from an inactive precursor (Werle *et al.* 1937). This substance was initially called substanz DK (darmkontrahierende substanz" or "gut-contracting substance"). This was later renamed kallidin and the inactive precursor, kallidinogen (Werle and Berek, 1948). At about the same time, Rocha e Silva *et al* (1949) demonstrated that the incubation of dog plasma with snake venom or trypsin produced a substance that was hypotensive and caused slow contraction of the guinea pig ileum. This substance was called bradykinin, because of the slowly initiated sustained contraction of the guinea-pig ileum. Since kallidin (lys-bradykinin) and bradykinin were formed under the same conditions and had similar pharmacological actions, it was suspected that they were closely related and derived from the same substrate. This was later confirmed pharmacologically (Schachter, 1956), and by

chemical synthesis (Boissonnas *et al.* 1960).

Kallikreins

The biological effects of the kallikrein-kinin cascade are mediated via the vasoactive peptides, bradykinin and lys-bradykinin, liberated from the kininogen substrate by kininogenases. Although trypsin, uropepsin and plasmin have kininogenase activity, the two most potent endogenous kininogenases are plasma and tissue kallikrein. The kallikreins differ from each other with respect to their molecular weights, isoelectric points, substrate specificity, immunological profiles and the type of bioactive peptide released. Plasma kallikrein acts on HK to produce the nonapeptide bradykinin, and tissue/glandular kallikrein releases the decapeptide kallidin (Lys-bradykinin) from LK (Fielder, 1979). Kallidin is converted to bradykinin by the action of an arginine aminopeptidase (Guimaraes *et al.* 1973).

Tissue Kallikrein

Tissue kallikreins are a group of serine proteases encoded by a multigene family (*KLK*) in several species (Evans *et al.* 1987). The human *KLK* locus is situated on chromosome 19q (Evans *et al.* 1988), and was thought to consist of three genes; *KLK1* (tissue kallikrein), *KLK2* (human glandular kallikrein), and *KLK3* (prostate specific antigen), each comprising 5 exons and 4 introns. The *KLK1* gene is expressed predominantly in the salivary glands, pancreas and kidney (Mahabeer and Bhoola, 2000), while *KLK2* and *KLK3* are expressed almost exclusively in the prostate (Yousef *et al.* 1999a). However, recently new genes have been mapped in the region of the kallikrein gene family, suggesting that the kallikrein gene family consists of 13 genes (Yousef *et al.* 1999a). These include zyme, normal

epithelial cell-specific 1 gene (NESI), stratum corneum chymotryptic enzyme, neuropsin, trypsin-like serine protease (TLSP) and five new kallikrein genes (KLK-L1 to KLK L5). KLK L1 is expressed in prostate tissue, testis, mammary gland, adrenals, uterus, thyroid, and salivary glands and has been demonstrated in the breast carcinoma cell line BT-474 (Yousef *et al* 1999b). KLK-L2 is expressed mainly in breast, brain, and testis and to a lesser extent in many other tissues and is upregulated by estrogens and progestins in the breast cancer cell line BT-474 (Yousef and Diamandis, 1999). KLK-L4 is expressed in a variety of tissues including prostate, salivary gland, breast, and testis and appears to be down regulated in breast cancer tissues and breast cancer cell lines (Yousef *et al.* 2000a). Similarly, TLSP is expressed in many tissues including cerebellum, prostate, salivary glands, stomach, lung, thymus, small intestine, spleen, liver, and uterus and it's expression appears to be regulated by steroid hormones in the breast carcinoma cell line BT-474 (Yousef *et al.* 2000b).

Tissue kallikreins are acidic glycoproteins with a molecular weight of 25-45 kDa and an isoelectric point of 3.5-4.4 (Pisano, 1975). Human urinary TK is synthesized bound to a 17 amino acid signal peptide, which is cleaved to produce an inactive proenzyme, prokallikrein. The further removal of seven amino acids forms the active enzyme. The proteolytic enzymes, trypsin and thermolysin (a bacterial metalloproteinase) have been shown to activate the proenzyme *in vitro* (Takada *et al.* 1985), as has plasmin and plasma kallikrein (Yamada and Erdos, 1982). However, the endogenous pathway for the activation of the proenzyme is not known.

Human urinary kallikrein is a single chain polypeptide of 238 amino acids with Ile at the amino terminal and Ser at the carboxy-terminal (Takahashi *et al.* 1988). The number and

position of glycosylation sites vary in the different species. Human urinary kallikrein has three asparagine linked glycosylation sites (Lu *et al.* 1989) and three oxygen glycosylation sites linked to two serine and one threonine residues (Kellerman *et al.* 1988). Tissue kallikrein immunoreactivity and/or enzyme activity has been demonstrated in numerous cells and biological tissues, including the saliva and salivary gland (Bhoola and Dorey, 1971), colon (Chen *et al.* 1995), endometrium and myometrium of the uterus (Clements *et al.* 1994), placenta and amniotic fluid (Malofiejew, 1973), cardiac tissue extracts (Nolly *et al.* 1981), bronchial lavage from asthmatics (Christiansen *et al.* 1987) and nasal secretions from patients with allergic rhinitis (Baumgarten *et al.* 1989). Of interest is that increased levels of salivary gland TK have been reported in patients with malignant tumours away from the oral cavity (Jenzano *et al.* 1986), and restriction of sodium intake increased both salivary and urinary TK levels (Horwitz *et al.* 1982). Additionally, TK has been identified in synovial fluid from arthritic joints (Worthy *et al.* 1990; Bhoola and Dieppe, 1991; Rahman *et al.* 1994), and in the circulating and synovial fluid neutrophils (Figueroa *et al.* 1989; Williams *et al.* 1997).

The primary function of TK is the cleavage of the kininogen substrate to release the decapeptide lys-bradykinin (kallidin). Although LK is the preferred substrate for TK, the enzyme releases the kinin molecule from both HK and LK (Carretero and Scicli, 1980). Release of kallidin from the kininogen molecule requires the hydrolysis of the Met-Lys bond at the amino terminal and the Arg-Ser bond at the carboxy terminal. In addition to its kininogenase activity, TK has been implicated in other biological actions such as processing of growth and peptide hormones (Mason *et al.* 1983), pathogenesis of acute pancreatitis, the homeostatic control of blood pressure and neutrophil diapedesis (Figueroa *et al.* 1989). In addition, tissue kallikreins are considered to process *in vitro* a variety of

promolecules that may include matrix metalloproproteinases (Tschesche *et al.* 1989), procollagenase (Eeckhout and Vaes, 1977) and progelatinase (Menashi *et al.* 1994). The potential activation of these proproteinases by TK, suggests that TK may play a role in endothelial cell migration, leucocyte aggregation and tissue remodeling.

Plasma kallikrein

Plasma kallikrein is a serine protease synthesized in the zymogen form, plasma pre-kallikrein (PPK) predominantly by the liver. However, PPK mRNA has also been detected in the kidneys, adrenal gland and placenta (Ciechanowicz *et al.* 1993) as well as the brain, heart, lung, trachea, endothelial cells, leukocytes and a variety of fibroblast and epithelial cell lines (Hermann *et al.* 1999). The PPK is encoded by a single gene of about 22 kilobases in length (Beaubien *et al.* 1991) that is located on chromosome 4 q34–q35, and structurally similar to the Factor X1 (thromboplastin antecedent factor) gene (Asakai *et al.* 1987). It consists of 15 exons, the first 2 exons encoding 5′–untranslated regions and the signal peptide, the next eight exons encode the heavy chain and the last five exons the light chain and protease portion of the molecule. A 75% homology exists between the human, rat and mouse plasma kallikreins. PPK is a single chain glycoprotein synthesized in the liver and is secreted into the circulation. In the plasma, it is present in two forms, 85 and 88 kDa, (Hojima *et al.* 1985; Veloso and Colman, 1991). The inactive PPK circulates as a heterodimer complex bound with Factor XI to its substrate HK through domain 6 of the kininogen molecule (Mandle *et al.* 1976). Following vascular damage, HK orientates PPK and factor X1 towards Hageman Factor (HF). Following activation of HF through either contact with anionic surfaces, autocatalytically or the negative feedback action of plasma kallikrein, activated HF (HFa) converts the inactive PPK to active plasma kallikrein, which consists of a 32 kDa light chain and 52 kDa heavy chain linked by disulphide bonds

(Kaplan *et al.* 1989). Plasma kallikrein subsequently cleaves bradykinin from HK through hydrolysis of the Lys-Arg and Arg-Ser bonds to produce a nonapeptide with arginine at both the amino- and carboxy-terminals (Fig. 1.5). This process is amplified in the presence of HK, which acts as a cofactor (Griffin and Cochrane, 1976). Although LK is a poor substrate for PK, it will form BK in the presence of neutrophil elastase. It is believed that neutrophil elastase cleaves a fragment from LK, from which plasma kallikrein releases bradykinin (Sato and Nagasawa, 1988).

Activated HF also converts factor XI (bound to HK) to activated factor XI (X1a) that initiates the coagulation cascade (Ratnoff *et al.* 1961). In addition, PK, factor X1a and HFa convert plasminogen to plasmin, and thereby promote fibrinolysis (Colman, 1969; Mandle and Kaplan, 1977). Plasma prekallikrein localizes on the outer surface of the cell membrane of non-fixed neutrophils, attached to domain 6 of HK (Henderson *et al.* 1994). Plasma kallikrein also participates in the inflammatory process since it is chemotactic for PMN leucocytes (Kaplan *et al.* 1972), and has the ability to cause aggregation of PMN leucocytes with a similar potency to that of the chemotactic peptide N-formyl-methionyl-leucyl-phenyl-alanine (N-fMLP) (Schapira *et al.* 1982). Purified PK has also been shown to cause significant release of elastase from neutrophils pre-treated with cytochalasin B (Wachtfogel *et al.* 1983), and to convert latent collagenase to its active form in vitro (Nagase *et al.* 1982). Activation of the plasma kallikrein/kinin system has also been demonstrated on endothelial cells (Rojkjaer and Schmaier, 1999). On endothelial cells, prekallikrein appears to be activated by an antipain sensitive protease and is dependent on the presence of high molecular weight kininogen and an optimal free Zn^{2+} concentration. The kallikrein formed on endothelial cell membranes is capable of cleaving its receptor and HK, thus liberating bradykinin and the HK PK complex from the endothelial cell surface

as well as activating pro-urokinase and plasminogen (Lin *et al.* 1997).

Kallikrein inhibitors

The presence of inhibitors of TK was first described in the circulation and in bovine organs (Kraut *et al.* 1930). More recently, kallistatin-binding protein, a specific endogenous inhibitor of TK, has been identified, purified and cloned (Chao *et al.* 1990; 1996). Kallistatin also binds to elastase and chymotrypsin, but not to PK, urokinase or collagenase. At the gene and protein level, kallistatins are considered members of the serpin (serine protease inhibitor) superfamily (Chao and Chao, 1995). Similarly, other members of the serpin family that show a high degree of homology to kallistatin, namely, protein C inhibitor (Espana *et al.* 1995), α_1 antitrypsin and α_1 antichymotrypsin also bind tissue kallikrein (Clements, 1997). The enzyme and inhibitor form a 1:1 stoichometric, heat stable complex of 92 kDa, which is emiocytosed by hepatocytes and cleared from the circulation. Aprotinin (trasylol) is an *in vitro* inhibitor of both PK and TK, and the substitution of an amino acid has resulted in the synthesis of [Val 15] aprotinin, which selectively binds PK and neutrophil elastase (Wenzel *et al.* 1986). Whereas, the actions of TK are inhibited by α_1 antitrypsin, PK is inhibited by α_2 macroglobulin and C_1 esterase inhibitor (Worthy *et al.* 1990). Fifty percent of PK is trapped within the protease inhibitor, α_2 macroglobulin (Suzuki *et al.* 1987). After the kallikrein-inhibitor complexes are formed, they are rapidly cleared from the circulation.

Kininogens

Kininogens are single chained glycoproteins synthesized primarily synthesized by hepatocytes (Takagaki *et al.* 1985), and secreted into the circulation. There are three

molecular species of kininogens with the HK and LK present in humans, while T-kininogen is unique to the rat. The kininogen molecule consists of an amino acid terminal heavy chain and a carboxy-terminal light chain, with the kinin moiety interleafed between the two polypeptides and linked to both terminals by single disulphide bonds (Kellerman *et al.* 1987). The heavy chain is common to both HK and LK, but the light chain is unique to either HK or LK.

A single kininogen gene consisting of 11 exons, transcribes a unique mRNA for HK and LK by alternative splicing (Kitamura *et al.* 1985). HK and LK share the coding region of the first nine exons, a part of exon 10 containing the bradykinin (BK) sequence and the first 12 amino acids after the carboxyterminal BK sequence. Exon 11 codes for the 4 kDa light chain of LK. The complete exon 10 contains the full coding sequence for the 56 kDa light chain of HK.

The kininogens are multidomain proteins. Although each domain has one or more specific functions (Fig. 1.6) the protein as a whole participates in several biological processes. The heavy chain consists of 3 domains (D1 - D3). Domain 1 has a low affinity Ca^{2+} binding site, the function of which is unknown (Ishiguro *et al.* 1987). Domains 2 and 3 contain the amino acid sequence, Glu-Val-Val-Ala-Gly found in cysteine protease inhibitors (Salvesen *et al.* 1986). Both HK and LK are potent cysteine protease inhibitors (Schmaier, 1997). Domain 2 is the calpain inhibitory region (Bradford *et al.* 1993). When platelets are activated, calpain translocates to the external membrane where it may be inhibited by plasma or externalised platelet α granule HK (Schmaier *et al.* 1986). Domain 3 inhibits cysteine proteases except calpains (Salvesen *et al.* 1986). Domain 4 contains the kinin moiety, released from the kininogen molecule by the enzymic action of kininogenases. In addition, domain 4 has the ability to inhibit α - thrombin induced platelet aggregation

(Hasan *et al.* 1996), and serves as a cell-binding site (Hasan *et al.* 1994). The carboxy terminal portion of BK and the amino terminal portion of the light chain participate as low affinity binding sites to endothelial cells. More importantly, the domain 4 cell-binding region holds the kininogens in the appropriate conformation for optimal cell binding. The light chain of LK is 4 kDa and consists of one domain (D5) of unknown function. The light chain of HK is 56 kDa and consists of domains 5 (D5H) and 6 (D6). Domain 5 contains two histidine and glycine rich regions, one at its carboxy-terminal and the other on its amino-terminal. It serves as a cell-binding site on platelets (Meloni *et al.* 1992), neutrophils (Wachtfogel *et al.* 1993) and endothelial cells, (Reddigari *et al.* 1993). In addition, the histidine and glycine-rich regions have the ability to bind to anionic surfaces such as Zn^{2+} (DeLa Cadena and Colman, 1992) and heparin (Bjork *et al.* 1989). Domain 6 of HK has a PPK and factor X1 binding site (Tait and Fujikawa, 1987). The procoagulant activity of HK is dependent on its ability to bind to PPK and factor X1, as well as its ability to bind to anionic surfaces. Inhibition of either inhibits the procoagulant activity of HK (Reddigari and Kaplan, 1989). The binding of PPK to bound HK initiates a sequence of events that lead to prekallikrein activation on biological surfaces. LK is a 66 kDa β-globulin with a plasma concentration of 160 μg/ml (2.4 μM) and an isoelectric point of 4.7. HK is a 120 kDa α - globulin with a plasma concentration of 80 μg/ml (0.67 μM) and an isoelectric point of 4.3 (Schmaier, 1997).

Kininogens are present in extracellular fluids and have been isolated on human platelets (Schmaier *et al.* 1986), the collecting ducts of human kidney (Figueroa *et al.* 1988), sweat glands, endothelial cells (van Iwaarden, 1988), and human neutrophils (Figueroa *et al.* 1992). Specific, reversible and saturable binding sites for kininogens have been reported on human neutrophils, platelets and endothelial cells. On the human neutrophil, the kininogen molecule is bound to PPK, and the substrate and enzyme exists as a complex.

The kininogens present on the human neutrophils probably originate from the plasma pool or are co-secreted from the hepatocytes and enter the circulation as a complex. Currently there is no evidence as to whether they are synthesized by the neutrophils.

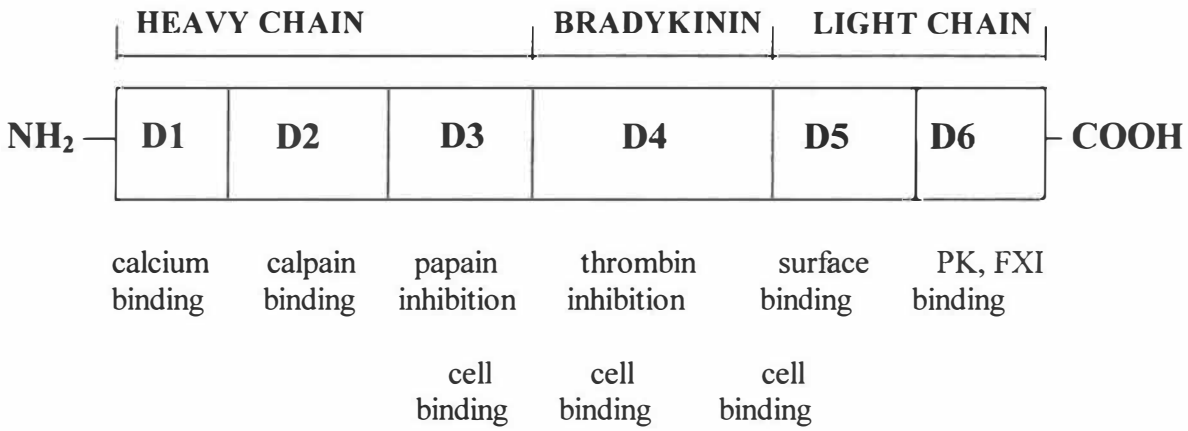


Figure 1.6 Domain structure of high molecular weight kininogen

Kinins

Bradykinin and lys-bradykinin are potent vasoactive peptides released from HK and LK by plasma and tissue kallikrein respectively. Kinins in plasma range from 10-5000 ρg/ml (Carretero and Scicli, 1988), and urinary excretion of kinins is about 24.6 ± 1.0 μg/day. They are thought to initiate the cardinal signs of inflammation namely pain, vasodilatation and oedema. The kinin peptides are potent pain-producing substances and cause pain through two mechanisms. Firstly, via the direct-stimulation of nociceptors (C and Aδ) fibres, and secondly, by inducing sensitisation of sensory fibres to physical and chemical stimuli (Rang *et al.* 1991).

When compared to 5′ hydroxytryptamine (5′HT), BK is at least 10 times more potent in causing pain on the blister base (Whalley *et al.* 1987). This algesic effect of BK is potentiated by thromboxanes, prostaglandins (Vane, 1978) and 5′-HT. Both bradykinin and kallidin cause vasodilatation and increase vascular permeability (Bhoola *et al.* 1960; Carter *et al.* 1974). It is postulated that the release of kinins could result in opening the junctions between the endothelial cells, and thus promote the local diapedesis of neutrophils and plasma extravastion, as seen in the inflammatory process (Figueroa *et al.* 1989) (Fig. 1.7). In addition, kinins stimulate the release of the pro-inflammatory cytokines, IL-1 and TNF (Tiffany and Burch, 1989), osteoclastic bone resorption (Lerner *et al.* 1987), and stimulate the release of several second-generation mediators, for example, platelet activating factor, leucotrienes, prostaglandins, substance P, acetlycholine and noradrenaline (Fig. 1.8).

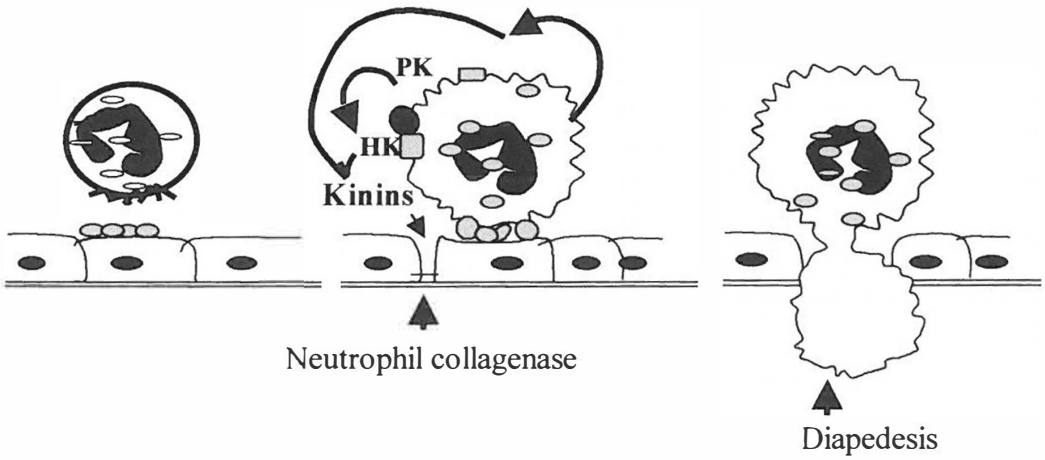


Figure 1.7 Role of kinins in neutrophil diapedesis

Kinins cleaved from HK by the protease PK (both present on neutrophils) are thought to result in endothelial cell retraction and neutrophil migration. This is enhanced by the simultaneous secretion of collagenase.

(HK, high molecular weight kininogen; LK, low molecular weight kininogen; PK plasma kallikrein; TK, tissue kallikrein)

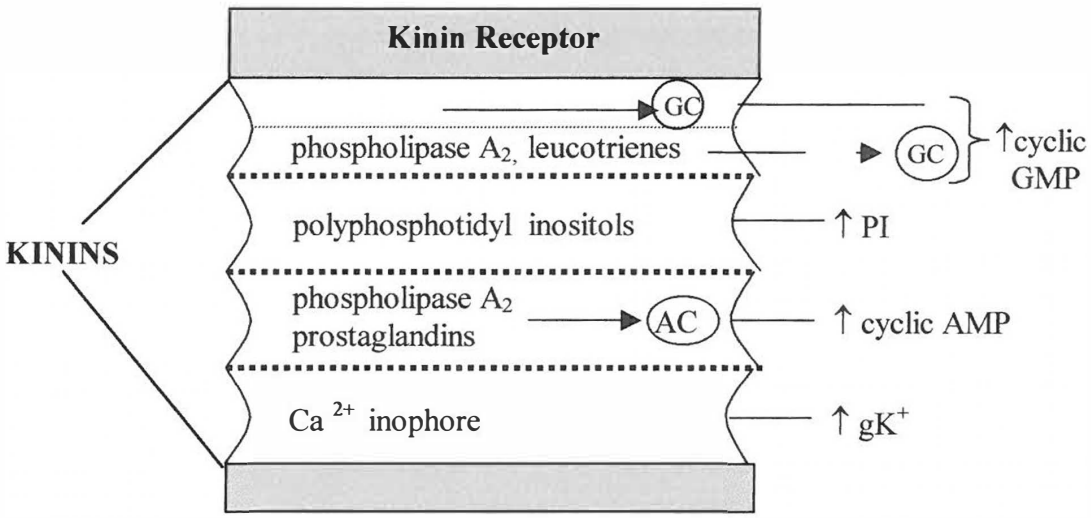


Figure 1.8 Kinin receptor coupling and signal transduction

(PI, phosphotidylinositol second messengers (diacyl glycerol and inositol triphosphate); gK^{2+}, potassium conductance; GC, guanylate cyclase, AC, adenly cyclase)

Several studies support the hypothesis that bradykinin sensitises primary afferent neurones to other chemicals present in inflamed tissues by recruiting additional sensory neurons thereby contributing to hyperalgesia. In a study using primary cultures of dorsal root ganglion neurons from neonatal rats, pre-treatment with bradykinin increased the proportion of "intermediate-size" dorsal root ganglion neurons that responded to capsaicin or a low pH (6.1) and "small-size" neurons that responded to low pH but not to capsaicin (Stucky *et al.* 1998). In addition B2 bradykinin receptors have been localised in neurones of the brain stem, basal nuclei, cerebral cortex, thalamus and hypothalamus, endothelial lining of the superior sagittal dural sinus and ependyma of the lateral and third ventricles, whereas B1 kinin receptors have been localised on neurones of the thalamus, spinal cord and hypothalamus (Raidoo and Bhoola, 1997). Increased expression of B2 and B1

receptor mRNA in the lumbar dorsal root ganglia following chronic constriction injury to a rat sciatic nerve has been observed after 48 hours and 14 days respectively (Levy and Zochodne, 2000). The increased expression of both receptors coincided with the analgesic effects of their antagonists in that HOE-140 (a potent B2 receptor antagonist) was analgesic at both 48 hours and 14 days, while the B1 receptor antagonist des-Arg(9), [Leu(8)]-BK had an analgesic effect only at 14 days (Levy and Zochodne, 2000). In another study, intramuscular infusions of BK and serotonin into the tibialis anterior muscle of volunteers produced significantly increased pain as measured by the visual analogue scale compared to an infusion of isotonic saline and the duration of pain was significantly increased by the combination of BK and serotonin (Babenko *et al.* 2000). In addition, the infusion of the non -peptide BK B2 receptor antagonist, FR173657, has been shown to reduce the nociceptive behavioural responses following intraplantar injection of BK in unanaesthetized rats (Griesbacher *et al.* 1998).

Other biological activities of kinins include the ability to lower systemic blood pressure, increase capillary permeability and produce natriuresis. Kinins also stimulate the secretion of renin from the kidney (Beierwaltes *et al.* 1985) and the release of vasopressin from the neurohypophysis (Baertschi *et al.* 1981). In the nervous system BK is involved in the central regulation of blood pressure, nociception and diuresis and it increases neuronal excitability (Nitsch *et al.* 1998). The cellular actions of kinins are mediated via the activation of specific kinin receptors situated on the surface membrane of many cells.

Kininases

Kinins are inactivated by peptidases called kininases (Bhoola *et al.* 1992). The kininase family comprises the kininase 1 carboxypeptidases; kininase 1 carboxypeptidase N (K1-

CPN) and kininase caboxypeptidase M (K1-CPM) and the kininase II peptidyl- peptidases, kininase II-angiotensin coverting enzyme (K11-ACE) and kininase II neutral endopeptidases (K11-KEP). In addition, the prolidase, aminopeptidase B and two endogenous kininases (kininase A and B) also hydrolyse, and thus inactive kinin.

Kininase I carboxypeptidases N (KI-CPN) is an arginine carboxypeptidase synthesised by the liver and secreted into the circulation where it accounts for 90% the bradykinin-hydrolysing activity (Zacest *et al.* 1974). The enzyme is a tetrameric molecule and comprises two heterodimers, each consisting of a 83 kDa non-catalytic unit and a 50 kDa catalytic unit (Levin *et al.* 1982). KI-CPN acts on the carboxy-terminal of the bradykinin molecule, and removes the Arg^9 residue to produce des[Arg^9]–BK that is a B_1 receptor agonist. Similarly, hydrolysis of the C-terminal arginine of kallidin results in the formation of the B1 receptor agonist, des[Arg^{10}]–kallidin. Levels of KI-CPN are decreased in cirrhosis of the liver and increased in pregnancy (Erdos and Skidgel, 1997). Elevated levels have also been reported in the blood and synovial fluid of patients with arthritis (Chercuiffe *et al.* 1987).

Kininase I Carboxypeptidase M (KI-CPM) is a membrane bound 62 kDa single chain glycoprotein (Skidgel *et al.* 1989), and is found in a wide variety of cells and tissues. High levels of CPM mRNA have been shown in human placenta, lung and kidney. The enzyme cleaves the C-terminal arginine of BK and Lys-BK to form des[Arg^9] BK and des[Arg^{10}] kallidin respectively. Since it is localised to the plasma membranes of a wide variety of cells and tissues, where kinin receptors are located, KI-CPM is likely to regulate the local actions of kinins (Erdos and Skidgel, 1997). Des[Arg^9]BK and des[Arg^{10}] kallidin are

agonists for the B_1 receptors, therefore KI-CPM may play a role at sites of inflammation where B_1 receptors may be upregulated (Bhoola *et al.* 1992).

Kininase II-Angiotensin I-converting enzyme (KII-ACE) is a single chain glycoprotein of about 140 to 170 kDa, and is present in vascular beds bound to the plasma membrane of endothelial cells as well as in a soluble form (Erdos and Skidgel, 1997). High levels are found in the pulmonary vascular beds, kidney , myocardial vascular endothelium small intestine choroid plexus and placenta (Erdos and Skidgel, 1997). Soluble ACE is found in urine, lung oedema, amniotic, seminal and cerebrospinal fluid and in homogenates of prostate and epididymis (Erdos and Skidgel, 1997). KII-ACE inactivates circulating kinins mainly during their passage through the lung (Bhoola *et al.* 1992) by hydrolysis of two separate bonds on the C-terminal end of the kinin molecule. First, it removes the dipeptide Phe–Arg and next splits the Ser–Pro bond (Zacest *et al.* 1974). In addition, KII-ACE hydrolyses enkephalins, neurotensin, substance P and lutenising hormone-releasing hormone (Erdos and Skidgel, 1997). Several inhibitors of ACE have been developed.

Kininase II-Neutral Endopeptidase 24.11 (KII-NEP 24.11) is a single chain protein consisting of 742 amino acids, and has a molecular weight of 88 kDa (Malfroy *et al.* 1988). After synthesis, the enzyme migrates to plasma membrane where it becomes translocated to be anchored on the outer surface of the fibroblast from which it may be secreted into the body fluids (Lorkowski *et al.* 1987). It inactivates kinins by removing the C-terminal Phe-Arg dipeptide (Gafford *et al.* 1983). In addition, it cleaves several other peptides including substance P, enkephalins, atrial natiuretic factor and neurotensin (Erdos and Skidgel, 1997). KII-NEP is present in high concentrations in the brush border of proximal tubules of the kidney, microvilli of the intestine, fibroblasts of the lung and brain placenta and male genital tract (Erdos and Skidgel, 1997). In addition, KII-NEP, has been reported on

the cell membrane of human neutrophils (Connelly *et al.* 1985). KII-NEP hydrolyses N-fMLP, a peptide that stimulates chemotaxis. Activation of neutrophil or antigen-antibody reactions triggers the internalisation of KII-NEP, with a rapid loss of its enzymic activity. This could have marked effect on the migration of neutrophils to sites of inflammation. It could also have a regulatory effect on the biological activity of kinins formed on the external surface of the neutrophil membrane on which HK, LK and plasma prekallikrein have been localised (Figueroa *et al.* 1992; Henderson *et al.* 1992).

Additional kinin peptidases: Aminopeptidase B which cleaves the Arg^{1}-Pro^{2} bond of the BK molecule has been described in erythrocytes, kidney and lung tissue (Erdös and Skidgel, 1997). Two additional endopeptidases, kininase A and B, have been purified from the rat brain (Oliviera *et al.* 1976). Kininase A cleaves the Phe^{6}-Ser^{8} bond and kininase B the Pro^{7}-Phe^{8} bond of bradykinin.

Kinin Receptors

The pharmacological activity of the carboxypeptidase metabolites, $desArg^{9}$ -BK and $desArg^{10}$ - kallidin, was initially demonstrated in isolated preparations of rabbit vascular smooth muscle (Regoli *et al.* 1977), and the receptor mediating these responses was classified as a B1 receptor. Subsequently two major types of receptors were identified, B1 and B2 (Regoli and Barabe, 1980), and their classification confirmed with selective agonists, gene cloning and receptor expression studies. Both these receptors belong to a superfamily of G-protein coupled rhodopsin-like receptors, and consist of a seven transmembrane spanning regions with three extracellular and four intracellular loops (Burch and Axelrod, 1987) (Fig. 1.9).

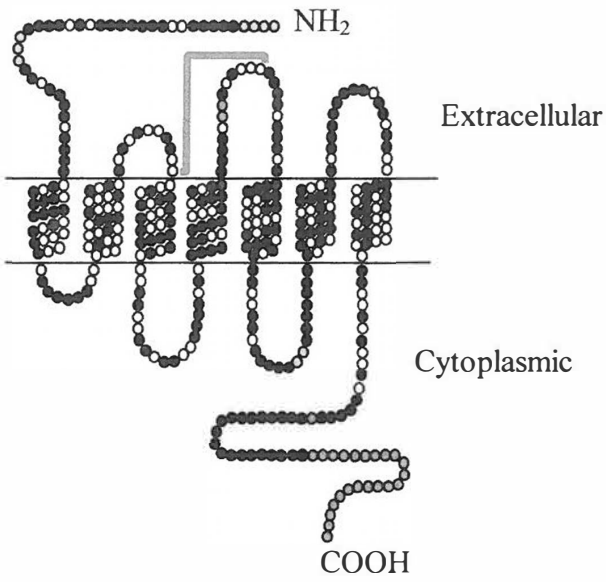


Figure 1.9 Structure of the kinin receptors

(NH_2, amino terminal; COOH, carboxy terminal)

B 1 Receptors

The cDNA clone encoding a human B1 kinin receptor has been isolated from a human embryonic lung fibroblast cDNA library by expression cloning (Menke *et al.* 1994). The mRNA encoding for the B1 receptor is approximately 2 kb shorter than that of the B2 receptor (Webb *et al.* 1994). The B1 receptor is composed of 353 amino acids, and has a 36% homology to the amino acid sequence of the B2 receptor. The potency order of of kinin receptor agonists for the B1 receptor in isolated tissues is desArg^9-BK > Tyr-(Me)^8-BK > BK. Des-Arg^9 -BK was the first described selective agonist for B1 receptors (Regoli *et al.* 1977). Subsequent studies have reported that desArg^9 -kallidin is a potent agonist (Menke *et al.* 1994), in contrast to BK, which is essentially inactive at the B1 receptor. The B1 receptors are apparently not present constitutively, but appear to be inducible by inflammatory mediators such as bacterial lipopolysaccharide and interleukins (Pesquero *et al.* 2000) in smooth muscle cells and fibroblasts in experimental inflammation or after exposure to noxious stimuli. Mice with a targeted deletion of the gene for the B1 receptor

are healthy, fertile, and normotensive and show a blunted hypotensive response to bacterial lipopolysaccharide and a reduced accumulation of PMN leukocytes in inflamed tissue. Under normal non-inflamed conditions, these animals are analgesic in behavioral tests of chemical and thermal nociception. Using whole-cell patch-clamp recordings it has been shown that the B1 receptor was not necessary for regulating the noxious heat sensitivity of isolated nociceptors. However, functional B1 receptors are present in the spinal cord, and their activation can facilitate a nociceptive reflex (Pesquero *et al.* 2000). Induction of B1 receptors was observed in the cardiovascular responses of rabbits injected with K11-ACE inhibitors (Nwator and Whalley, 1989), on the isolated rabbit aorta as a selective time-dependent response to in vitro incubation (deBlois *et al.* 1991), inflamed porcine vascular tissues as compared to healthy tissue (Schremmer-Danniger *et al.* 1996), and in the porcine aortic smooth muscle layer in LPS induced inflammation (Schremmer-Danniger *et al.* 1998). Moreover, while B2 receptor blockade has been shown to attenuate LPS- induced porcine end toxin shock; B1 receptor blockade appeared to counteract the beneficial effects of B2 blockade (Siebeck *et al.* 1996). These findings suggest that the upregulation of the B1 receptors may be a mechanism of host defence. B1 receptor upregulation has also been reported in an in vivo model of myocardial infarction (Tschope *et al.* 2000), in endotoxin-induced renal inflammation in the rat (Marin-Castano *et al.* 1998), as well as in the peripheral T cells from patients with multiple sclerosis (Prat *et al.* 1999). In addition, cytokines, notably IL-1β, released during the inflammatory response, have been shown to induce the expression of B1 receptors (Perkins and Kelly, 1994). TNF α and IL-1 β have been shown to increase the expression of both B1 and B2 receptor expression in a human embryonic lung fibroblasts and this upregulation was inhibited by dexamethasone (Haddad *et al.* 2000). IL-8 has also been shown to increase B1 receptor expression and mRNAs in human lung fibroblasts (Bastian *et al.* 1998). In the naïve rat, infusions of IL-1β or TNF α

increased the paw oedema induced by a B1 agonist (Campos *et al.* 1998). In addition, the paw oedema was inhibited by a B1 antagonist and not by the B2 antagonist. The induction of B1 receptors by the pro-inflammatory cytokines is thought to be due to the release of other cytokines, activation of protein kinase C and tyrosine kinase pathways, co-ordinated with the activation of MAP-kinase and nuclear factor kappa B (Campos *et al.* 1999). Conversely, stimulation of the B1 receptor on macrophages has been reported to release IL-1 and TNF (Tiffany and Burch, 1989). This led to the hypothesis that B1 receptors essentially mediate kinin responses in inflamed and injured tissues (Marceau, 1995).

B1 receptor expression or activity has also been demonstrated in cardiovascular system (Pruneau *et al.* 1996), urinary (Butt *et al.* 1995), intestinal (Rhaleb and Carretero, 1994) and lung tissues (Nadar *et al.* 1996), as well as cultured vascular cells (Tropea *et al.* 1993), embryonic calvarium bones (Ljunggren and Lerner, 1990), tracheal smooth muscle (Marsh and Hill, 1994) and fibroblasts (Marceau and Tremblay, 1986).

B1 receptors stimulate phosphatidylinositol hydrolysis in smooth muscle leading to mobilization of intracellular Ca^{2+} (Butt *et al.* 1995), phospholipase C or phospholipase A (Burch and Axelrod, 1987), and appear to cause biosynthesis and release of prostaglandins (Schneck *et al.* 1994). In *in vivo* studies, B1 receptors have been implicated in kinin induced persistent hyperalgesia (Perkins *et al.* 1993), and plasma extravasation (Cruwys *et al.* 1994).

B2 Receptors

The human B2 receptor gene is localised on chromosome 14 (14q32 band), comprising more than 28 kb, and organised in 3 exons and 2 introns. The B2 receptor protein consists

of 364 amino acids, is highly glycosylated, and exists in multiple isoforms at 69 kDa with an isoelectric point of pH 6.8 - 7.1. The BK binding site is located at the amino-terminal part of the third extracellular loop (Abd Alla *et al.* 1996). Most of the actions of BK and kallidin are mediated through B2 receptors (Regoli and Barabe, 1980; Marceau, 1995). The potency order of kinin receptor agonists for the B2 receptor is Tyr-$(Me)^8$-BK > BK > desArg^9-BK. B2 receptors are ubiquitous and are expressed in most tissues. Their effects have been demonstrated in the gastro-intestinal and respiratory (Field *et al.* 1992), genitourinary (Butt *et al.* 1995), cardio -vascular (Gaudreau *et al.* 1981) and neuronal tissues (Babbedge *et al.* 1995), as well as the eye (Everett *et al.* 1992), the plasma membrane of neutrophils (Haasemann *et al.* 1994) and synovial tissue (Bathon *et al.* 1992a; Uhl *et al.* 1992) and a higher density of B2 receptors have been reported in RA compared to osteoarthritis (OA). In the normal human kidney B2 receptors are present in the entire nephron and are thought to be responsible for most of the physiological effects of BK (Naicker *et al.* 1999). However, in the diseased kidney B2 receptors appear to be reduced. These receptors have also been identified in normal skin (Schremmer-Danninger *et al.* 1999) myometrium and endometrium of the uterus (Murone *et al.* 1999), human embryonic lung fibroblasts (Haddad *et al.* 2000), retina (Takeda *et al.* 1999) and human deciduas derived cells (Buchinger and Rehbock 1999). Upregulation of the B2 receptors has been reported following myocardial infarction (Tschope *et al.* 2000) and in response to cytokines. TNF-α and IL-1β both induced a rapid and transient increase in B1 and B2 receptor mRNA expression in human embryonic lung fibroblasts (Haddad *et al.* 2000) and IL-1β has been shown to increase the expression of B2 receptors in human deciduas derived cells (Rehbock *et al.* 1999). In addition immunolabeling for B2 has been observed on the astrocytic cells in patients with astrocytomas (Raidoo *et al.* 1999) and oesophageal carcinoma (Dlamini *et al.* 1999).

Like the B1 receptors, B2 receptors are coupled to G-proteins, and promote several signal transduction events at the cellular level (Bhoola *et al.* 1992). These include calcium mobilisation, chloride transport, activation of phosphotidylinositol-specific phospholipase C, formation of nitric oxide, activation of phospholipase A2 and stimulation of adenyl cyclase. Thus, the acute nociceptive and inflammatory responses as well as the vasoactive properties elicited by BK appear to be mediated via B2 receptors. *In vivo*, the B2 receptors have been implicated in hypotension (Benetos *et al.* 1986), acute bronchoconstriction and oedema formation (Sakomoto *et al.* 1994).

Receptor Antagonists

The understanding of the critical role the kinin receptor plays in health and disease has been greatly enhanced by the development of several antagonists. The first selective antagonist of the B1 receptor was desArg^{9} [leu^{8}] BK (Regoli *et al.* 1977). Other antagonists such as desArg^{10}[leu^{9}] kallidin, with higher affinity and a longer duration of action have been reported (Regoli *et al.* 1990). The "first generation" competitive antagonists of B2 receptors were bradykinin analogues, in which the proline at position 7 was replaced with D-phenylalanine (D Phe) and the two phenylalanine residues at position 5 and 8 were replaced with thienyalanine.

The prototype [D Phe^{7}] BK was moderately potent as an antagonist.

The "second generation" antagonists introduced two new unnatural amino acids into the first generation structure; D tetrahydroisoquinolone-3-carboxylic acid (Tic) at position 7 and octrahydroindole-2-carboxylic acid (Oic) at position 8 (Wirth *et al.* 1991). DArg–[Hyp^{3}, Thi^{5}, Dtic^{7}, Oic^{8}]–BK (Hoe 140 or icatibant) proved more potent than the first generation antagonists with a longer in vitro lifetime and has been efficacious in clinical

trials for allergic asthma and rhinitis.

The "third generation" single chain BK antagonists (B-9224, B-9430 and B-9668) were introduced with the new amino acid, α-2-indanyl-glycine (Igl) at positions 5 (L-isomer) and 7 (D-isomer) of the HOE 140 structure (Stewart *et al.* 1999). The antagonist B 9430 is effective at both B2 and B1 receptors and is not degraded by lung and kidney homogenates. The next important advance is the introduction of the pentafluorophenylalanine (f5f) residue at position 7. These "fourth generation antagonists" have the highest potency compared to the previous antagonists and show combined B2-B1 antagonist activity.

Non-peptide BK antagonists

Non-peptide antagonists have been developed for several substances, including substance P, angiotensin II and vasopressin. The first non-peptide antagonist for the B2 kinin receptor, WIN64338, had muscarinic cholinergic activity. Subsequently several orally available non-peptide antagonists for the kinin receptors have been developed. Three non-peptide antagonists, FR 173657, 165649 and 167344 have been developed. These have been found to be potent against B2 binding, with no effect on B1 binding. *In vivo*, they blocked human B2 receptor mediated phosphatidylinositol hydrolysis, BK induced bronchoconstriction in guinea pigs and carrageenin-induced paw oedema in rats (Asano *et al.* 1999). In addition, a non-peptide antagonist for the B1 receptor (PS020990) has also been developed (Horlick *et al.* 1999).

The effects of B1 and B2 antagonists have been studied in several animal models. The B2 antagonists have been shown to reduce BK-induced effects on vascular tone and oedema

formation in the rabbit hind limbs (Breil *et al.* 1995) and LPS-induced hyperalgesia (Walker *et al.* 1996). Indomethacin also reduced the LPS-induced hyperalgesia supporting the hypothesis that kinins mediate hyperalgesia via the stimulation of B2 receptors and the formation of prostanoids. Similarly, the non peptide B2 antagonist (FR173657) has also been shown to abolish BK stimulated release of prostaglandins in the isolated rabbit ear (Griesbacher *et al.* 1997) and HOE 140 to inhibit BK induced release of arachidonic acid, interleukin-6 (IL-6), and interleukin-8 (IL-8) in cultured decidua-derived cells (Buchinger and Rehbock, 1999). B1 and B2 receptor antagonists have also been shown to inhibit PMN elastase release and the resultant increase in permeability of endothelial cells (Carl *et al.* 1996) and HOE 140 to reduce the LPS induced increase in vascular permeability (Ueno *et al.* 1996) and to suppress growth of solid tumours (Wu *et al.* 1998). Further, B2 receptor antagonists inhibited the allergic like inflammatory pleurisy triggered by BK, which is characterized by acute mast cell degranulation, protein leakage and pleural eosinophil infiltration (Bandeira-Melo *et al.* 1999). In addition blockade of the B1, B2 and IL-1 β receptors have been shown to substantially modulate capsaicin and substance P induced hyperalgesia (Davis and Perkins, 1996) and both B2 and B1 receptor antagonists had an analgesic effect following peripheral nerve injury (Levy and Zochodne, 2000) and zymosan-induced hyperalgesia (Belichard *et al.* 2000). Bradyzide, a rodent-selective non-peptide B (2) bradykinin antagonists inhibited BK-induced inositol trisphosphate (IP3) formation, blocked BK-induced hypotension and plasma extravasation and reversed Freund's complete adjuvant-induced mechanical hyperalgesia in the rat knee joint to the same degree as morphine and diclofenac, but was more potent than paracetamol (Burgess *et al.* 2000).

1.2.3.2.2 Cytokines

Cytokines are a family of low molecular weight soluble molecules that are responsible for direct cell-to-cell communication in a non-antigenic, but specific manner. They are important for both physiological responses and the pathophysiology of a range of diseases, including the initiation and perpetuation of inflammation and share the following common characteristics:

- Low molecular weight
- Involvement in immunity and inflammation where they regulate the amplitude and duration of response
- Produced transiently and locally, acting in a paracrine or autocrine manner
- Extremely potent, acting at picomolar concentrations
- Interaction with high affinity cell surface receptors specific for each cytokine
- Their cell surface binding ultimately leads to a change in the pattern of cellular RNA and protein synthesis and altered cell behaviour
- Individual cytokines have multiple overlapping cell regulatory actions
- Cytokines interact in a network by inducing each other, transmodulating cell surface receptors and by synergistic, additive or antagonistic interactions on cell functions.

A variety of cells, macrophages, lymphocytes, fibroblasts and endothelial cells secrete cytokines. The cytokines may be either pro-inflammatory or anti-inflammatory. Interleukin 1 (IL-1), IL-6, IL-8 and TNFα are considered to be the most potent pro-inflammatory cytokines and interact in a network with TNFα stimulating the production of IL-1, IL-6 and IL-8 and IL-1 stimulating the production of IL-6 and IL-8 in the pathogenesis of synovitis (Brennan *et al.* 1992a) (Fig. 1.10). The major targets for these

cytokines in joint inflammation are the vessels, symposium, cartilage and bone (Table 1.2). The net result of activation of the cytokine system results in new blood vessel formation and infiltration of the symposium by inflammatory cells that contribute to the development of sinusitis and the subsequent bone and cartilage destruction.

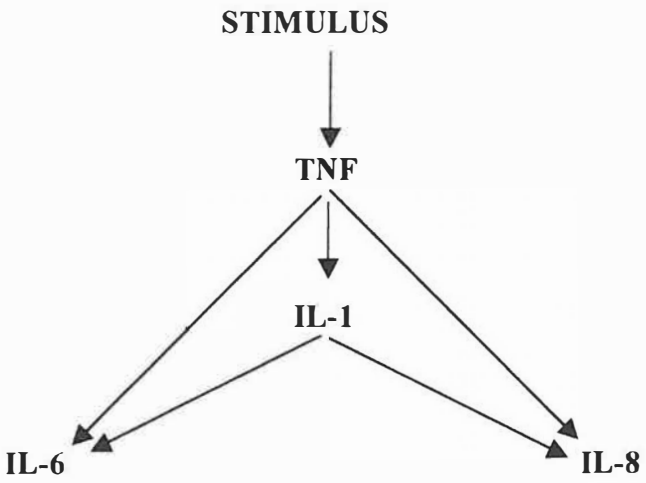


Figure 1.10 Interaction of the pro-inflammatory cytokines

TABLE 1.2 PROINFLAMMATORY CYTOKINES IN RHEUMATOID ARTHRITIS

Cytokine	Source	Actions
IL- 1	macrophages, fibroblasts synovial lining cells T and B lymphocytes endothelial cells	• recruits cells to site of inflammation • stimulates production of IL-6 & TNF∝ • augments T cell proliferation & B cell activation • induces hepatic production of acute phase proteins • activates neutrophils to synthesise and release PGs • increases binding of lymphocytes and monocytes to endothelial cells • induces endothelial cell proliferation
TNF	macrophages, monocytes	• stimulates production of IL-1,6 and 8 • enhances PG-dependent bone resorption • inhibits collagen synthesis • increases PGE_2 and collagenase production • increases plasminogen activity • increases release of FGF • modulates PMN function such as the release of oxygen metabolites, phagocytosis, adhesion to endothelium and the ability to degrade cartilage
IL-6	neutrophils, monocytes, fibroblasts, T and B cells endothelial cells	• stimulates the release of hepatic acute phase • induces activated B cells to differentiate into plasma cells
IL-8	neutrophils, fibroblasts, hepatocytes, epithelial and endothelial cells	• stimulates and attracts neutrophils
GM-CSF	macrophages, fibroblasts, endothelial cells and activated lymphocytes	• stimulates secretion of IL-1, TNF∝ & PGE_2 • amplifies cartilage destruction by IL-1 & TNF∝ • activates chemotaxis, phagocytosis, antibody cytotoxicity and oxidative metabolism in granulocytes • induces HLA-DR expression on monocytes

ABBREVIATIONS : IL : interleukin; TNF : tumour necrosis factor; PG : prostaglandin; FGF : fibroblast growth factor, GM CSF : granulocyte macrophage colony stimulating factor

Interleukin-1

The IL-1 family consists of IL-1α, IL-1β and IL-1 receptor antagonist (IL-1RA), which are structurally related to each other and have similar affinity for IL-1 receptors on cells (Dinarello, 1994). IL-1α and IL-1β are potent agonists that elicit various biological responses, whereas IL-1RA blocks the effects of the agonists by competing for binding sites on the cell surface receptors (Arend, 1993).

IL-1α, IL-1β and IL-IRA are encoded by separate genes; designated as *ILIA, ILIB, and ILIRN* respectively. The three genes are clustered on the long arm of human chromosome 2 in a region (q13-q21) that spans more than 430 kilobases (Nicklin *et al.* 1994). The human *ILIA* and *ILIB* genes are approximately 10.2 and 7.5 kb in size respectively, and have 7 exons each that encode similar regions on the respective proteins. The *ILIRN* gene contains 8 exons, spanning approximately 17 kb, and gives rise to both secretory IL-IRA (sIL-IRA) and intracellular (icIL-1RA) (Eisenberg *et al.* 1991). Transcription is induced rapidly in response to various stimuli, including bacterial cell wall components, cytokines, BK, immune stimuli and inflammatory mediators, and does not require *de novo* protein synthesis. The mRNAs for IL-1∝ and IL-1β are translated into 31 kDa polypeptides on free polysomes (Fenton, 1992),

IL-1α has an isoelectric point of 5 and is functional in the propeptide state, whereas IL-1β has an isoelectric point of 7 and has to be processed to an active 17 kDa protein by the specific proteinase interleukin converting enzyme (ICE) (Kostura *et al.* 1989). These molecules share 50% homology at the gene level and 26% homology at the protein level.

Three related cell surface proteins are involved in IL-1 binding and signalling; type 1 IL-

1R (receptor), type 2 IL-1R and the IL-1R associated protein (Sims and Dower, 1994). The IL-1R genes are members of the large immunoglobulin supergene family, and are located in the same region of human chromosome 2 as their ligands. Type 1 IL-1R is an 85 kDa glycoprotein consisting of 552 amino acids and is divided into a 319 residue extracellular ligand-binding domain, a 20-residue transmembrane ligand-binding domain, a 20-residue transmembrane-spanning region, and a 213-residue cytoplasmic tail (Tocci and Schmidt, 1997) (Fig. 1.11). The type 1 IL-1R binds all three members of the IL-1 family, but has the greatest affinity for IL-1RA (Sims and Dower, 1994). The type 2 IL-1R is a 68 kDa glycoprotein consisting of 386 amino acids, a 332-residue extracellular domain, a 20-residue transmembrane region and a short 29-residue cytoplasmic tail. Type 2 receptors do not transduce a signal upon IL-1 binding, instead the type 2 IL-1R is shed from the cell surface as a soluble form. The soluble IL-1R (sIL-1R) type 2 is found circulating in the blood of healthy individuals, and its cell surface expression is increased by a variety of inflammatory mediators, and is thought to function as a constitutive natural antagonist (Colotta *et al.* 1993). The IL-1R-associated protein (IL-1RAP) has not been shown to bind IL-1 directly, but increases the affinity of the type 1 receptor for IL-1α and IL-1β approximately three fold.

IL-1 is produced during antigen presentation and is secreted by macrophages, fibroblasts, endothelial cells, synovial lining cells and T and B lymphocytes (Lorenzo, 1991). It has a wide range of biological actions and acts via modulating gene expression in target cells. Both IL-1α and IL-1β possess co-mitogenic properties, recruit cells to the site of inflammation, and stimulate the production of pro-inflammatory mediators, including IL-6 and TNF. IL-1 has been shown to augment T-cell proliferation and B-cell activation in response to antigenic challenge (Dinarello *et al.* 1986), induce hepatic production of acute

phase proteins (Dinarello, 1984), and activate neutrophils to synthesize and release prostaglandins (Rossi *et al.* 1985). It also increases binding of lymphocytes and monocytes to endothelial cells, induces endothelial cell proliferation and neovascularization. IL-1 has been shown to be arthritogenic in animals (Chandrasekhar *et al.* 1990), and causes a transient synovitis when injected directly into the joints. In man, high levels of IL-1 are present in serum and synovial fluid of patients with RA (Arend and Dayer, 1995), and immunoreactive IL-1 has been identified in the synovial lining cells and subintimal mononuclear cells (Brennan *et al.* 1991). On exposure to IL-1 cultured fibroblast-like synoviocytes proliferate, produce metalloproteinases (such as collagenase and stromelysin), and secrete cytokines such as GM- CSF (Alvaro-Gracia *et al.* 1989). In addition IL-1 dramatically increases prostaglandin production (Dayer *et al.* 1986). Interleukin 1 is a potent inducer of bone resorption (Boyce *et al.* 1989). This effect may be mediated through PGE_2, which is known to resorb bone (Klein and Raisz, 1970) or other mechanisms such as the inhibition of collagen synthesis, and the stimulation of plasminogen activator (Boyce *et al.* 1989). In addition, IL-1 is responsible for cartilage destruction (Dinarello, 1994). The increase in secretion of plasminogen activator stimulated by IL-1 converts plasminogen to plasmin, a neutral protease that causes cartilage destruction (Campbell *et al.* 1988). Interleukin-1 also has chemo-attractant properties and increases neutrophil accumulation in joints (Pettipher *et al.* 1986), and has the capacity to increase the lifespan of neutrophils *in vitro* and *in vivo* (Barrett, 1994). Interleukin-1 interacts with cells through its receptor. The specific inhibitor, IL-1 receptor antagonist, inhibits IL-1 related processes *in vitro* and *in vivo* (Dinarello *et al.* 1991). It is thought that lower levels of IL-1 receptor antagonists relative to that of the total production of IL-1 may be responsible for the IL-1 induced effects in RA (Firestein *et al.* 1994a).

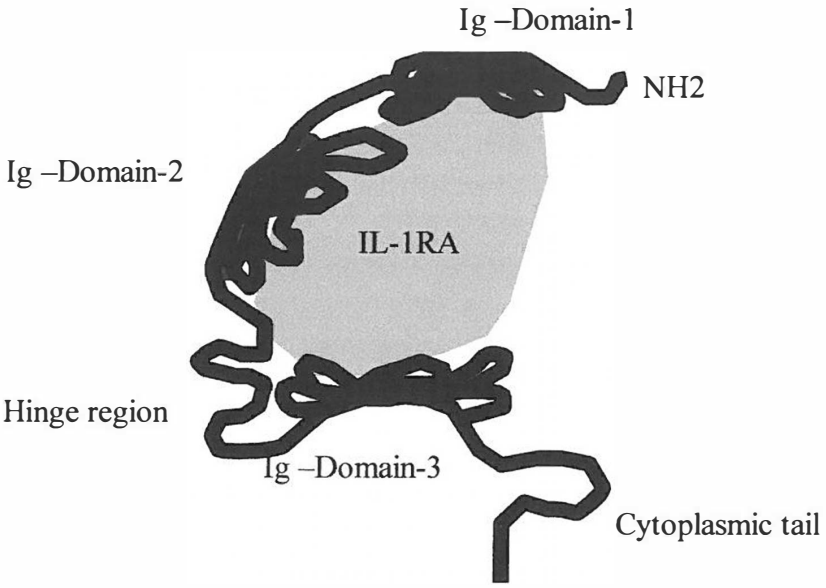


Figure 1.11 Structure of the IL-1 receptor

(COOH, carboxy; Ig, immunoglobulin;IL-1RA, interkeukin 1 receptor antagonist NH_2, amino)

Tumour necrosis factor (TNF)

The human TNF gene is located on the short arm of chromosome 6 close to the HLA locus. The coding region spans approximately 3 kb and includes 4 exons (Nedwin *et al.* 1985). The TNF gene family also includes lymphotoxin-α, which was previously known as TNF β. Human TNF is initially translated as a prohormone consisting of 233 amino acids and is subsequently modified by acylation with myristic acid for insertion into membranes (Stevenson *et al.* 1992). The membrane bound portion of 26 kDa retains biological activity. During activation and induction of TNF secretion, the membrane form is cleaved by a serine protease, to a 17 kDa monomer composed of 157 amino acids and three secreted monomers associate into a biologically active trimer (Smith and Baglioni, 1987).

TNF is produced principally by macrophages and monocytes. The production of TNF is

stimulated by several factors, including LPS, IL-1, GM-CSF and TNF-itself, hypoxia, oxygen radicals and complement activation. TNF mediates its effects by interaction with two related membrane receptors, TNF-R1 and TNF-R2 (or type 1 and type 11) with molecular masses of approximately 55 and 75 kDa (Brockhaus *et al.* 1990) (Fig. 1.12). Signaling occurs when two or three receptors mingle around a single TNF trimer and results in activation of secondary signal cascades involving prostaglandins, leukotrienes, calcium, ceramides, and tyrosine kinases. The cell surface receptors are cleaved by proteolytic enzymes. This results in the down-regulation of the membrane receptors and the formation of soluble receptors (sTNFR), which, by competing for TNF, block its function.

TNFα is thought to be the controlling element in the 'cytokine network' and is responsible for the production of other cytokines for example IL-1, IL-6 and IL-8 (Brennan *et al.* 1992a). The cellular actions of TNFα include a PG-dependent stimulation of bone resorption (Lorenzo *et al.* 1991), inhibition of collagen synthesis (Canalis, 1987), increase in PGE_2 and collagenase production (Dayer *et al.* 1985), increase in plasminogen activity and increased release of fibroblast growth factor from synovial and endothelial cells (Takahashi *et al.* 1991). Several studies have shown that TNFα modulates PMN function such as degranulation, release of oxygen metabolites, phagocytosis, adhesion to the endothelium and the ability of neutrophils to degrade cartilage in vitro (Kowanko *et al.* 1990). The TNFα molecule binds with both the p55 and p75 receptors, both of which are upregulated in RA synovial tissue (Brennan *et al.* 1992b). TNFα -containing cells have been localised to the cartilage-pannus junction (Chu *et al.* 1991), suggesting production near the site of tissue destruction. In addition, increased levels of soluble TNF receptors in synovial fluid compared to serum have been reported in RA patients (Cope *et al.* 1992),

supporting local production of these molecules. High concentrations of TNF are present in serum and synovium of patients with RA (Tetta *et al.* 1990, Manicourt *et al.* 1993) and RA patients with detectable levels of TNFα have a higher erythrocyte sedimentation rate and synovial fluid leucocyte count (Saxne *et al.* 1988) suggesting that high TNFα activity is associated with inflammation that is more active.

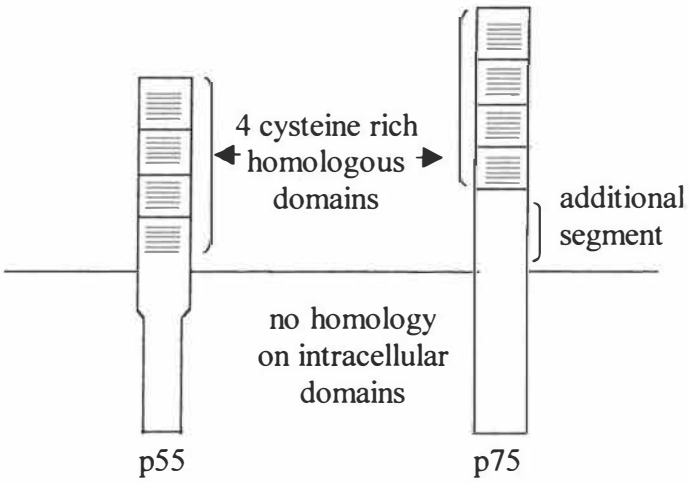


Figure 1.12 Schematic representation of the tumour necrosis factor receptors

Interleukin 6

IL-6 is a glycoprotein produced by most nucleated cells, including macrophages, monocytes, fibroblasts, T and B cells, and endothelial cells (Mizel, 1989). The human IL-6 gene, located on chromosome 7p21 (Bowcock *et al.* 1988), is approximately 5 kb in length and consists of 5 exons and 4 introns (Yasukawa *et al.* 1987). The gene encodes for a 212 amino acid protein that is cleaved to a mature protein of 183-185 amino acids and a molecular weight of 21 kDa (Van Snick, 1990). Gene expression is upregulated by IL-1, TNF, platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and lipopolysaccharide (Akira *et al.* 1990; Northemann *et al.* 1989). Treatment with corticosteroids, on the other hand, inhibits the expression of the IL-6 gene (Tobler *et al.*

1992). The effects of IL-6 are mediated via the IL-6 receptor, which is a 80 kDa glycoprotein with structural homology to immunoglobulin supergene family, is specific for IL-6 and is present on monocytes, hepatocytes and T and B lymphocytes. This can be shed from the cell surface and can be found in a soluble form in biological fluids and cell supernatants. IL-6 binds to either the soluble or membrane bound receptors to form a complex which is then associated with a transmembrane 130 kDa glycoprotein required for signal transduction (Cox and Gauldie, 1997).

IL-6 is produced by monocytes, T lymphocytes and fibroblasts, and the synthesis of this cytokine is induced by ILI and TNFα (Wong and Clark, 1988). IL-6 stimulates the release of acute phase proteins from the liver (Gauldie *et al.* 1987), and induces activated B cells to differentiate into plasma cells which produce immunoglobulins (Arend and Dayer, 1990). This mechanism may account for the production of rheumatoid factor. High levels of IL-6 have been detected in the synovial fluid of patients with RA (Van Leeuwen *et al.* 1995) and the level of IL-6 has been shown to correlate with disease activity (Waage *et al.* 1989). In addition, synovial cells in culture have been shown to spontaneously produce IL-6. Further, this production is augmented by IL-1 and TNF (Guerne *et al.* 1989). In contrast, IL-4 suppresses IL-6 production (Suzuki *et al.* 1993).

Interleukin 8

IL-8 is a member of the chemokine supergene family. The chemokines belong to two related polypeptide families, C-X-C and CC chemokines, as defined by the location of the two cysteine residues at the amino terminus. In the C-X-C family, the cysteine residues are separated by a non-conserved amino acid and, in the CC family the cysteine residues are in juxtaposition (Baggiolini *et al.* 1994). The C-X-C chemokines are clustered on

human chromosome 4. IL-8 is an 8 kDa peptide of the C-X-C chemokine family. It is a potent neutrophil attractant and stimulator, and is produced by neutrophils, fibroblasts, hepatocytes, epithelial and endothelial cells (Baggiolini *et al.* 1989). Interleukin-1, TNF, and LPS stimulated neutrophils exhibit increased expression of IL-8 mRNA and IL-8 production (Strieter *et al.* 1992). Other chemotactic factors such as, NfMLP, C5a, and platelet activating factor also increase IL-8 production by neutrophils and together these factors account for the influx of large numbers PMN leucocytes into the synovial cavity in an autocrine manner. The activity of IL-8 is mediated by its binding to high affinity receptors present on neutrophils. These are G-protein coupled receptors with 7 transmembrane regions (Baggiolini *et al.* 1994). The expression of the receptors is down regulated by IL-8, GM-CSF and TNFα (DeMarco and Zurier, 1997). High levels of IL-8 have been demonstrated in the synovial fluid of patients with RA (Brennan *et al.* 1990) and gout (Terkletaub *et al.* 1991). Human synovial cells stimulated by IL-1 have also been shown to express IL-8 in RNA (DeMarco *et al.* 1991) and immunoreactive IL-8 has been localised in the sublining macrophages and in scattered lining cells (Koch *et al.* 1991).

Granulocyte-macrophage colony stimulating factor (GM CSF)

GM-CSF is a predominant growth factor that may be synthesized by macrophages, fibroblasts, endothelial cells and activated lymphocytes (Groopamn *et al.* 1989). The gene for GM-CSF is located on the long arm of chromosome 5 (Wong *et al.* 1985), and encodes for a glycoprotein consisting of 174 amino acids with a molecular weight of 18.6 kDa. Although it was first characterized based on promotion of growth and differentiation granulocytes and macrophages, it also has several pro-inflammatory properties and may be involved in the pathogenesis of RA. GM-CSF stimulates the secretion of IL-1 and enhances the secretion of TNFα and prostaglandin E_2 (PGE_2) from macrophages (Fischer

et al. 1988; Heidenreich *et al.* 1989), and amplifies cartilage destruction by IL-1 and TNF (Alvaro–Gracia *et al.* 1989). In addition, GM-CSF activates chemotaxis, phagocytosis, antibody dependent cytotoxicity and oxidative metabolism in granulocytes (Firestein, 1994), and induces HLA-DR expression on monocytes (Xu *et al.* 1989). Furthermore, IL-1 and TNFα have been shown to induce GM-CSF production in the synovium and antibodies to TNFα to decrease its production (Haworth *et al.* 1991). Significant levels have been demonstrated in rheumatoid synovial fluid and rheumatoid synovial explants have been shown to produce GM-CSF *in vitro* (Xu *et al.* 1989).

1.2.3.2.3 Role of immune and inflammation signalling cells

As the initiating event antigen is engulfed by the macrophage or antigen presenting cell, processed and then expressed on the surface of the cell in the groove of Class II major histocompatibility complex (MHC) molecules, where it interacts with specific receptors on the T cells (Panayi, 1992). The macrophage releases a number of cytokines, IL-1, TNFα, IL-6, colony stimulating factors, coagulation factors, lysozomal enzymes, plasminogen activator, collagenases, leucotriene B4 (LTB4), prostaglandin E2 (PGE2), and oxygen free radicals which are necessary for the activation of T cells. The activated T cells then proliferate as helper cells, generate cytotoxic T cells and induce B cells to proliferate and differentiate to antibody producing plasma cells. In addition, the activated T cells stimulate the production of a variety of cytokines and other inflammatory mediators, which are responsible for the synovial proliferation and joint damage. While the T cells are implicated in the initiation of the inflammatory process a number of other cells such as the macrophages, neutrophils, fibroblasts, endothelial cells and chondrocytes participate in the chronic disease process, and may be responsible for the perpetuation of the synovitis (Figs. 1.13 and 1.14).

Lymphocytes

Following recognition of foreign antigen, T cells proliferate into either CD4 or CD8 cells. The CD4 cells stimulate B cell proliferation via the secretion of IL2, IL-4 and IL5, enhance cytotoxic T cells, and induce the expression of MHC antigens, IL-3 and GM-CSF. A large number of lymphocytes are found in the synovium. Following the release of B cell growth factor and B cell differentiating factor by activated T cells, B cell proliferate and differentiate into plasma cells which produce immunoglobulins and rheumatoid factors. Synovial plasma cells have been shown to produce RF (Milgrom, 1988), and deposits of IgM and IgG RF are found in the cartilage matrix from patients with RA, but not in normal or osteoarthritic cartilage (Jasin, 1985). Rheumatoid factors play a role in perpetuating and amplifying the immune response by their ability to activate the complement cascade, initiate local damage and act as antigen presenting cells.

Neutrophils

Neutrophils infiltrate the diseased joints in huge numbers and can exceed 100,000/mm^3, and have the potential to cause tissue damage. This may result either from the release of granule contents and or by the generation of reactive oxygen species involving nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidase, present on their surface. The neutrophil granules contain several potent proteolytic enzymes and hydrolases that may induce tissue damage (Spitznagel, 1984). These include elastase, cathepsin G, collagenase, gelatinase, glucuronidase, N-acetyl glucosaminidase, acid phosphatase, phospholipase A2 and tissue kallikrein. Other constituents include myeloperoxidase, lysozome, C5 cleaving enzyme, plasminogen activator, cationic proteins and lactoferrins. A second mechanism by which neutrophils are capable of tissue injury is through the generation of reactive oxygen metabolites such as hydrogen peroxide and

hydrochlorous acid (Elsbach and Weiss, 1988).

Normally, neutrophils circulate in a non-activated state and do not attach to endothelial cells, whereas activated neutrophils have an increased adhesiveness, motility, chemotaxis, phagocytosis, degranulation and activation of surface NADPH oxidase. The neutrophil surface contains a variety of receptors including those for complement chemotactic peptide (C5a), leucotriene B4 (LTB4) (Sherman *et al.* 1988), platelet activating factor (PAF) (Ng and Wong, 1986) and kallikreins and kininogens (Haasemann *et al.* 1994). Following receptor binding there is activation through, a guanine-nucleotide-binding (G) protein of phospholipase A2 that cleaves phosphoinositide to generate inositol triphosphate (IP3) and diacyglycerol (DAG), both of which function as intracellular second messengers. DAG activates protein kinase C and IP3 increases intracellular calcium through release from stores. These actions facilitate phosphorylation of several intracellular proteins and subsequent alteration in cell shape and function. In addition, neutrophils can be activated by several other factors including IL-8, GM-CSF, immune complexes and plasma kallikrein. Activated neutrophils have the ability to degrade cartilage in vitro (Kowanko *et al.* 1989) and this ability is increased by TNFα (Kowanko *et al.* 1990). TNFα also modulates other neutrophil function such as degranulation, release of oxygen metabolites (Shalaby *et al.* 1987), phagocytosis and adhesion to the endothelium (Gamble *et al.* 1986). Activated neutrophils attach firmly to and damage the endothelial cells following oxidant generation. The activated neutrophils induce phospholipase A2 to release arachidonic acid metabolites from membrane phospholipids to produce prostaglandins and leucotrienes (Fig. 1.14). Kinins, by activating phospholipase A augment this process (Sharma, 1988). Prostaglandin E_2 (PGE_2) and PGF_2 induce oedema, vasodilatation and increase sensitisation of pain in response to bradykinin. Leucotrine B is a chemo-attractant, and

attracts quiescent neutrophils to the site of activation (Pillinger and Abramson, 1995). In addition, neutrophils secrete a number of cytokines including IL-8, which may play an autocrine role in attracting further neutrophils to the synovial fluid, thus perpetuating the inflammatory process. Similarly, the endothelial cell can also be activated to facilitate interactions with neutrophils and other lymphocytes.

Macrophages and mast cells

Macrophages are thought to play an important role both in the initiation of RA, as antigen presenting cells, and in chronic inflammation (Burmester *et al.* 1997). A large number of activated macrophages are found in rheumatoid synovium and within the destructive pannus tissue. The macrophage infiltration in the synovium correlates with both disease activity (Soden *et al.* 1991), and radiological progression.(Mulherin *et al.* 1996). Macrophages produce large amounts of prostaglandins (Seitz and Hunstein, 1985), proinflammatory cytokines (Hahn *et al.* 1993; Firestein *et al.* 1990), and the proteolytic enzymes stromelysin, gelatinase B and elastase (Burmester *et al.* 1997).

A small number of mast cells are present in the normal synovium. However, in the inflamed synovium, there is an increase in the number of mast cells in the synovial fluid as well as at the cartilage-pannus junction (Tetlow and Woolley, 1995a; 1995b). An increase in mast cells has been reported with flares in disease activity. In contrast, corticosteroid therapy and clinical improvement is associated with a decrease (Malone *et al.* 1987). Mast cell granules contain a number of mediators, including tryptase, chymase and histamine. Tryptase has been detected in the synovial fluid of patients with arthritis (Buckley *et al.* 1997). In addition, mast cells have the ability to generate LTB4 from membrane phospholipids and secrete a number of cytokines such as IL-1, TNFα, GM-CSF, IL-8 and

IL-6. Tryptase has also been shown to induce the release of bradykinin either through the activation of prekallikrein or direct release from kininogen (Imamura *et al.* 1996). This process is enhanced by neutrophil elastase (Kozik *et al.* 1998). Thus the mast cells are thought to play a role in angiogenesis, fibroblast proliferation and neutrophil chemotaxis (Austen KF, 1994).

Endothelial cells

One of the earliest changes in the inflamed synovium is endothelial cell damage (Koch, 1998). While the primary cause of the endothelial cell damage is not known, it is believed to result from the activity of cytokines, complement activation components, arachidonic acid metabolites, histamine, serotonin and other inflammatory mediators. In inflammation, the microvasculature undergoes two distinct morphological changes. Firstly, there is an increase in high endothelial venule (HEV) formation that facilitates lymphocyte trafficking to the synovium and T cells accumulate in a perivascular distribution. Secondly, there is new blood vessel formation or angiogenesis that may contribute to cartilage destruction (Koch, 1998). Angiogenesis is stimulated by a variety of cells, such as macrophages, lymphocytes, PMN leucocytes, mast cells and fibroblasts as well as by various soluble molecules, including transforming growth factor α, TNF-α, heparin, PGE2 and platelet activating factor. Kinin receptors have been identified on the endothelial cells of elastic arteries and veins, as well on the endothelial cells of muscular arteries and arterioles (Raidoo *et al.* 1997). These findings suggest that kinins may modulate vascular permeability.

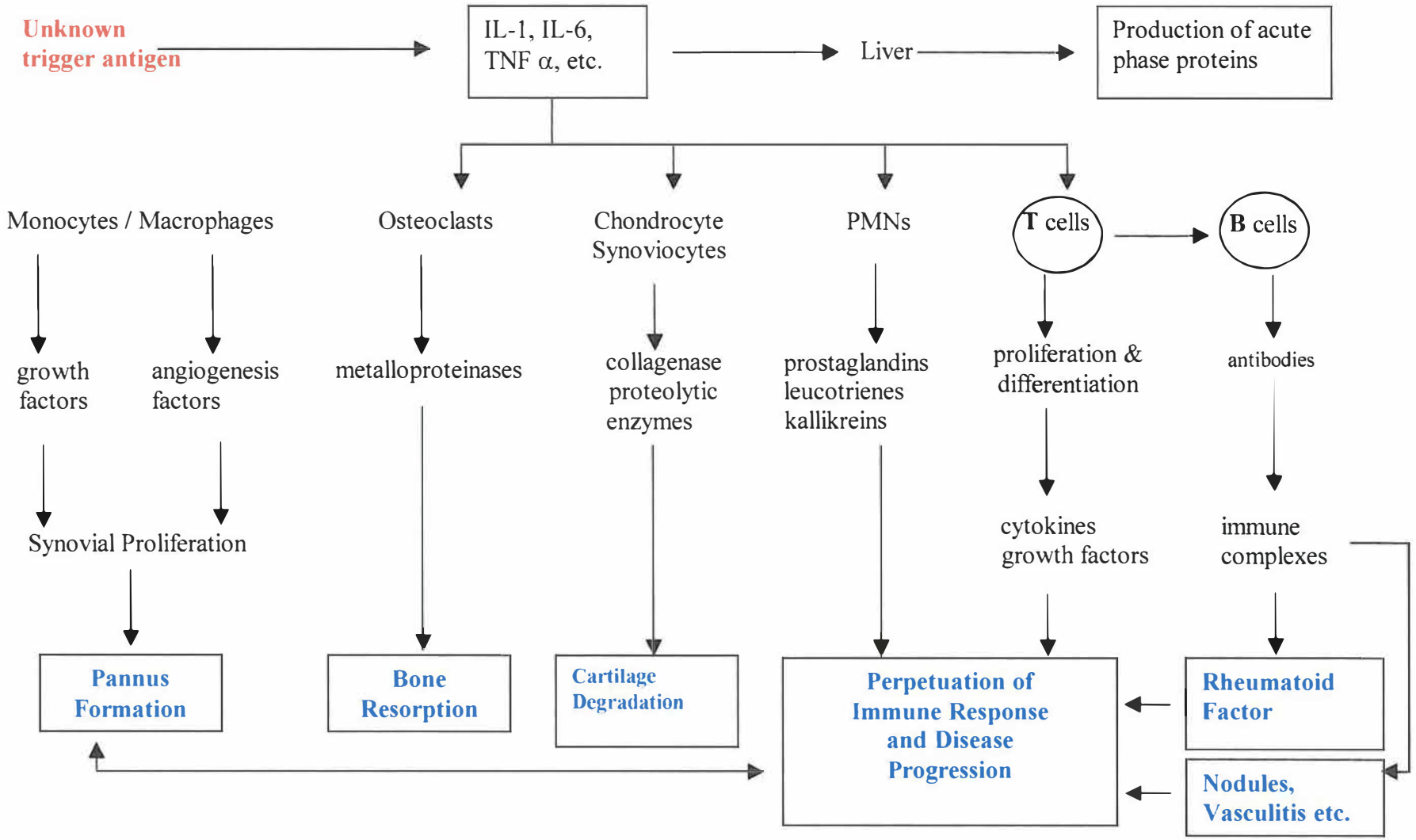


Figure 1.13 Role of inflammatory cells in pathogenesis of rheumatoid arthritis.

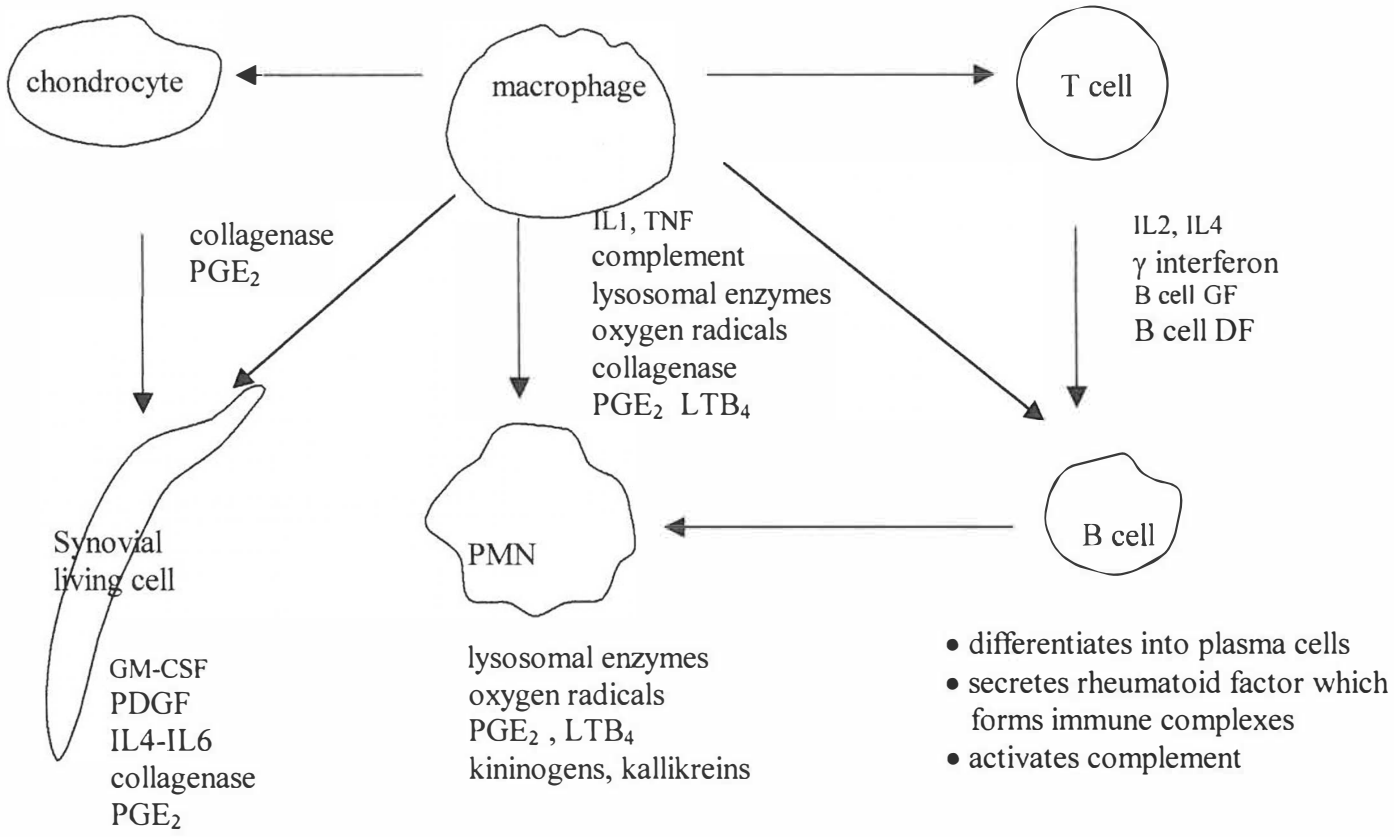


Figure 1.14 Cellular interactions in the pathogenesis of rheumatoid arthritis

(DF, differentiation factor; GF, growth factor; GM CSF, granulocyte macrophage colony stimulating factor; IL, interleukin; LTB4, leucotriene B4; PGE_2 , prostaglandin E_2; TNF, tumour necrosis factor)

1.2.3.3 Secondary mediators of synovitis

Complement proteins

Complement proteins may cause direct damage to the cell membrane or cytokine-like effects, for example C5a is a neutrophil chemotactic factor. Complement proteins are mainly produced in the liver, but may also be produced locally by the synovial macrophages and/or fibroblasts (Littman *et al.* 1989; Katz and Strunk, 1988) and induced by cytokines such as IL-1 and TNF (Perlmutter *et al.* 1986). Activation of the complement pathway is initiated when immune complexes activate C1 with subsequent cleavage of C4 and C2 culminating in the activation of C3 that binds to the cell surface and facilitates phagocytosis. Alternatively, activated C3 may activate the remainder of the complement pathway by cleavage of C5 leading to the formation of the membrane attack complex by sequential addition of C6, C7, C8 and C9 (Fig. 1.15). This complex is inserted into cell membranes, and produces cell lysis. C3a is a potent vasodilator, whereas C5a is chemotactic for neutrophils and triggers neutrophil oxidative burst, promotes endothelial adherence and stimulates LTB4 synthesis. Both C3a and C5a cause histamine release from mast cells.

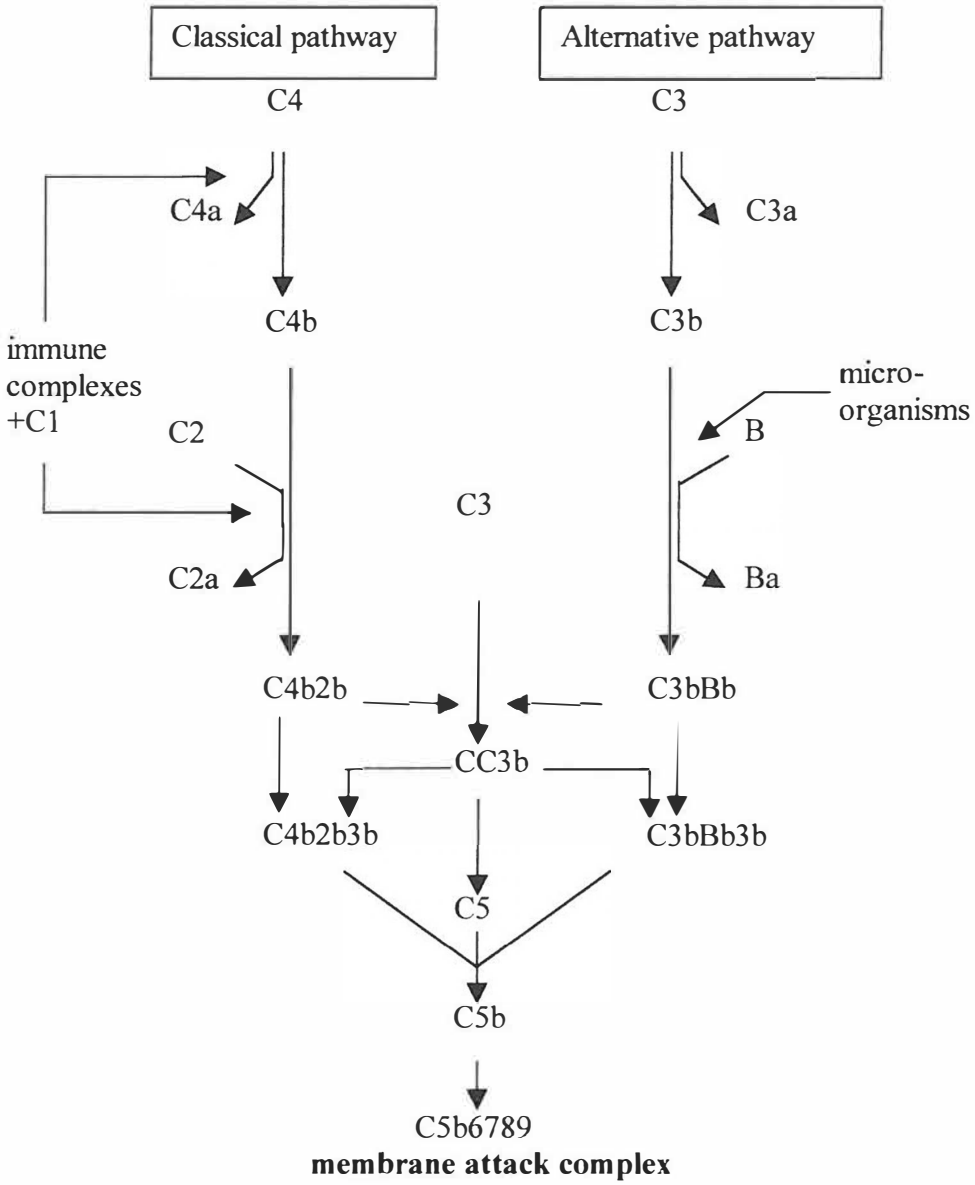


Figure: 1.15 Complement pathways

Arachidonic acid metabolites

Activation of phospholipase A2 cleaves arachidonic acid from membrane phospholipids. Further metabolism via the cyclo-oxygenase pathway results in the production of prostaglandins or leucotrienes via the lipo-oxygenase pathways (Fig. 1.16). Prostaglandins are potent vasodilators that act synergistically with other mediators including, complement fragments, histamine and kinins to increase vascular permeability. In addition, PGE_2 and PGI_2 stimulate osteoclastic bone resorption. Leucotriene B4 (LTB_4) is a chemo-attractant for leucocytes and promotes the adherence of leucocytes to endothelial cells. It also activates the secretion of reactive oxygen species and degradative enzymes from neutrophils. Interleukin-1 and TNFα increase the production of PG from synoviocytes *in vitro* (Rossi *et al.* 1985; Dayer *et al.* 1985).

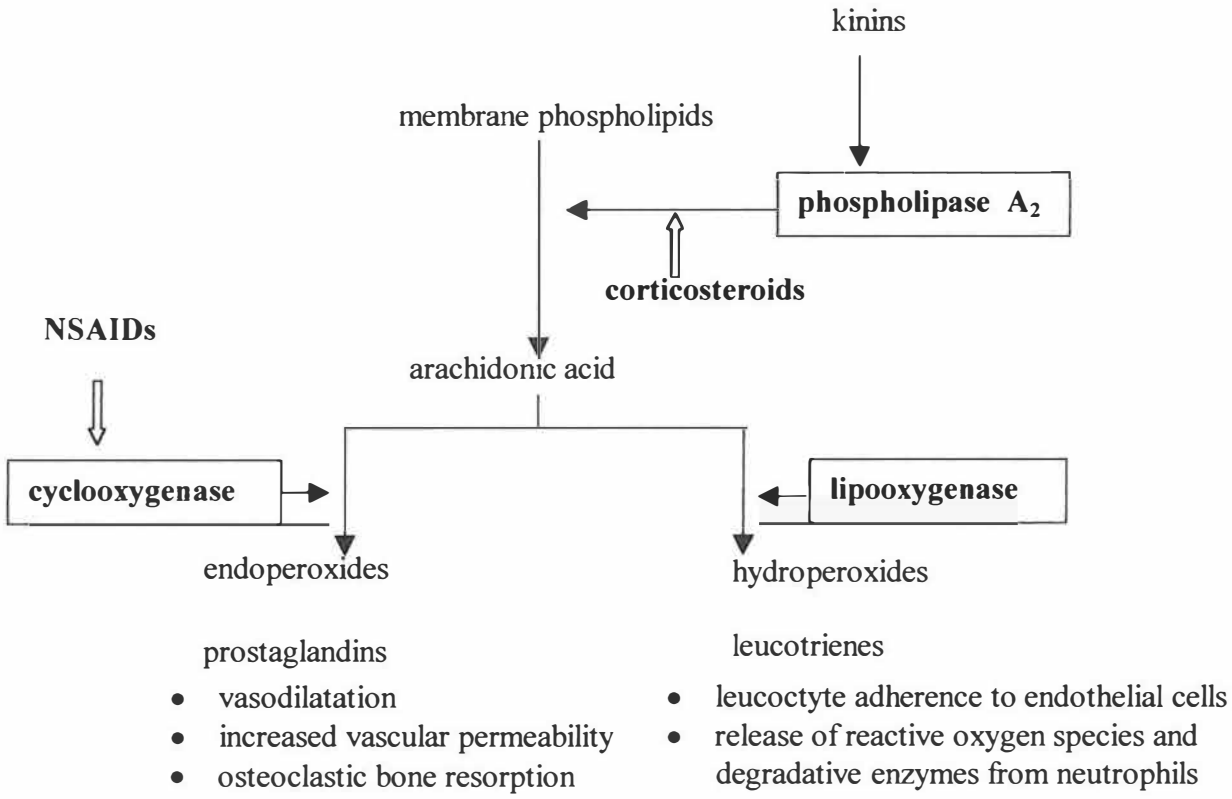


Figure 1.16 Arachidonic acid metabolism

Neuropeptides

Peptide mediators produced by neurons (neuropeptides) such as substance P and met-enkephalin also play a role in the pathogenesis of RA.

Substance P, an 11 amino-acid peptide, is a member of a structurally related family of peptides called tachykinins. Mammalian tachykinins are encoded by two distinct genes; the pre-protachy-kinin (PPT)-A and PPT-B genes. Substance P is encoded by mRNAs arising from the PPT-A gene (Schaffer *et al.* 1998) and is synthesised in the cell bodies of the dorsal root ganglion. It is thereafter distributed to central and peripheral nerve terminals by a fast axonal transport system. The tachykinins mediate their actions by activating the neurokinin (NK) receptors, NK1, NK2 and NK3. All three receptors are G-protein coupled receptors that, on stimulation activate phospholipase C and thus lead to the generation of inositol triphosphate and diacyl-glycerol. Substance P is able to induce vasodilatation because of direct action on vascular smooth muscle and enhanced production of nitric oxide by the endothelium (Schaffer *et al.* 1998). In addition it increases histamine release from mast cells, and causes activation of macrophages and neutrophils by stimulating the release of PG and lysozomal enzymes. Substance P plays an important role in synovitis because of its ability to increase monocyte production of cytokines such as IL-1 and IL-6 and to stimulate fibroblasts-like synoviocytes to proliferate and produce collagenase (Lotz *et al.* 1987).

Met-enkephalin is a pentapeptide that binds to opiate receptors. It is synthesized in the adrenal gland as a large precursor molecule, proenkephalin. In addition, human T cells and macrophages have also been shown to produce met-enkephalin (Plotnikoff *et al.* 1997).

Three opiod receptors have been identified, μ, κ, and δ. Met-enkephalin binds with high affinity to δ receptor. Receptors for met-enkephalin have been reported on human T lymphocytes and phagocytic leukocytes. Met-enkephalin enhances B cell antibody production and macrophage oxygen radical generation. It is also a macrophage and lymphocyte chemotactic factor. Immunoreactive met-enkephalin has been detected in synovial fluid.

Vasoactive amines and nucleotides: Histamine, serotonin and adenosine are low molecular amines, which are important inflammatory mediators. Histamine, a decarboxylation product of histidine, is stored in mast cells and released on mast cell activation. Its biological effects include vasodilatation and enhanced permeability of postcapillary venules. Serotonin, stored in the dense bodies of platelets, enhances microvascular permeability and fibrosis by promoting collagen synthesis by fibroblasts. Adenosine, a nucleotide generate during mast cell activation, enhances the phagocytosis of immune complexes and superoxide formation by neutrophils at low concentrations.

Effector molecules: enzymes causing cellular damage: Effector molecules differ from cytokines and growth factors in that they have the ability to alter the environment directly without the need to bind to cell surface receptors. Of these metalloproteinases (MMPS) are matrix degrading enzymes, which participate in acute inflammation, and include collagenase, stromelysin and gelatinase (Woessner, 1991). They are proenzymes that require proteolytic cleavage for activation. *In vitro* this proteolysis has been shown to be accomplished by many enzymes, including trypsin, plasmin, tryptase and other proteases. Collagenase degrades proteoglycan when in a triple helical structure, whereas gelatinase

degrades denatured collagen and stromelysin degrades proteoglycan and is able to cleave and activate collagenase. Collagenase and stromelysin are both produced by synovial lining cells, and their production is induced by IL-1 and TNFα (Walakovits *et al;* 1992, Dayer *et al.* 1986). High levels of MMPS have been noted in the serum and synovial fluid of patients with RA (Clark *et al.* 1993; Manicourt *et al.* 1995). The activity of MMPs is inhibited by tissue inhibitors of metalloproteinases (TIMP); natural inhibitors produced locally by chondrocytes and fibroblasts (Vincent *et al.* 1994). TNFα significantly inhibits TIMP production in endothelial cells, synoviocytes and chondrocytes (Shingu *et al.* 1993.)

1.2.4 Therapeutic management of RA

The exact aetiology of RA remains elusive thus curative treatment has not been discovered. The goals of treatment have been to relieve pain and decrease joint inflammation, prevent joint destruction, restore function of disabled joints, correct deformities and maintain quality of life. To achieve this a multidisciplinary approach is required.

Medical management:

Three main classes of drugs have been used to treat RA: -

1) Non steroidal anti-inflammatory drugs (NSAIDs)
2) Corticosteroids
3) Disease–modifying anti-rheumatic drugs (DMARDs)

NSAIDs reduce pain and inflammation by inhibiting the action of cyclo-oxygenase (COX) and therefore decrease the production of the vasoactive prostaglandins and leucotrienes. However, NSAIDs do not significantly alter the course of disease. Recently more specific NSAIDs, the COX_2 inhibitors have been developed. This class may have less

gastrointestinal toxicity.

Corticosteroids have immunosuppressive and anti-inflammatory effects. Their main effect is through the increased synthesis of lipocortin, which inhibits the enzyme phospholipase A_2, thereby reducing the production of PGs and LTs. (Fig. 1.16). Corticosteroids also inhibit pro-inflammatory cytokine production, including that of IL-1 and TNF and the synthesis of a variety of pro-inflammatory enzymes, including collagenase, elastase and plasminogen activator (Kirwan, 1994). Although systemic steroids provide effective symptomatic relief, substantial toxicity with long-term use precludes the use of high dose oral steroids on a long-term basis (Saag, 1997). However, recent studies have suggested that the early use of low steroids may significantly retard bone and joint damage (Kirwan, 1995). The local use of corticosteroid preparations, normally intra-articular injections of long-acting steroids is well established.

Disease modifying anti rheumatic drugs or "remittive" therapy are thought to retard the progression of the disease. These include gold, sulphasalazine, penicillamine, hydroxychloroquine, methotrexate (MTX) and other cytotoxics. These drugs provide symptomatic and clinical improvement and have been shown to retard radiological progression (Sharp *et al.* 2000), however there is insufficient evidence that they alter long-term outcome. Their onset of action is usually delayed for weeks to months. The mechanism of action of this class of drugs is not entirely known, and their effect is variable. Several studies have suggested that these drugs may extend their benefit via their effects on cytokines. Methotrexate inhibits IL-1 activity and production (Chang *et al.* 1992), and has been shown to decrease the concentrations of TNFα receptors with an associated clinical improvement (Barrera *et al.* 1993), to decrease synovial TNFα

production (Firestein *et al.* 1994b) and to suppress the synthesis of IL-6 and the subsequent generation of reactive oxygen species in synoviocytes (Sung *et al.* 2000). In addition, MTX has also been shown to decrease the number of synovial fluid neutrophils by inhibiting neutrophil migration (Kraan *et al.* 2000). *Sulphasalazine* treatment has been associated with a fall in serum IL-1 levels (Danis *et al.* 1992). Cyclosporin A suppresses IL-1 secretion (Cranney and Tugwell, 1998) and significantly reduces serum IL-6 levels in RA patients (Crilly *et al.* 1995). Most of these drugs have serious potential side effects and require long-term monitoring. Recently, a new class of drug, leflunomide, has been developed as a DMARD in the treatment of RA. Leflunomide acts by inhibiting *de novo* pyridimidine synthesis and is thought to be superior to placebo and comparable to MTX and sulphasalazine in efficacy (Sharp *et al.* 2000). Like MTX, Leflunomide has also been shown to decrease neutrophil migration (Kraan *et al.* 2000).

Immunotherapy: With the advances in the understanding of the pathogenesis of RA, several newer therapeutic agents have been developed. These include agents that target cell surface receptors and cytokine function, such as recombinant IL-1 receptor antagonist (IL-1RA) and monoclonal antibodies directed against TNFα. Recombinant IL-1RA has been shown to decrease the number of tender joints and serological indices of inflammation, in clinical trials in RA patients (Bresnihan and Cunnane, 1998). The monoclonal antibodies against TNFα appear to be the most promising in view of their integral role in the pathogenesis of RA. Treatment with both a recombinant TNF receptor (p75)-Fc fusion protein and a chimeric TNF monoclonal antibody (CA2) has been associated with significant clinical and laboratory improvement (Keystone, 1999). The addition of anti-TNFα monoclonal antibody to MTX in patients with persistent activity has been found to be safe and effective in reducing the inflammation (Kavanaugh *et al.* 2000).

1.3 THE ROLE OF KININ PEPTIDES IN INFLAMMATORY JOINT DISEASE

Kinins are potent vasoactive peptides, with the cellular actions of stimulation of nociceptive C fibres, extravasation of vascular fluid, release of neurotransmitters and stimulation of the release of second messengers including cytokines, prostaglandins and leucotrienes. Kinins are potent pain producing substances when applied to a blister base (Bhoola *et al.* 1961) or when injected intradermally (Ferreira, 1972). In addition, they increase capillary permeability (Bhoola *et al.* 1960, Imamura *et al.* 1984). Various components of the kinin system have been identified in synovial fluid and synovium, thereby supporting their role in inflammatory joint disease. The first isolation of a pain producing substance, resembling BK was reported in synovial fluid of patients with RA (Armstrong *et al.* 1957). Subsequently elevated kinin levels were reported in acute gouty arthritis (Eisen, 1966; Melmon *et al.* 1967). In a later study, Hargreaves *et al* (1988) compared circulating levels of immunoreactive BK in patients with RA with volunteer subjects. Higher levels of immunoreactive BK were found to be present in the patients with RA. In the same study the addition of soybean trypsin inhibitor (a serine protease inhibitor of PK) blocked the formation of BK *in vitro* in a rat model, and produced analgesia. In addition, both BK and kallidin appear to enhance bone resorption via prostaglandin formation (Lerner *et al.* 1987; Gustafson *et al.* 1998). These studies support the role of kinins as mediators of the inflammatory response and joint damage in inflammatory arthritis. Additionally, in patients with RA, elevated KI-CPN levels have been reported in the blood and synovial fluid (Chercuiffe *et al.* 1987). Increased expression of KII-NEP 24.11 has been reported on the cell membranes of circulating neutrophils (Connelly *et al.* 1985). Activation of the neutrophils or antigen-antibody reactions triggers the internalization of KII-NEP 24.11, with a rapid loss of its enzymic activity. Since KII-NEP 24.11, hydrolyses N-fMLP (a chemotactic factor), this could have

a marked effect on neutrophil migration to sites of inflammation. KII-NEP 24.11 has also been identified on human synovial fibroblasts suggesting that it may play a role in regulating peptide levels in the joint (Bathon *et al.* 1992b).

Plasma kallikrein was first implicated in gout (Kellermeyer and Beckenridge, 1965) and later in RA (Jasani *et al.* 1969). PK has been identified in the synovial fluid from patients with RA (Rahman *et al.* 1995). Higher activity has been reported in synovial fluid from patients with RA compared to OA (Suzuki *et al.* 1987), and in the synovial fluid compared to plasma of patients with RA (Volpe-Junior *et al.* 1996). In addition, PK has been shown to be chemotactic for PMN leucocytes (Kaplan *et al.* 1972), and to induce aggregation of PMN leucocytes (Schapira *et al.* 1982). It also, primes neutrophils for superoxide production in the joint and converts latent collagenase to its active form *in vitro* (Nagase *et al.* 1982). These findings suggest that PK plays a role in neutrophil mediated tissue damage in arthritis. This is further supported by studies of the effects of PK inhibitors on synovitis. The intra-peritoneal injection of PKS-527, a new selective PK inhibitor, suppressed collagen-induced arthritis in mice and reduced levels of PK (Fujimori *et al.* 1993). In another study, pretreatment with a specific PK inhibitor modulated peptidoglycan polysaccharide induced synovitis and joint erosions in the genetically susceptible Lewis rats (Colman *et al.* 1997).

Tissue kallikrein has also been described in inflammatory exudates, and in human and animal synovial biopsy specimens (Thomas and Zietlin; 1983, Al- Haboubi, 1986; Selwyn *et al.* 1989). Active enzyme levels were reported to be higher in patients with RA compared to OA (Selwyn *et al.* 1989). Additionally, TK has been identified in synovial fluid from arthritic joints (Worthy *et al.* 1990; Bhoola and Dieppe, 1991) and in circulating

and synovial fluid neutrophils (Figueroa *et al*; 1989, Rahman *et al.* 1994). In a study comparing PMNs leucocytes from synovial fluid of patients with RA to blood PMN leucocytes from healthy volunteers, there was reduced TK immunoreactivity on the SF neutrophils (Williams *et al.* 1997). This suggests that TK is released from the granules of RA synovial fluid PMNs and cleaves the kinin moiety from the kininogen molecule on the surface of the PMNs. TK also processes a variety of substrates *in vivo*, including the matrix pro-proteinases, procollagenase and progelatinase, which degrade collagen (Eeckhout and Vaes, 1977; Tschesche *et al.* 1989; Menashi *et al.* 1994). In addition, saliva of patients with connective tissue diseases including RA has been shown to contain increased levels of immunoreactive TK (Greaves *et al.* 1989).

Both HK and LK have been reported in synovial fluid (Jasani *et al.* 1969). The presence of HK, LK, and specific high affinity reversible binding sites for HK on neutrophils (Figueroa *et al.* 1992; Gustafson, 1989), together with the accumulation of large numbers of neutrophils in the synovial fluid and synovial membranes (Dularay *et al.* 1990) provides strong circumstantial evidence for the role of kininogens as a substrate for kininogenases in arthritis.

Kinin B2 receptors have been identified on synovial tissue by autoradiography (Bathon *et al.* 1992a) and Scatchard analysis (Uhl *et al.* 1992). A higher density of receptors was seen in RA compared to OA (Bathon *et al.* 1992a), and treatment with IL-1 enhanced the number of binding sites. The B2 receptor antagonist, HOE 140, has been shown to reduce BK induced plasma extravasation in rat arthritic joints (Cruwys *et al.* 1994), and to suppress knee swelling in adjuvant induced arthritis in rats (Sharma and Wirth, 1996). The B1 agonist, desArg^9 BK induced slight but significant plasma extravasation, which was

inhibited by desArg^{9} (Leu^{8}) BK, the B1 receptor antagonist (Cruwys *et al.* 1994). The authors suggest that while most of the inflammatory responses are mediated by B2 receptors; a small number of B1 receptors may be present in normal joints.

1.4 INTER-RELATIONSHIP BETWEEN THE CYTOKINE AND KININ CASCADES

Both the cytokines and kinins are potent pro-inflammatory molecules, and evidence exists that cytokines may potentiate bradykinin activity and vice versa. Des Arg^{9}-BK is a potent stimulator of IL-1 and TNF release from macrophages (Tiffany and Burch, 1989). Conversely, cytokines for example, IL-1 and TNF have been shown to induce responsiveness of cells to BK to evoke release of PGE_2 (Burch and Tiffany, 1989; Bathon *et al.* 1989). Synovial cells from patients with RA pretreated with IL-1 and TNF showed increased responsiveness to BK by release of prostanoids (Bathon *et al.* 1989). In a study to determine the ability of IL-1, TNF and BK to release PGE_2 from human synovial cells, IL-1 was most potent and BK weakest. However, pretreatment of cells with IL-1, potentiated the BK and TNF and induced PGE_2 release (O'Neill, 1989). Additionally, pre-treatment with IL-1 enhanced the number of BK binding sites on human synovial fibroblasts (Bathon *et al.* 1992a). Similarly, a relationship between cytokines and kinins has been noted in other conditions. In human decidua cells cultured with and without BK, IL-6 and IL-8 secretion was enhanced by BK (Rehbock *et al.* 1997). Cultured human bronchial smooth muscle cells treated with IL-1, elicited a rapid and transient increase in the density of B2 receptors through an increase in B2 receptor MRNA level (Schmidlin, 1998).

These studies support an inter-relationship between the cytokine and kinin systems. In the pathogenesis of inflammatory joint disease there exists a possibility of a positive loop where BK stimulates IL-1 and TNFα release from the macrophages and IL-1 in turn stimulates the induction of BK receptors (Fig. 1.17). This may account for the intensity and perpetuation of the inflammatory process.

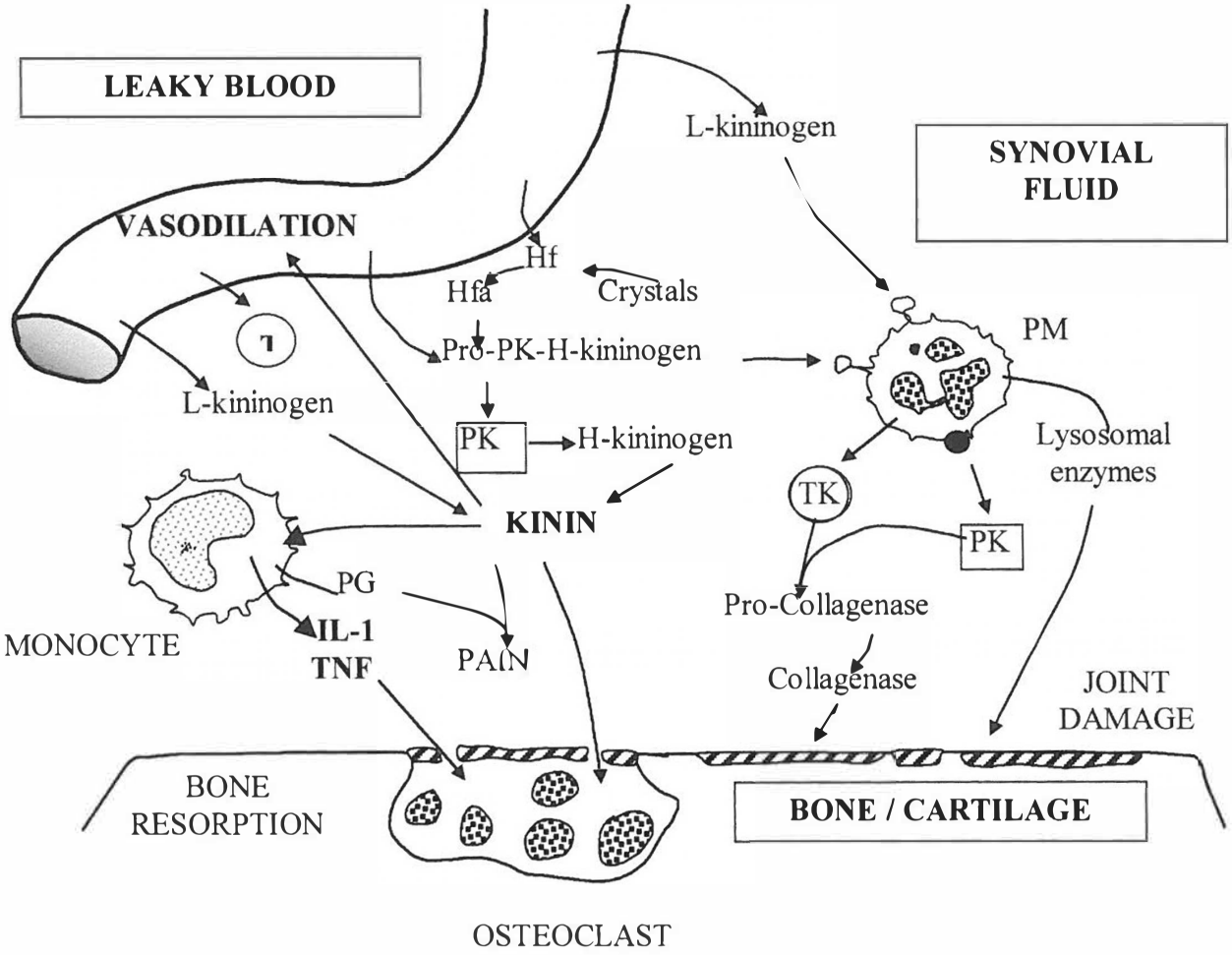


Figure 1.17 Interaction between the kinin and cytokine cascades in inflammatory arthritis

1.5 SUMMARY

Rheumatoid arthritis is a chronic multisystem disease of unknown aetiology. The current hypothesis is that an unknown antigen triggers an autoimmune response in a genetically susceptible individual. The predominant pathological change is that of an inflammatory synovitis, characterized by a cellular infiltration and angiogenesis with subsequent bone and cartilage destruction. These pathological changes occur as a result of the activation of a variety of cells, inflammatory mediators and effector molecules. The pro-inflammatory cytokines appear to play a central role in the pathogenesis of RA. Sufficient evidence also exists for a key role for the kallikrein-kinin system. In addition, there appears to be an inter-relationship between cytokines and kinins in the inflammatory process. Kinins induce the release of cytokines and cytokines have been shown to augment the effects of kinins. This may lead to an enhancement and perpetuation of the inflammatory process.

Clinically, RA presents with an insidious onset of symmetrical polyarthritis affecting both the small and large joints. Active disease is manifest by swollen, tender joints clinically, elevation in acute phase proteins and functional disability. The extent of joint involvement, levels of laboratory indices of inflammation and degree of radiological damage may be used to assess the severity of the disease. These markers are also used to initiate and monitor therapy.

Due to the lack of an exact aetiology, curative treatment for RA is not available. Treatment is therefore aimed to reduce joint inflammation and pain and to prevent further joint damage. Despite significant symptomatic effects, current treatments do not appear to alter the bone and cartilage changes. Recent advances in the understanding of the pathogenetic mechanisms, has led to the development of biological agents that are promising.

Further advances in the understanding of the pathogenesis are necessary and may lead to alternative and perhaps more effective treatment options. Several previous studies have a suggested a role for the kallikrein-kinin system in the inflammatory process and joint damage in rheumatoid arthritis. In addition, the inter-relationship between the cytokine and kinin systems and other effector molecules has been studied *in vitro*. However, the correlation of the markers of disease activity in rheumatoid arthritis (clinical, biochemical and radiological) with components of the kallikrein-kinin system has not been studied previously.

1.6.1 HYPOTHESIS

Kinins and cytokines influence the disease activity in rheumatoid arthritis and there is an inter-relationship between cytokines and kinins.

1.6.2 AIMS OF THE STUDY

1. To visualize the presence of tissue kallikrein and bradykinin receptors on synovial tissue samples.

2. To correlate tissue kallikrein activity in the synovial fluid and urine with measures of disease activity in patients with rheumatoid arthritis.

3. To demonstrate evidence of kinin generation by demonstrating kinin activity in synovial fluid and to correlate the kinin generating capacity with disease activity.

4. To demonstrate the presence of cytokines (IL-1β and TNFα) in the synovial fluid and to correlate the cytokine levels with tissue kallikrein activity and kinin generation.

5. To visualize the presence of tissue kallikrein, kinin moiety and bradykinin receptors on circulating and synovial fluid neutrophils from patients with rheumatoid arthritis and to correlate their immunoreactivity with clinical and biochemical markers of disease activity.

CHAPTER 2

METHODS

2.1 SAMPLE COLLECTION

2.1.1 Ethical approval and Patient/Guardian Consent

Ethical permission for this study was obtained from the Ethics Committee of the Medical School, University of Natal. Permission for the collection of post-mortem tissue samples was obtained from Professor M A Dada, Head - Department of Forensic Medicine, University of Natal. Informed consent was obtained from all subjects (Appendix 2.1). Control samples (blood) were obtained from age and sex matched healthy subjects. Control synovial tissue was collected at autopsy with the co-operation of the attending forensic surgeon.

2.2 PATIENT SELECTION

Patients attending the arthritis clinic with a diagnosis of rheumatoid arthritis were enrolled in the study. The following inclusion and exclusion criteria were applied.

Inclusion criteria:

Patients who fulfilled the revised 1987 American College of Rheumatology criteria for RA (Appendix 2.2), and who had active synovitis of at least one knee joint as judged by the presence of pain and a palpable warm effusion.

Exclusion criteria:

Intra-articular steroid injection within the last three months.

Change in disease modifying antirheumatic drug therapy within the last three months.

Oral steroids therapy in a dose of more than 10 mg daily.

2.3 ASSESSMENT OF DISEASE ACTIVITY

At the time of entry into the study a full history and clinical assessment was undertaken. Demographic data, including age, sex, duration of disease and age at onset of disease were recorded. The presence or absence of extra-articular manifestations of RA as well as the duration and treatment regimen was noted. In addition, the following parameters were assessed to determine the degree of disease activity and or disability:

- Duration of morning stiffness
- Swollen and tender joint counts
- Pain
- Physician's global assessment of disease activity
- Patient's global assessment of disease activity
- Local activity index
- Modified health assessment questionnaire (HAQ)
- Erythrocyte sedimentation rate
- Disease activity score

2.4 SAMPLE COLLECTION

2.4.1 Blood

Whole venous blood (10 ml) was obtained by venipuncture from the RA patients and volunteer subjects. Each sample was drawn into a vacutest tube containing 0.5 ml of 3.8 % (w/v) sodium citrate. For CRP measurements, blood was centrifuged at 4°C at 1000 g for 10 min and the serum stored at -70° C.

2.4.2 Synovial fluid

Synovial fluid (SF) was collected from the knee joint of the RA patients requiring therapeutic arthrocenthesis. Approximately 10- 15 ml of synovial fluid was aspirated using a sterile technique. All samples were centrifuged at 4°C, 1000 g for 10 min, within 3 h of collection. For measurement of basal kinin and cytokine levels, the SF was mixed immediately with an equal volume of the cocktail that prevented both the formation and destruction of the peptides, and the kinin generating cocktail for measuring the capacity of the synovial fluid to form kinins (Appendix 2.3). Thereafter samples were aliquotted into eppendorf tubes and stored at –20°C until assayed.

2.4.3 Synovial tissue

Synovial tissue obtained from patients with acute synovitis attending the early Arthritis Clinic at St Vincent's Hospital, Dublin were kindly supplied by Dr D Kane and Professor B Bresnihan. Ethical approval was obtained from the local ethics committee. Synovial tissue was obtained from the knee by needle biopsy under local analgesia, thoroughly washed in cold sterile saline (physiological saline; 0.9 % NaCl, Sabax, Adcock Ingram, SA) at 4°C to remove excess blood, and thereafter suspended in 5 % formal saline (41 % formaldeyde/0.9 % NaCl, 1: 8 v/v) for 48 h at room temperature (RT).

Control synovial tissue was obtained at post-mortem, performed within 24 h of death, from individuals who had died because of trauma not involving the joints or sudden unexplained death. The corpses had been refrigerated immediately, and had been maintained at 4°C in the state morgue, prior to removal of tissue. Histologically undiagnosed, visually *normal* synovial tissue was removed and washed in cold sterile normal saline (physiological saline; 0.9 % NaCl, Sabax) at 4°C to remove excess blood, before being divided into approximately

1 cm^3 wedges. This divided tissue was then suspended in 5 % formal saline (41 % formaldehyde/ 0.9 % NaCl, 1:8 v/v) for 48 h at RT.

2.5 SAMPLE PROCESSING AND STORAGE

2.5.1 Neutrophil isolation

The SF and anticoagulated blood (3.8 % sodium citrate) samples were mixed with an equal volume of phosphate buffered saline (PBS: 150 mM sodium chloride, 200 mM sodium dihydrogen, pH 7.2). Neutrophils were isolated by careful layering of blood on 3.5 ml histopaque 1119/1077 (Sigma, UK), and centrifugation at 1000 g for 30 min at 20°C. Neutrophils were harvested with a pasteur pipette from the band formed on centrifugation, mixed with an equal volume of PBS, centrifuged and washed twice with PBS. The cell pellet was resuspended in PBS and spotted onto glass slides, air-dried overnight, fixed in 4 % paraformaldehyde for 5 min at RT and stored at 4^0 C.

2.5.2 Tissue processing: fixation and wax embedding for light microscopy

Formalin-fixed synovial tissue samples were orientated and set in plastic tissue cassettes. The tissue samples were then processed by routine fixation and embedding techniques. Briefly, the tissue samples were dehydrated using absolute ethanol and xylene, and then embedded in paraffin wax under sterile conditions, in an automatic tissue processor (Shandon, UK). These wax-embedded samples were used for both light and fluorescent microscopy. The automated fixation, dehydration, clearing, infiltration and embedding was carried out by the Department of Histopathology, University of Natal, SA (Appendix 2.4). The wax-embedded tissue samples were stored at RT for future microscopic and immunohistochemical analysis.

2.5.3 Histology

2.5.3.1 Haematoxylin and Eosin (H&E) staining of wax-embedded tissue

Ultra-thin (3 μm) sections of the wax-embedded tissue were cut on a rotary microtome (Jung RM2035, Leica, Germany) and floated onto poly-L-lysine (10 % in distilled water v/v; Sigma, St. Louis) coated glass slides (LASEC, SA). The slides were allowed to air-dry and then stained with haematoxylin and eosin (H&E) (Appendix 2.5) to confirm the presence/absence of disease in post-mortem synovium, to determine the histological diagnosis in arthroscopic samples, and to ensure that tissue processing was optimal. The following staining method was performed at RT.

2.6 IMMUNOCYTOCHEMISTRY

2.6.1 Neutrophils

The SF and circulating neutrophils from RA patients and circulating neutrophils from control subjects (section 2.5.1) were immunostained to detect the presence or absence of tissue kallikrein, the kinin moiety in the kininogen molecule and B1 and B2 receptors. The specific antibody being studied was the primary antibody, and the secondary antibody was a conjugated antibody labelled with fluorescein isothiocyanate (FITC, 525 nm emission; Sigma, St Louis) visualised by confocal microscopy.

Primary and secondary antibodies.

The primary and secondary antibodies used for the immunolocalisation of the kinin moiety in the kininogen molecule, TK, B1 and B2 receptors are detailed below:

Kinin moiety:	Primary antibody:	monoclonal mouse antibradykinin antibody [(diluted 1:200 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)] (donated by M. Webb, Sandoz, UK)
	Secondary antibody:	FITC labelled anti-mouse IgG (Sigma, St Louis) [(diluted 1:32 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)]
TK:	Primary antibody:	polyclonal goat anti-human recombinant TK IgG [(diluted 1:1000 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)] (Raidoo et al., 1996)
	Secondary antibody:	FITC labelled sheep anti-goat IgG (Sigma, St Louis) [(diluted 1:32 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)]
B1 receptors:	Primary antibody:	rabbit anti-human B1 receptor IgG (donated by F. Hess) [(diluted 1:200 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)]
	Secondary antibody:	FITC labelled anti-rabbit IgG (Sigma, St Louis). [(diluted 1:32 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)]
B2 receptors:	Primary antibody:	rabbit anti-human B2 receptor IgG (donated by W. Müller-Esterl and K.Jarnagin) [(diluted 1:200 with 0.01 M phosphate buffer (pH 7.2)/1% BSA)]
	Secondary antibody:	FITC labelled sheep anti rabbit IgG [(diluted 1:200 with 0.01 M phosphate buffer (pH 7.2)/1 % BSA)]

Anti -TK antibody: The polyclonal anti-TK antibody was raised in rabbit and goat host animals (Raidoo et al., 1996). Antibody generation was directed against recombinant tissue kallikrein (rTK) generated in *E.coli* transfected with human TK cDNA. Dr. Michael Kemme kindly supplied this rTK (Institute for Biochemistry, Technical University of Darmstadt, Darmstadt, Germany). The lyophilised rTK protein was reconstituted in sterile physiological saline (0.9 % NaCl) at a concentration of 1 μg/ul, frozen in 50 ul aliquots, and maintained at -20°C.

Anti B2 receptor antibodies: Of the eight polyclonal antibodies, fully characterised for specificity, raised to synthetic peptides of the amino-terminal and loop regions encoded by the rat B2 receptor cDNA, and based on the homogeneity between these regions with the human receptor, only four were shown to react strongly with human epithelial cells and neutrophils (Haaseman *et al.* 1994). Of these, a combination of antibodies directed to intracellular domain one (ID1, LHK, 280), intracellular domain two (ID2, DRY, 277) and the fourth extracellular domain (ED4, DTL, 283) were used in this study as the match between the peptide sequences of these regions in the rat and human receptors was 80 %, 100 % and 75 % respectively. Werner Müller-Esterl and Kurt Jarnagin (Germany) kindly provided the anti-peptide antibodies and the corresponding peptides.

Anti-B1 antibodies: A polyclonal antibody directed at the following C-terminus synthetic peptide of the kinin B1 receptor (Ile-Ser-Ser-His-Arg-Lys-Glu-Ile-Phe-Gln-Leu-Phe-Trp-Arg-Asn) (I-S-S-S-H-R-K-E-I-F-Q-L-F-W-R-N) was raised in rabbit. It was kindly supplied by Fred Hess (Merck Research Laboratories, R80M-213, Rahway), USA having been fully characterised for specificity.

Confocal microscopy

Protocol for immunolabelling-neutrophils: Fc receptors in the neutrophil preparations and other non-specific sites were blocked with 1% human IgG (Sigma, St. Louis) for 10 min at RT. The slides were then incubated with the specific goat or rabbit primary antibody diluted with 0.01M phosphate buffer (pH 7.2)/ 1 % BSA, at 4°C, under humid conditions, for 18 h. Finally, the bound primary-antibody-complex was conjugated to a FITC labelled secondary antibody for 30 min at RT. The labelled slides were then mounted onto glass coverslips with an aqueous mountant (90 % glycerol/10 % PBS, v/v), and the edges of the coverslips sealed with commercial fingernail varnish. All washing steps, after each incubation, were carried out in 0.01 M phosphate buffered saline (pH 7.2) (Sigma, St. Louis). Fluorescent emission was analysed using the Leica TD4 confocal microscopy system (Leica, Germany). Between incubations the sections were washed thoroughly by submerging the slides in PBS [0.01 M phosphate buffer (pH 7.2) containing 0,0027 M potassium chloride, 0.137 M sodium chloride; Sigma, St. Louis] for 5 min. In the method controls the primary antibody was replaced with buffer. All dilutions were made up in 0.01 M PBS/1 % BSA (Appendix 2.6). The cell preparations were not allowed to dry out at any stage and labelled slides were stored in the dark.

Image analysis

Images were generated with immunolabelled neutrophils, using the Leitz DM IRB confocal microscope (Leica, Germany) attached to Diamond Pro 17 (Mitsubishi) and Diamond PRO 21T (Mitsubishi) monitors. The pixel density for these 8 bit false colour images was 225 x 225. The grey scale ranged from 0 to 256, and was divided into 8 equal phases (POLI Look-Up-Table), with each phase having a lower and upper threshold value on the grey scale (Fig 2.1). Pixels with a grey scale value between 156 and 256 were considered to indicate specific

immunofluorescence intensity.

Grey values for the 8 grey scale phases	Representative immunolabelling intensity
(8) 221 - 253 (7) 188 – 220 (6) 155 – 187	HIGH
(5) 122 – 154 (4) 89 – 121	MEDIUM
(3) 56 – 88	LOW
(2) 33 – 55 (1) 0 – 32	ZERO

Figure 2.1 Grey scales for immunolabelling intensity

The amount of antigen was estimated by analysis of the computer-generated images. Using the Analysis 2.1 Pro system (Soft-Imaging Software GmbH, 1996, Germany) the regions of interest (ROI) in each image were encircled. Within these areas the number of pixels falling within each phase, as well as the area analysed, was established. This data, was exported to Microsoft Excel to give an indication of the relative intensity of immunolabelling in (n) number of cells, by calculating the mean value of the grey scale in the range 155 – 256, and expressing the values as pixels x $10^2/\mu m^2$.

2.6.2 Protocol for immunolabelling : synovial tissue

Three-micron sections of the wax-embedded tissue were adhered onto adhesive coated (poly-L-lysine; Sigma, St. Louis) slides. Detection of immunoreactive TK, B1 and B2 receptors was performed by standard histochemistry techniques using the relevant specific primary antibody (see section 2.6.1.), and the appropriate anti-species secondary antibody conjugated with peroxidase-antiperoxidase (PAP) immuno-enzyme complex. The final reaction involved

the hydrolysis of diaminobenzidine (DAB), the chromogen substrate, and the product precipitate (dark brown) was visualised by light microscopy.

The PAP method as modified by Figueroa *et al.* (1988) was used for the immunolabelling of TK and the kinin receptors. The three-micron thin sections of the wax-embedded tissue sections on glass slides were heated on a heating block (Clifton, UK) at 60°C (to melt the wax) for 5 min. The peroxidase-anti-peroxidase (PAP) method required dewaxing the tissue sections in analytical grade xylene (analytical grade; Saarchem, SA) twice for 10 min each, rehydration with graded ethanolic solutions (100 %, 90 %, 70 % and 50 % in distilled water, v/v) and distilled water as the final rehydrant. When the tissue was approximately 50 % rehydrated, it was immersed in absolute methanol (analytical grade, Saarchem, SA) for 20 min to quench endogenous tissue-peroxidase activity. Following rehydration, the tissue was boiled in a metal-salt solution, 0.1M sodium citrate, pH 6.0 (analytical grade, Saarchem, SA) at 80°C for 10 min (antigen retrieval) (Shi *et al.* 1999) in a conventional microwave oven (R-4A52; Sharp Corp., Japan). The tissue in the boiling solution was allowed to cool down to RT (approximately 20 - 25 min). The tissue-sections were then further blocked with 10 % H_2O_2 (analytical grade, Saarchem, SA)/90 % absolute methanol (v/v) for 20 min to quench endogenous peroxidases. Incubation with the primary antibody was performed at 4°C for 18 h in a humidified chamber. Then, the tissue was treated with a peroxidase-anti-peroxidase (PAP) streptavidin-biotin conjugating system (LSAB K0690; Dako, USA) for 20 min. each. This entailed incubating the tissue with a universal (goat, rabbit, mouse) IgG-biotin link for 20 min at RT in a humidified chamber. Next, the tissue sections were covered with a strepavidin-peroxidase conjugate for 20 min at RT in a humidified chamber. The labelled antibody bound to the PAP immuno-enzyme complex was visualised by incubating the sections, in the dark, for 5 to 7 min with liquid

diaminobenzidine (DAB) precipitant (DAB K3465; Dako, USA). The sections were counterstained with Mayers Haematoxylin (Sigma, St. Louis,) for 3 min and intensified under running tap water for 5 min. Sections were then dehydrated by a reversal of the dehydration process from distilled water, through the increasingly concentrated ethanolic solutions into xylene, and finally mounted with a permanent medium (Entellen, Merck) (Appendix 2.7). Results were viewed by conventional light microscopy under a Nikon photo-microscope (Nikon Optiphot; Nikon, Japan). All incubations were carried out in a humidifying chamber. Between incubations the sections were washed thoroughly by submerging the slides in PBS [0.01 M phosphate buffer (pH 7.2) containing 0,0027 M potassium chloride, 0.137 M sodium chloride; Sigma, St. Louis] for 5 min. Tissue sections were not allowed to dry out at any time during the labelling process. The labelled slides were stored in the dark.

Positive tissue and method controls for immunocytochemistry (ICC)

As the kinin moiety within the kininogen molecule has been previously localised on control circulating neutrophils, these cells were used for the positive and method controls.

For the TK studies control human salivary gland tissue was used a positive control tissue to demonstrate localisation, upon previous evidence of abundant labelling for TK in such tissue (Schachter *et al.* 1980). Samples of fresh control human salivary gland were collected at post-mortem, fixed in 5 % formalin and embedded in paraffin wax. During each immunolabelling run, this tissue, demonstrating the presence of TK in the ducts of the human salivary gland, served as an appropriate method control for the labelling procedure.

Sections of fresh control human kidney collected at post-mortem, served as positive controls for B2 receptors. During each labelling run, this appropriate positive control tissue demonstrated the presence of B2 receptors in tubules and connecting ducts (Naidoo *et al.* 1996a). Human spinal cord was used as positive control tissue for B1 receptor immunolocalisation. During each labelling run, the presence of immunoreactive B1 receptors was demonstrated in the neurons in the substantia gelatinosa of the spinal cord (Raidoo and Bhoola, 1997). All labelled slides were stored in the dark to minimise bleaching of fluorescence and fading of precipitant immunolabels.

Negative method controls for ICC

The absence of positive specific immuno-labelling following pre-adsorption of the primary antibody with an excess of the specific antigen demonstrated the specificity of the antibody utilised. The primary antibody was diluted 1:500 with 0.01 M phosphate buffer (pH 7.2) and added to a 2 mg/ml stock solution of antigen to yield a final concentration of 1 mg/ml antigen. This antibody-antigen conjugate was incubated overnight at 4°C to allow maximum formation of antigen-antibody complexes. Following removal of the preadsorbed antigen by centrifugation (2200 x g, 4°C, Heraeus Biofuge 1.3 R, Germany) the supernatant was used for the immunolabelling experiments instead of the primary antibody.

For the immunolabelling of controls for the synovial tissue the primary antibody was replaced with goat non-immune serum diluted in 0.01 M phosphate buffer (pH 7.2)/1 % BSA. In the method controls the primary antibody was replaced with buffer. All dilutions made up in 0.01 M PBS/1 % BSA (pH 7.2).

2.7 MEASUREMENT OF TISSUE KALLIKREIN IN SYNOVIAL FLUID

Assays for tissue kallikrein

In the synovial fluid samples, the presence of enzymatically active TK was determined by an amidolytic assay, while the enzyme linked immunosorbant assay (ELISA) measured immuno-reactive TK. Commercially purified human urinary tissue kallikrein (HUK, Calbiochem, USA) was used to validate both the amidolytic assay and the ELISA. These results were used to calculate both the intra- and inter-assay coefficients of variation for both assays.

Synovial fluid

Before TK measurement of synovial fluid was possible, it was necessary to reduce the viscosity of the fluid by incubation with hyaluronidase. Next, $\alpha 2$ macroglobulin and rheumatoid factor were removed.

Hyaluronidase digestion: For measurement of basal kinin levels, frozen synovial fluid (to which both kinin generating and kininase cocktails had been added at the time of aspiration) was thawed and digested with hyaluronidase (Sigma, 22.5 U per ml synovial fluid) at 37°C for 30 min. The treated synovial fluid was centrifuged (7,000 g, 4°C, 10 min), and the kinins in the supernatant extracted with acid-alcohol (section 2.8.1). For the kinin generation studies, frozen synovial fluid (collected without the addition of inhibitors) was thawed and centrifuged (7,000 g, 4°C, 10 min). The supernatant was incubated with an equal volume of kininase inhibitors (10 μm phosphoramidon, 10 μm captopril, 6 mm 1,10 μM phenoanthroline and 60 mm EDTA in phosphate-buffered saline, pH 8) and hyaluronidase (22.5 U/ml synovial fluid), at 37°C.

Removal of α2 macroglobulin: α2 macroglobulin (α2 M) was removed from SF by adsorption with anti-α2 macroglobulin IgG (Dakopatts, Dako, UK) coupled to CnBr Sepharose. SF was incubated with an equal volume of anti α2-M Sepharose and 0.2 vol of 0.1 M Tris/0.5M NaCl pH 8.5 for 30 min at RT. The mixture was centrifuged and supernatant removed.

Removal of rheumatoid factor: SF was incubated with 0.3 vol of heat aggregated rabbit IgG coupled to CnBr Sepharose (Sigma, UK) and 0.2 of vol 0.1 M Tris/0.5 M NaCl pH 8.5 for 30 min at RT.

2.7.1 Enzymic assay (amidolytic micro-assay)

This end-point chromogenic assay was used to measure functionally active TK in the synovial fluid samples using a microtitre plate. The amount of functionally active TK was measured by assessing the amidolytic activity of the enzyme TK on a selective, synthetic substrate, H-D-Val-Leu-Arg-pNA (S2266; Kabivitrum, Sweden) (Amundsen *et al.* 1979) in the presence of SBTI (Sigma, St. Louis), and ethylenediaminetetraacetic acid (EDTA; Sigma, St.Louis) as modified by Selwyn *et al.* (1989), and further developed as an end-point assay in a microtitre plate by Rahman *et al.* (1994). In this amidase assay, the enzymic activity of TK on H-D-Val-LeuArg-pNA releases para-nitroaniline (pNA), which has a peak absorbance at 405 nm. A standard curve was set up using commercially available purified human urinary kallikrein, HUK (Calbiochem, USA) from which the concentration of TK in the sample extracts were calculated using Biorad Microplate Manager software (Biorad, UK). Following the measurement of total protein in an aliquot of the extract, using the Bradford Protein Determination method (Bradford 1976) (Appendix 2.8), the enzymic

activity of TK was expressed as ng TK/ug protein. Control urine was included in each assay run to determine inter-assay coefficients of variation. During each assay, 2 sets of plates were processed simultaneously, one being the measure of basal activity of TK at zero time, and the other measuring the enzymic activity of TK after a 3 h substrate incubation. The method was as follows.

Protocol: For the standard curve 600 ul of 400 ng/ml HUK standard, stored at -20°C as 1200 ng/ml aliquots, was prepared in amidase buffer (0.2 M Tris-Cl, pH 8.2). 300 µl of this standard was serially diluted to 6.25 ng/ml in amidase buffer. 50 µl (in triplicate) of each standard dilution was added to two 96-well tissue culture plates (Corning Cell Wells™, Corning, USA) designated *blank* and *substrate* plates. For the control curve, pooled urine was serially diluted down to 1/64 in amidase buffer (0.2 M Tris-Cl, pH 8.2) and 50 µl of each dilution (in triplicate) was added to both plates. 50 µl of each sample was loaded (in triplicate) onto each plate. Next, 50 µl of assay buffer (60 mg/ml SBTI, 75 mg/ml EDTA in amidase buffer) was added to each well in both plates. To the *blank* plate 50 µl of dH_20 was added to each well, and the absorbance of this plate read at 405 nm on a Biorad Microplate Reader 3550 at RT. To the *substrate* plate 50 µl of substrate S2266 (1.5 mM in deionised distilled water, as per manufacturer's instructions) was added to each well, and the plate incubated for 3 h at 37°C. The absorbance of this *substrate* plate was then read at 405 nm on a Biorad Microplate Reader 3550 at RT (Appendix 2.9). To determine the amidolytic activity of TK, the blank absorbance values were subtracted from the standards, controls and samples, on each plate. Then, the values of each standard, control and sample from the *blank* plate (zero activity) were subtracted from the *substrate* plate (enzymic activity). The net enzymic activity of the HUK standards was used to set up a plot of absorbance versus HUK concentration using Biorad Microplate Manager software (Biorad, UK).

2.7.2 Enzyme linked immunosorbant assay (ELISA).

An aliquot of the synovial fluid samples was used to measure the total TK by a basic sandwich ELISA using goat anti-human rTK IgG and rabbit anti-human rTK IgG. The anti-species antibody, anti-rabbit IgG (Sigma, St. Louis), conjugated to the enzyme alkaline phosphatase, interacts with the rabbit anti-human rTK IgG. The conjugated alkaline phosphatase then cleaves the chromogenic substrate disodium p-nitrophenyl phosphate (pNPP) to produce a yellow colour that has peak absorbance at 405 nm.

Protocol: A 96 well, flat-bottomed polystyrene ELISA plate (Corning, USA) was loaded 100 µl/well with primary antibody, goat anti-human rTK IgG (30 ng/ml goat anti-human rTK IgG in coating buffer) and incubated at 4^0C overnight. The wells were then washed with 200 µl 0.01 M PBS/0.5 %Tween three times for three min each at RT. The wells were incubated with 200 ul 5 % milk blocker twice for 30 min each at RT, with a 0.01 M PBS/0.5 %Tween wash in-between. Next, a 1200 ng/ ml aliquot of HUK (Calbiochem, USA), stored at -20^0C, was thawed at 4^0C and then serially diluted from 40 ng/ml to 0.625 ng/ ml in 0.01 M PBS (pH 7.2), and 100 µl of each standard (in triplicate) added to the wells. Blank wells (in triplicate) were filled with 100 µl of 0.01M PBS (pH 7.2) and control wells were filled (in triplicate) with 100 µl/well pool urine (see section 2.4.4.5) diluted 1/8 to 1/512 in 0.01 M PBS (pH 7.2). In addition, 100 µl of sample (in triplicate) was loaded onto the plate. The plate was then incubated at 37^0C for 1 h in a shaking water bath (Tecator, UK). The wells were then washed with 200 µl 0.01 M PBS/0.5% Tween three times for three min each at RT. Next, 100 µl of 25 ng/ml secondary antibody rabbit anti-human rTK IgG (in 0.01 M PBS, pH 7.2) was loaded into every well and the plate incubated at 37^0C for 1 h in a shaking water bath (Tecator, UK). The wells were then washed with 200 µl 0.01 M PBS/0.5 % Tween three times for three min each at RT. Next, all the wells were

loaded with 1/250 anti-rabbit phosphate conjugate (diluted in 0.01 M PBS, pH 7.2) and incubated for 1 h at 37^0 C in a shaking water bath. The plate was washed with 200 µl 0.01 M PBS/0.5 % Tween for 3 min each at RT. Finally, the wells were all loaded with 100 µl of a 1 mg/ml chromogenic pNPP substrate (Sigma, St. Louis, 1 tablet in 5 ml of $MgCl_2$/10 % diethanolamine, pH 9.6 substrate buffer) and the colour was allowed to develop until the highest absorbances peaked at 1 to 1.5 absorbance units (Appendix 2.10). Readings were taken at RT with a Biorad Microplate Reader 3550 (Biorad, UK) using Biorad Microplate Manager software. The mean blank absorbance was subtracted from the standards, controls and samples, and a curve of absorbance versus HUK concentration was plotted, values expressed as ng/ml.

2.8 KININ GENERATION ASSAYS

The capacity to form kinins from the kininogen content of SF was determined. Initially, endogenous TK was activated by incubating the SF samples with an equal volume of the generating cocktail for 60 min at 37^0 C, in the presence of kininase inhibitors to limit the degradation of the generated kinins. The kinins generated were thereafter extracted with acid-alcohol and measured by a competitive ELISA.

Before kinin measurement of synovial fluid was possible, it was necessary to reduce the viscosity of the fluid by incubation with hyaluronidase. Next, α2 macroglobulin and rheumatoid factor were removed (section 2.7). Thereafter, kinins were extracted by the acid-alcohol extraction procedure, modified from that of Albano *et al.* (1976), and measured by a competitive ELISA.

Acid-alcohol extraction The synovial fluid was mixed with an equal volume of 0.003 % HCI in absolute ethanol, vortexed and then incubated at - 20°C for 90 min. This was then centrifuged (7,000 g, 4°C, 10 min) and the supernatant decanted. The precipitate was washed with one volume of acid alcohol (diluted 1:1 in distilled water) and after recentrifugation, the corresponding supernatants pooled, evaporated to dryness at 55°C and the dry residue was reconstituted in the required volume of assay buffer, centrifuged and the clear supernatant used for measurement.

Kinin ELISA

To optimise binding of bradykinin (BK) to the plate it was necessary to first conjugate BK to a large protein. Cytochrome C was used for this purpose.

Conjugation of bradykinin to cytochrome C: Bradykinin (Sigma, UK) was conjugated to cytochrome C via a linker molecule, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Sigma, UK), according to a method modified from that of Carlsson et al. (1978). SPDP (13 μM in 0.1 M phosphate buffer pH 8.6, containing 20 % ethanol) was mixed with an equal volume of cytochrome C (type IIS from Saccharomyces cerevisiae, Sigma, UK, 10 μM in phosphate buffer pH 8.6) and incubated at 22°C for 60 min. The mixture was then filtered through ultra-free filters, (Millipore, UK) with 10,000 nominal molecular weight cut off, to remove any unconjugated SPDP. The cytochrome C-SPDP conjugate was resuspended in the two volumes of 0.3 M phosphate buffer pH 6. One volume of bradykinin (1 mM) was then added and this was incubated at 22°C for 60 min. Before use in the ELISA, each new batch of conjugate was tested to determine the optimum concentration for coating of the ELISA plate.

ELISA procedure: The acid-alcohol extracts were reconstituted to the original volume of the synovial fluid in PBS, and kinin concentration measured by an ELISA method as follows. Nunc Immulon Maxisorp (Nunc, UK) microtitre plates were coated overnight at 4°C with 100 µl/well of 3 to 4 µg/ml bradykinin-cytochrome C conjugate in sodium carbonate buffer, pH 9.6. Also overnight at 4°C, the sample or standard was preincubated with an equal volume of anti-rabbit anti-bradykinin monoclonal antibody (kindly donated by M. Webb, Sandoz, London) (Phillips and Webb, 1989) at 9.4 µg/ml. The next day, empty sites on the plate were blocked with 1 % bovine serum albumin (BSA< Roche, Germany) for 30 min at RT. The preincubated samples or standards (100 µl/well) were then incubated on the plates for 3 h at 37°C. In the next step, alkaline phosphatase labelled anti-mouse IgG (Sigma, UK) was added (diluted 1:250 in 0.01 M PBS, pH 7.2)) and then incubated for 2 h at 37°C. Finally, the substrate, disodium p-nitrophenyl substrate (Sigma, UK) was added, incubated at RT for approximately one hour until sufficient colour developed (i.e. maximum absorbance at 405 nm). Incubations were carried out in a humid box. Between each step the wells were washed three times for 3 min with PBS containing 0.05 % Tween (Sigma, UK). All dilutions were carried out in PBS containing 0.1 % BSA, unless stated otherwise. Nonspecific binding to the plate was determined for each plate by incubation of anti-bradykinin antibody (preincubated with buffer only) in wells that had been coated with coating buffer containing no bradykinin conjugate, and subtracted from all absorbance values. The absorbance values obtained for the standard BK was used to plot a standard curve of absorbance versus log concentration from which the basal and generated kinin contents of the SF samples were determined (Bio Rad microplate manager software). The basal and generated kinin contents of control urine were also measured during each run to determine the inter- and intra-assay coefficients of variation.

An important aspect of this assay is the use of two kininase/protease inhibitor cocktails. Firstly, aliquots of all samples and control urine were stored in inhibitor cocktail. This contained proteins that inhibited the enzymic activities of TK, PK and other trypsin-like proteases ((10 μM aprotonin and 10 μM SBTI), thereby preventing the release of kinin from endogenous kininogen. It also contained 10 μM captopril, an inhibitor of the KII family of kininases, and phosphoramidon, another kininase inhibitor, to ensure that the basal kinins present in the samples were not destroyed.

A second aliquot of each sample and control urine was stored in kinin generating cocktail, which was similar to the inhibitor cocktail except that it lacked aprotonin and SBTI. The absence of these protease inhibitors ensured that kinin could be released from endogenous kininogen and once formed, was protected by inhibitors of the peptidases. The detailed stepwise method for the competitive ELISA is tabulated in appendix 2.11.

2.9 CYTOKINE MEASUREMENTS

An aliquot of the synovial fluid samples was used to measure interleukin-1β (IL-1β) and tumour necrosis factor α (TNF) levels by a quantitative sandwich enzyme immunoassay technique using a commercial ELISA kit (Quantikine HS, R&D Systems).

Tumour necrosis factor α: A monoclonal antibody specific for TNF α is precoated onto a microplate. Standards and samples are pipetted into the wells and any TNFα present is bound by the immobilised antibody. Thereafter, an enzyme-linked (alkaline phosphatase) polyclonal antibody specific for TNFα is added. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an

incubation period, an amplifier solution is added and colour develops in proportion to the amount of TNFα bound in the initial step.

Protocol: A 96 well, flat bottomed polystyrene ELISA plate had been precoated with a murine monoclonal antibody against TNFα. 50 µl of assay diluent HD1-11 was added to each well. The standard solution was reconstituted to produce a stock solution of 32 pg/ml and thereafter serially diluted from 32 pg/ml to 0.5 pg/ml. 200 µl of standard or sample was added to the wells. The plate was then incubated for 14-20 h at 2^0-8^0 C. The wells were then washed using an automated AW1 Anthos washer. Next, 200 µl of TNFα conjugate was added to each well. The plate was then incubated for 3 h at RT. The wells were washed as before. Next, 50 µl of substrate solution (NADPH) was added to each well. The plate was covered and incubated for 3 h at RT. 50 µl of amplifier solution was added to each well. The plate was covered and incubated for 30 min at RT. Next, 50 µl of stop solution was added to each well. The optical density of each well was determined within 30 min using microplate reader set at 490 nm with correction to 650 nm (Appendix 2.12). A standard curve was plotted using the absorbance of the standards versus the concentration of the standards.

Interleukin-1β: A monoclonal antibody specific for IL-1β is precoated onto a microplate. Standards and samples are pipetted into the wells and any IL-1β present is bound by the immobilised antibody. Thereafter, an enzyme-linked (alkaline phosphatase) polyclonal antibody specific for IL-1β is added. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, an amplifier solution is added and colour develops in proportion to the amount of IL-1β

bound in the initial step.

Protocol: A 96 well, flat bottomed polystyrene ELISA plate had been precoated with a murine monoclonal antibody against IL-1β. 100 µl of assay diluent HD1-11 was added to each well. The standard solution was reconstituted to produce a stock solution of 8 pg/ml and thereafter serially diluted from 8 pg/ml to 0.125 pg/ml. 150 µl of standard or sample was added to the wells. The plate was then incubated for 14-20 h at RT. The wells were then washed using an automated AW1 Anthos washer. Next, 200 µl of IL-1β conjugate was added to each well. The plate was then incubated for 3 h at RT. The wells were washed as before. Next, 50 µl of substrate solution (NADPH) was added to each well. The plate was covered and incubated for 45 min at RT. 50 µl of amplifier solution was added to each well. The plate was covered and incubated for 45 min at RT. Next, 50 µl of stop solution was added to each well. The optical density of each well was determined within 30 min using microplate reader set at 490 nm with correction to 650 nm (Appendix 2.13).

2.10 STATISTICAL ANALYSIS

Results are presented as the mean and standard deviation of mean. A two-tailed, paired Student's t-test, unpaired for unequal variance, the Kruskal Wallis and one way analysis of variance (ANOVA) tests calculated significance. Levels of significance were determined using a 95% confidence interval; a p-value < 0.05 was taken to be statistically significant. All correlations were performed using the Pearson's correlation co-efficient.

Appendix 2.1 Patient information/consent form

As you know, you are suffering from arthritis, which has resulted in the accumulation of fluid in your knee. To allow us to understand why patients develop arthritis we would like to take blood, urine and fluid from your knee joint for testing in the laboratory. Blood will be taken from a vein in your arm and the fluid will be aspirated from your knee. You may feel slight pain. In addition you may be given an injection in of cortisone plus local anaesthetic into your knee to reduce the inflammation and pain. After the injection we will like to remove fluid again to determine the effect of the injection. You are free to refuse to any of these procedures and your refusal will not alter your future treatment.

Njengoba wazi, unesifo samathambo esesibangela ukwanda kometshezi edolweni. Ekukusizeni ukuba siqondisise ukuthi kwenziwa yini iziguli ziphathwe isifo samthambo, sithanda ukutha igazi, umchamo kanye noketshei olusedolweni kuhlolisiswe endlini yokucwaninga. Igazi lizothathwa emthanjeni wegalo, bese kuthi uketshezi lukhitshwe edolweni. Kungenzeka kube khona ubuhlungwana. Ngaphezu kwaloku uyonikezwa umjovu we cortisone kanye nondikindiki edolweni kwenzelwe ukuhlisa ukuvuvuka kanye nobuhlungu. Emuveni komujovo siyophinda sikhiphe uketshezi edolweni kona sizbona ukusebenza komjovo. Unenkululeko yokwenqaba ukuba kwenziwe lolucwaningo futhi ukwenqaba kwakho ngeke kuthikameze inqubo yokulashwa kwakho.

Appendix 2.2 The 1987 revised criteria for the classification of rheumatoid arthritis*

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joints	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joints
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in (2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on postero-anterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

*For classification purposes a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks

Appendix 2.3 Composition of inhibitor and buffer solutions

a) *Measurement of kinin generation: inhibitor solutions*

10 μM phosphoramidon, 10 μM captopril, 6 mM 1, 10 μM phenanthroline (Sigma, UK) and 60 mM EDTA in phosphate-buffered saline, pH 8

b) *Measurement of basal kinin and cytokine levels: inhibitor solutions:*

40 mg/ml aprotinin, 4 mg/ml SBTI, 10 μM phosphoramidon, 10 μM captopril, 6 mM1, 10 μM phenanthroline (all Sigma, UK) and 60 mM EDTA in phosphate-buffered saline, pH 8

c) *Measurement of tissue kallikrein*

200 μl bacitracin/SBTI

d) *ELISA coating buffer:* $Na_2HCO_3/NaHCO_3$ pH 9.6

1.59 g Na_2HCO_3/2.93 g $NaHCO_3$ per L dH_2O

e) *Amidase buffer*: 0.2 M Tris – HCl pH 8.2

24.22 g Trizma base per L dH_2O

f) *Assay buffer*

300 mg SBTI, 375 mg EDTA/ml amidase buffer

g) *Substrate buffer*: 5 mM $MgCl_2$/10 % diethanolamine pH 9.6

0.1016 g MgCl/10 ml Diethanolamine per l dH_2O

h) *0.1 M Sodium citrate* pH 6.0

29.4 g Tri-sodium citrate/l dH_2O

Appendix 2.4 **Fixation, Dehydration and Embedding schedule for light microscopy**

STEP	PROCEDURE	TEMP	TIME
1.	Fixation – 5 % formal saline	24°C	1 h
2.	Fixation – 5 % formal saline	24°C	1 h
3.	Dehydration - absolute ethanol	24°C	1 h
4.	Dehydration - absolute ethanol	24°C	1 h
5.	Dehydration - absolute ethanol	24°C	1 h
6.	Dehydration - absolute ethanol	35°C	1 h
7.	Dehydration - absolute ethanol	35°C	1 h
8.	Dehydration - absolute ethanol	35°C	1 h
9.	Dehydration - absolute ethanol	35°C	1 h
10.	Clearing - xylene	35°C	1 h
11.	Clearing - xylene	35°C	1 h
12.	Vacuum infiltration 1 - paraffin wax	60°C	1 h
13.	Vacuum infiltration 2 - paraffin wax	60°C	1 h
14.	Embedding - paraffin wax	20°C	20 min

Appendix 2.5 H&E Staining of wax-embedded tissue

STEP	PROCEDURE	TIME
1.	Dewax - Xylene	2 x 5 min
2.	Rehydrate - Absolute ethanol	2 x 1 min
3.	Rehydrate – 90 % ethanol	1 min
4.	Rehydrate – 70 % ethanol	1 min
5.	Rehydrate - water	1 min
6.	Haematoxylin - immerse slides in Mayer's Haemotoxylin (Sigma, St. Louis)	5 min
7.	Blue - rinse slides in running tap water	5 min
8.	Eosin - immerse slides in Eosin (Sigma, St. Louis)	2 min
9.	Rinse - by quickly immersing slides in 95 % ethanol	30 s
10.	Dehydrate - Absolute ethanol	2 x 1 min
11.	Dehydrate - Xylene	1 min
12.	Mount - maintain in xylene until mounting in Entellen (Merck)	

Appendix 2.6 Immunocytochemical localisation by immuno-fluorescence

STEP	PROCEDURE	TEMP	TIME
1.	Dewax - Xylene (analytical grade; Saarchem, SA)	RT	2 x 10 min
2.	Rehydrate – 100 % EtOH (analytical grade; Saarchem, SA)	RT	2 x 5 min
3.	Rehydrate – 90 % EtOH (in distilled water, v/v)	RT	2 x 4 min
4.	Rehydrate – 70 % EtOH (in distilled water, v/v)	RT	1 x 3 min
5.	Rehydrate - distilled water	RT	1 x 5 min
6.	Antigen retrieval - 0.1 M Sodium Citrate (pH 6.0) in microwave. Then allow to cool to RT for ± 20min	Boil at 80^0C	3 min high & 5 min low
7.	Wash - 0.01 M PBS pH 7.2	RT	
8.	Block non-specific binding sites – 1 % Human IgG	RT	2 min
9.	Wash - 0.01 M PBS (pH 7.2)	RT	5 min
10.	Primary antibody diluted with 0.01 M Phosphate buffer (pH 7.2) [0.1 M PBS/1 %BSA]	4°C	18 h
11.	Wash - 0.01 M PBS (pH 7.2)	RT	5 min
12.	FITC conjugated secondary antibody - (1/32 dilution in 0.01 M PBS/1 %BSA)	RT	30 min
13.	Wash - 0.01 M PBS (pH 7.2)	RT	5 min
14.	Mount - 10% PBS/90 % glycerol		

Appendix 2.7 Immunocytochemical localisation by immunoprecipitation

STEP	PROCEDURE	TEMP	TIME
1.	Dewax - Xylene (analytical grade; Saarchem, SA)	RT	2x10 min
2.	Rehydrate – 100 % EtOH (analytical grade; Saarchem, SA)	RT	2x5 min
3.	Quench endogenous peroxidase – 100 % MeOH (analytical grade; Saarchem, SA)	RT	1x20 min
4.	Rehydrate – 90 % EtOH (in distilled water, v/v; analytical grade; Saarchem, SA)	RT	2x4 min
5.	Rehydrate – 70 % EtOH (in distilled water, v/v analytical grade; Saarchem, SA)	RT	1x3 min
6.	Rehydrate - distilled water	RT	1x5 min
7.	Antigen retrieval - 0.1 M Sodium-Citrate (pH 6.0) in microwave. Then allow to cool to RT for ± 20 min	Boil at 80^0C	3 min high & 5 min low
8.	Wash - 0.01 M PBS (pH 7.2)	RT	
9.	Block endogenous peroxidase – 10 % H_2O_2/ 90% MeOH	RT	20 min
10.	Wash - 0.01 M PBS (pH 7.2)	RT	5 min
11.	Block non-specific binding sites – 1 % non-immune goat serum in buffered physiological saline		20 min
12.	Wash - 0.01 M PBS (pH 7.2)	RT	5 min
13.	Primary antibody diluted with 0.01 M Phosphate buffer (pH 7.2) [0.1 M PBS/1 %BSA]	4°C	18 h
14.	Wash - 0.01 M PBS (pH 7.2)	RT	5 min
15.	Incubate with anti-goat IgG Biotin link (Dako K0690)	RT	20 min
16.	Wash - 0.01M PBS (pH 7.2)	RT	
17.	Secondary antibody- Strepavidin-peroxidase (Dako K0690)	RT	20 min
18.	Wash - 0.01M PBS (pH 7.2)	RT	5 min
19.	Chromogen - liquid DAB (Dako, K3465)	RT	2-5 min
20.	Counterstain - Mayers' Heamatoxylin (Sigma, St. Louis)	RT	3-5 min
21.	Intensify - Wash in tap H_2O		5 min
22.	Dehydrate – 70 % EtOH (in distilled water, v/v)	RT	1 min
23.	Dehydrate – 90 % EtOH (in distilled water, v/v)	RT	1 min
24.	Dehydrate – 100 % EtOH (analytical grade; Saarchem, SA)	RT	1 min
25.	Dehydrate - Xylene (analytical grade; Saarchem, SA)	RT	1 min
26.	Mount - Entellen (Merck)		

Appendix 2.8 Measurement of total protein (Bradford 1976)

STEP	PROCEDURE	TEMP.	TIME
1.	Protein standard - 100 ml of 1mg/ml Bovine Serum Albumin (BSA, Fraction V, Boehringer) was prepared and stored in 1 ml aliquots at -20C. A single 1 ml aliquot of this standard was serially diluted with distilled water from 1000 µg/ml to 16 µg/ml. 30 µl of each dilution was added to a microassay plate, in triplicate.	RT	
2.	Blank - 30 µl distilled water was added to 3 wells of the plate	RT	
3.	Samples - were added in triplicate to the plate	RT	
4.	Biorad Protein Assay Reagent (Biorad, UK) - was diluted 1/5 in distilled water and 300 µl was added to each well of the plate	RT	
5.	Read absorbance at 595 nm.	RT	
6.	Calculation- subtract the absorbance of the blanks from the absorbance of the standards and samples. Plot absorbance versus concentration of BSA standards. Read protein concentration of samples from this graph.	RT	
7.	TK enzymic activity is expressed as ng TK/µg protein	RT	

Appendix 2.9 Enzymic / amidolytic micro-assay for Tissue Kallikrein

STEP	PROCEDURE	TEMP.	TIME
1.	HUK standards - prepare 600 ul of 400 ng/ml HUK from a 1200 ng/ml aliquot stored at -20°C using amidase buffer [0.2 M Tris-Cl (pH 8.2)]. Use 300 µl of this and double diluted down to 6.25 ng/ml in amidase buffer. Add 50 ul of each dilution in triplicate to two 96-well tissue culture plates (Corning Cell Wells™ 25860, Corning, New York)	RT	
2.	Blank - add 50 µl amidase buffer (triplicate) to the two microtitre plates	RT	
3.	Controls - add 50 µl control urine, from section 2.4.4.5 serially diluted to 1:64 (with amidase buffer), in triplicate to both plates	RT	
4.	Samples - add 50 µl synovial fluid samples in triplicate to the remaining wells	RT	
5.	Assay buffer - add 50 µl assay buffer (60 mg/ml SBTI, 75 mg/ml EDTA in amidase buffer) to each well of both plates	RT	
6.	Incubate	37°C	30 min
7.	Zero activity - add 50 µl distilled water to each of the wells of one plate and read absorbance at 405 nm on the Biorad Microplate Reader 3550.	RT	
8.	Enzymic activity - add 50 µl S-2266 (1.5 mM in deionised distilled water) to each well of the second plate and incubate	37°C	3 h
9.	Read absorbance at 405 nm after 3 h	RT	
10.	Calculation - for each plate subtract the absorbance of the blanks from the absorbance of the standards, controls and samples. Then subtract the zero activity absorbance from the enzymic activity and use this value to plot absorbance versus concentration of HUK standards. Read concentration of samples from this graph.		

Appendix 2.10 Tissue Kallikrein ELISA

STEP	PROCEDURE	TEMP	TIME
1.	Primary antibody - Coat the ELISA plate (Corning) with 100 ul of 30 ng/ml Goat a-human rTK IgG in coating buffer	4^0C	ON
2.	Wash the plate with 0.01M PBS/0.5 %Tween	RT	3x3min
3.	Block 1 - add 200 µl of 5 % milk blocker to each well	RT	30 min
4.	Wash the plate with 0.01M PBS/0.5 %Tween	RT	3x3min
5.	Block 2 - add 200 µl of 5 % milk blocker to each well	RT	30 min
6.	Wash the plate with 0.01M PBS/0.5 %Tween	RT	3x3min
7.	Standards - a 1200 ng/ml aliquot of HUK standard stored at -20°C was double diluted 40 ng/ml to 0.625 ng/ml, and 100 µl of each dilution was added in triplicate		
8.	Blanks - 100 µl 0.01M PBS (pH 7.2) was added to 3 wells		
9.	Controls -control urine from section 2.4.4.5 was serially diluted from 1/8 to 1/512 in 0.01M PBS (pH7.2), and 100 µl of each dilution added in triplicate. Also, known samples are rerun to test for inter-assay variation		
10.	Sample - 100 µl of sample was loaded onto the remaining wells of the plate in triplicate		
11.	Incubate	37^0C	60 min
12.	Wash the plate with 0.01 M PBS/0.5 %Tween	RT	3x3min
13.	Secondary antibody - add 100 µl of 25 ng/ml rabbit anti- human rTK to each well and incubate	37^0C	60 min
14.	Wash the plate with 0.01 M PBS/0.5 %Tween	RT	3x3min
15.	100 µl of anti-rabbit IgG (Sigma, St. Louis) diluted 1:250 with 0.01 M PBS was added to each well and incubated	37^0C	60 min
16.	Wash the plate with 0.01 M PBS/0.5 %Tween	RT	3x3min
17.	Chromogen - 100 µl of 1 mg/ml pNPP substrate was added to each well (1 tablet in 5 ml of substrate buffer) and allow colour to develop	37^0C	0-60 min
18.	Calculation - subtract the absorbance of the blanks from the absorbance of the standards, controls and samples. Plot absorbance versus concentration of HUK standards. Read TK concentration of samples and controls from this graph.		

Appendix 2.11 Kinin generation ELISA

STEP	PROCEDURE	TEMP	TIME
1.	Coat the ELISA plate (Nunc Immulon) with 100 µl of 3 to 4 µg/ml bradykinin-cytochrome c conjugate in sodium carbonate buffer, pH 9.6	4^0C	ON
2.	Primary antibody : pre-incubate sample or standard with equal volume of bradykinin monoclonal antibody at 9.4 µg/ml	4^0C	ON
3.	Wash the plate with 0.01M PBS/0.5 %Tween	RT	3x3min
4.	Block with: bovine serum saline	RT	30 min
5.	Wash the plate with 0.01M PBS/0.5%Tween	RT	3x3min
6.	Incubate standards and samples	37^0 C	3h
7.	Wash the plate with 0.01M PBS/0.5 %Tween	RT	3X3 min
8.	Secondary antibody: add alkaline phosphatase labelled anti mouse IgG (diluted1:250) and incubate	37^0 C	2h
9.	Wash the plate with 0.01M PBS/0.5 %Tween	RT	3X3 min
10.	Chromogen: add disodium p-nitrophenyl substrate (Sigma, UK) and allow colour to develop	37^0 C	0-60 min
11.	Calculation - subtract the absorbance of the blanks from the absorbance of the standards, controls and samples. Plot absorbance versus basal and generated kinin contents of urine and synovial fluid samples Read kinin concentration of samples and controls from this graph		

Appendix 2.12 Tumour necrosis factor α ELISA

STEP	PROCEDURE	TEMP.	TIME
1.	Standard solution – reconstitute standard solution to produce a stock solution of 32 pg/ml and serially dilute from 32 pg/ml to 0.5 pg/ml	RT	
2.	Add 50 µl of assay diluent HD1-11 to each well of a 96 well, flat bottomed polystyrene ELISA plate precoated with a murine antibody against TNF α	RT	
3.	Add 200 µl of standard or sample to each well	RT	
4.	Incubate	2°–8°C	14-24 h
5.	Wash wells using an automated AW1 Anthos washer		
6.	Add 200 µl of TNF α conjugate to each well		
7.	Incubate	RT	3h
8.	Wash wells using an automated AW1 Anthos washer		
9.	Add 50 µl of substrate solution (NADPH) to each well		
10.	Incubate	RT	3h
11.	Add 50 µl of amplifier solution to each well		
12.	Incubate	RT	30 min
13.	Add 50 µl of stop solution to each well		
14.	Read absorbance at 490 nm with a correction to 650 nm within 30 min		
15.	Plot a standard curve using the absorbance of the standards versus the concentration of the standards. Read the concentration of the samples from this graph		

Appendix 2.13 Interleukin 1 β ELISA

STEP	PROCEDURE	TEMP.	TIME
1.	Standard solution – reconstitute standard solution to produce a stock solution of 8 pg/ml and serially dilute from 8 pg/ml to 0.125 pg/ml	RT	
2.	Add 100 µl of assay diluent HD1-11 to each well of a 96 well, flat bottomed polystyrene ELISA plate precoated with a murine antibody against IL -1β	RT	
3.	Add 150 µl of standard or sample to each well	RT	
4.	Incubate	RT	14-20 h
5.	Wash wells using an automated AW1 Anthos washer		
6.	Add 200 µl of IL-1β conjugate to each well		
7.	Incubate	RT	3h
8.	Wash wells using an automated AW1 Anthos washer		
9.	Add 50 µl of substrate solution (NADPH) to each well		
10.	Incubate	RT	45 min
11.	Add 50 µl of amplifier solution to each well		
12.	Incubate	RT	45 min
13.	Add 50 µl of stop solution to each well		
14.	Read absorbance at 490 nm with a correction to 650 nm within 30 min		
15.	Plot a standard curve using the absorbance of the standards versus the concentration of the standards. Read the concentration of the samples from this graph		

CHAPTER 3

RESULTS

3.1 DEMOGRAPHICS

3.1.1 Patients with rheumatoid arthritis

For the synovial fluid and neutrophil studies, 50 patients with rheumatoid arthritis who fulfilled the inclusion criteria were included in the study (refer 2.2). There were 42 females and 8 males. The mean age was 49.5 ± 9.8 years with a range from 21 to 72 years. The mean duration of arthritis was 9.1 ± 7.2 years with a range from 0.8 to 36 years. All the patients fulfilled the ARA criteria for RA. Rheumatoid nodules were present in 4 patients (8 %) and the rheumatoid factor was positive in 37 patients (66%). Thirty patients (60 %) were receiving disease modifying anti-rheumatic therapy, while 20 (40 %) patients were on low dose oral prednisone (≤ 10 mg per day). Hypertension was the commonest co-morbid condition and was present in 13 patients (26 %). In addition, 4 patients (8%) were on treatment for diabetes and 3 patients (6 %) had hypothyroidism (Table 3.1). Full details appear in Appendix 3.1.

3.1.2 Controls: Blood and urine

Blood samples were obtained from 50 healthy volunteers who did not have any known illness, and were on not regular medication. They were matched with the RA patients for age and sex. There were 42 females and 8 males. The mean age was 49.5 ± 9.8 years with a range from 21 to 74 years (Table 3.1 and Appendix 3.2.)

Table 3.1 Demographics: Patients and Healthy volunteers.

	RA patients	Healthy volunteers
Number	50	50
Female: Male	42: 8	42: 8
Mean age	49.5 ± 9.8 yrs (range: 21 - 72 yrs)	49.5 ± 9.8 years (range: 21 - 74 years)
Mean duration of RA	9.1 ± 7.2 yrs (range: 0.8 - 36 yrs)	
Rheumatoid nodules	4 patients (8%)	
Positive RF	37 patients (66%)	
No. of ARA criteria		
7	3 (6%)	
6	24 (48%)	
5	19 (38%)	
4	4 (8%)	
DMARD therapy	36 (60%)	
Low dose corticosteroid therapy	20 (40%)	
Co-morbid diseases		
Hypertension	13 (26%)	
Diabetes	4 (8%)	
Hypothyroidism	3 (6%)	

3.1.3 Synovial tissue: patients with inflammatory arthritis

For the synovial tissue study, samples were obtained at arthroscopy from 6 patients with rheumatoid arthritis. These samples were kindly provided by Dr D Kane and Professor B Bresnihan, (St. Vincent's Hospital, Dublin). The mean age was 45.5 years, range 27 – 63 years (Appendix 3.3).

3.1.4 Synovial tissue: controls

Control synovial tissue was obtained from 10 patients in whom an autopsy was performed within 24 h of sudden death. There were 9 males and one female; with a mean age of 36 years, range 18 to 48 years. The causes of death were stab wounds (n = 4), gun shot injuries (n = 2), suicide by hanging (n = 2), blunt injury and acute myocardial infarction. These patients had no evidence of joint disease or trauma or surgery to the joints (Appendix 3.4).

3.2 SYNOVIAL TISSUE IMMUNOCYTOCHEMISTRY

The control (autopsy) and inflamed synovial tissue samples were initially stained with haematoxylin and eosin to verify the tissue morphology. The control synovial tissue demonstrated a single layer of synoviocytes and an absence of inflammatory cells in the subintimal layer (Fig. 3.1). In the inflamed synovium, there was hypertrophy of the lining layer and infiltration of the sublining layer with inflammatory cells, including lymphocytes, macrophages and plasma cells (Fig. 3.2).

Tissue kallikrein activity has been reported in the synovial fluid from arthritic joints (Worthy *et al.* 1990) and the salivary glands of patients with Sjogren's syndrome

secondary to RA (Hernandez *et al.* 1998). In addition, TK has been implicated in neutrophil diapedesis (Figueroa *et al.* 1989) and in the processing of promolecules that are involved in the pathogenesis of inflammatory arthritis (Tschesche *et al.* 1989; Menashi *et al.* 1994). The kinin B2 receptor has been identified on human synovial tissue by autoradiography (Bathon *et al.* 1992a) and Scatchard analysis (Uhl *et al.* 1992). Administration of kinin B1 antagonists and the B2 receptor antagonists, HOE 140, has been shown to reduce BK induced plasma extravasation in rat arthritic joints (Cruwys *et al.* 1994). This study was therefore undertaken to determine the expression of TK, B1 and B2 receptors on normal and inflamed synovial tissue.

Both the control and inflamed synovial tissue samples were thereafter immunostained to detect the presence or absence of (i) TK and the (ii) kinin B1 and B2 receptors. The slides were viewed and the intensity of the immunolabelling was quantified by image analysis in pixels x $10^2/\mu m^2$ (see Methods).

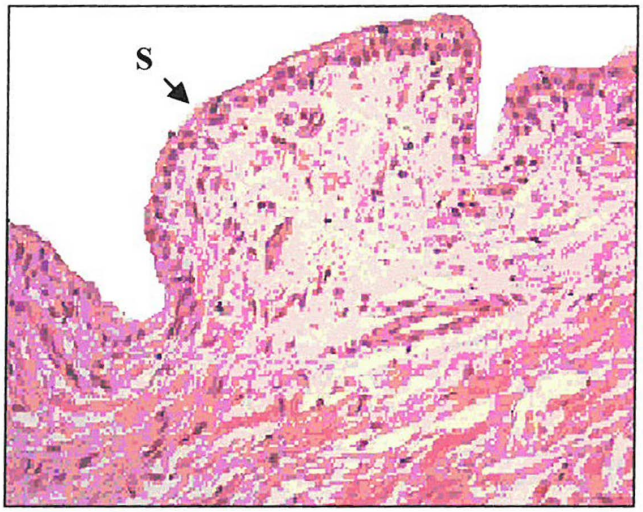


Figure 3.1 Photomicrograph of control synovial tissue

Haematoxylin and eosin stain demonstrating a single layer of synovial cells (S) and the absence of inflammatory cells in the sublining layer. Magnification x 400.

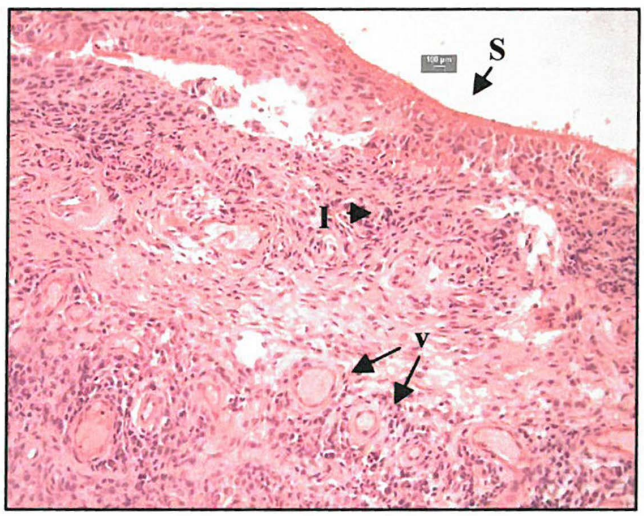


Figure 3.2 Photomicrograph of rheumatoid synovium

Haematoxylin and eosin stain demonstrating hypertrophy of the synovial lining layer (S) and a dense inflammatory infiltrate in the sublining layer (I) and increased vascularity (V). Magnification x 200.

3.2.1 Immunolocalisation of tissue kallikrein

The localization of immunoreactive TK in the cells of the apical region of the duct cells of the human sub-mandibular salivary glands (Schachter *et al.* 1980) was used as a positive control (Fig.3.3A). The loss of immunolabelling following the preabsorption of the antibody with an excess of rTK and replacement of the primary antibody with PBS served as the negative method control at each labeling run (Fig 3.3B).

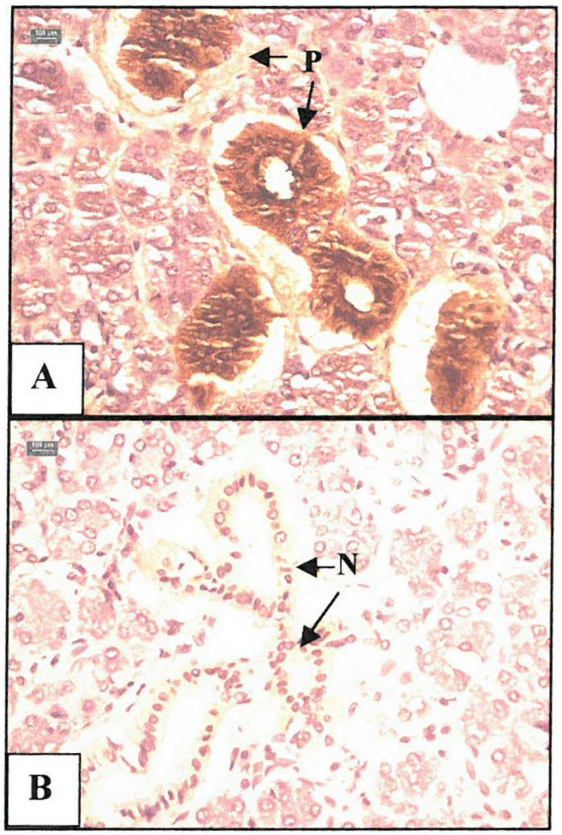


Figure 3.3 Photomicrographs of control human salivary gland

Positive labeling for TK (P) is present in the duct cells (A). There is an absence of labeling (N) in the method control (B). Immunoperoxidase stain. Magnification x 400.

The TK labeling in both the control and rheumatoid synovial tissue is shown in Fig. 3.4. Intense labeling was observed in the synovial lining cells, endothelial cells, and the subintimal macrophages. The intensity of labeling was quantified by image analysis. There was a significant increase in labeling in the endothelial cells of the RA patients compared to controls ($p < 0.05$) (Table 3.2 and Fig. 3.5).

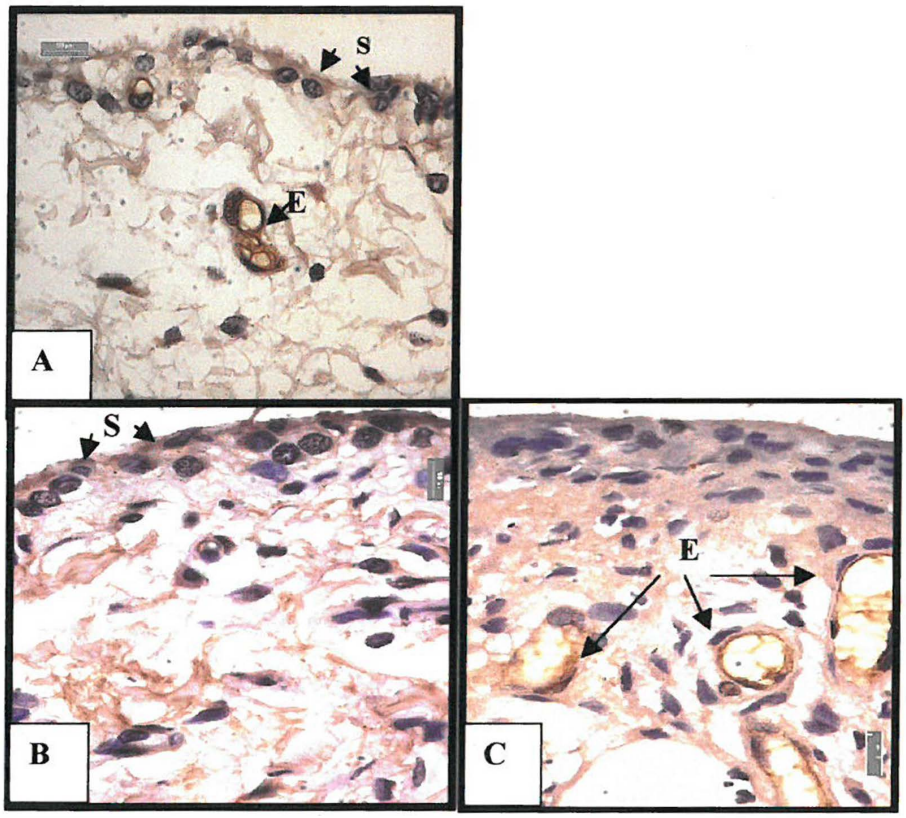


Figure 3.4 Immunovisualisation of tissue kallikrein in control and rheumatoid synovial tissue

Intense labeling for TK is seen in the synovial lining (S) and endothelial cells (E) in the normal (A) and rheumatoid synovium (B&C). Immunoperoxidase stain. Magnification x 1000.

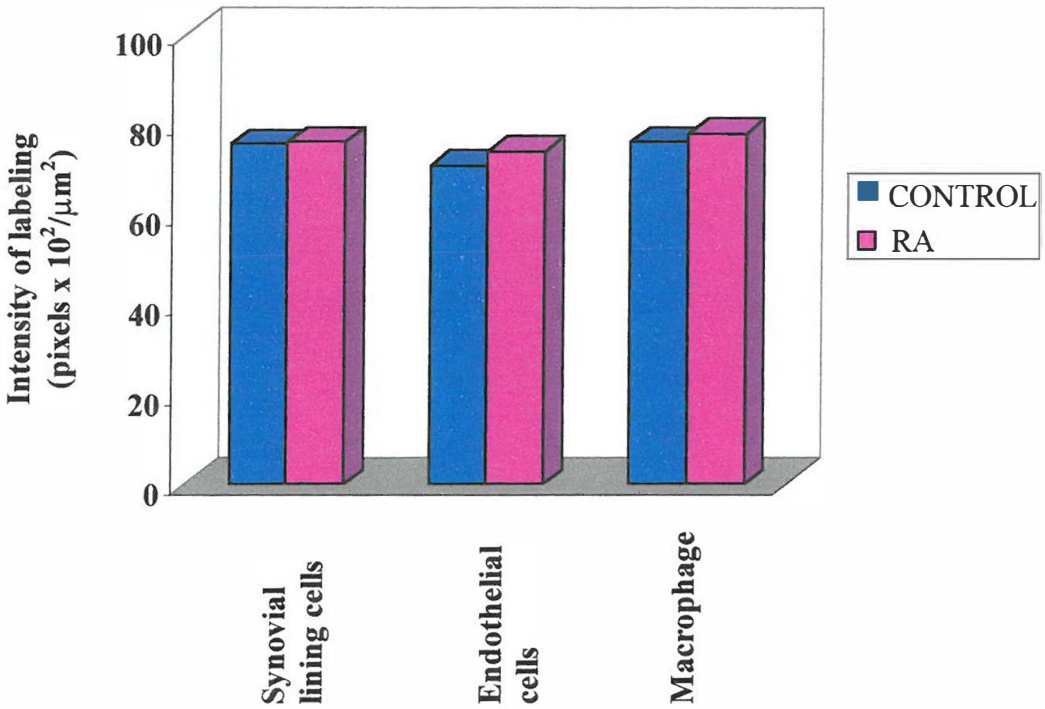


Figure 3.5 **Histogram of tissue kallikrein labeling on synovial tissue**

Table 3.2 **Intensity of labeling for tissue kallikrein in synovial tissue**

(pixels x $10^2/\mu m^2$)

	Synovial lining cells		Endothelial cells		macrophages	
	Control	**RA**	**Control**	**RA**	**Control**	**RA**
n	65	89	57	37	35	54
Mean	77	76	71	75	76	78
SD	12	11	8	10	8	8
*t-test	p > 0.05		p = <0.05		p > 0.05	

RA; rheumatoid arthritis, SD; standard deviation.
*students t-test for unequal variance

3.2.2 Immunolocalisation of the kinin B1 receptor

The kinin B1 receptors have been demonstrated in the neurons of the substantia gelatinosa in the spinal cord (Raidoo and Bhoola, 1997). Sections of the human spinal cord were therefore used as positive control tissue during the labeling runs (Fig. 3.6).

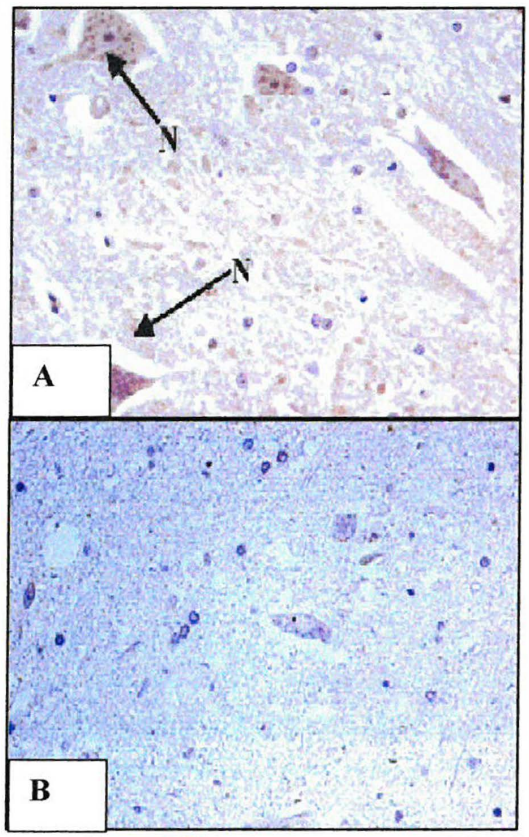


Figure 3.6 Photomicrograph of normal human spinal cord

Showing positive labeling for kinin B1 receptors in the neurones (N) of the substantia gelatinosa (A) and absence of labeling in the method control (B). Immunoperoxidase stain. Magnification x 400.

The labeling for the kinin B1 receptors in normal and rheumatoid synovium is shown in Fig. 3.7). Although visually there appeared to be an absence of labeling for the kinin B1 receptor on the normal synovial tissue, immunolabelling was quantified using image analysis. In both the normal and rheumatoid synovial tissue, labeling was demonstrated in the synovial lining cells, endothelial cells and subintimal fibroblasts and macrophages. There was a significant increase in the intensity of labeling in the synovial lining cells in the rheumatoid tissue compared to the control synovial tissue ($p < 0.01$) (Table 3.3 and Fig. 3.8).

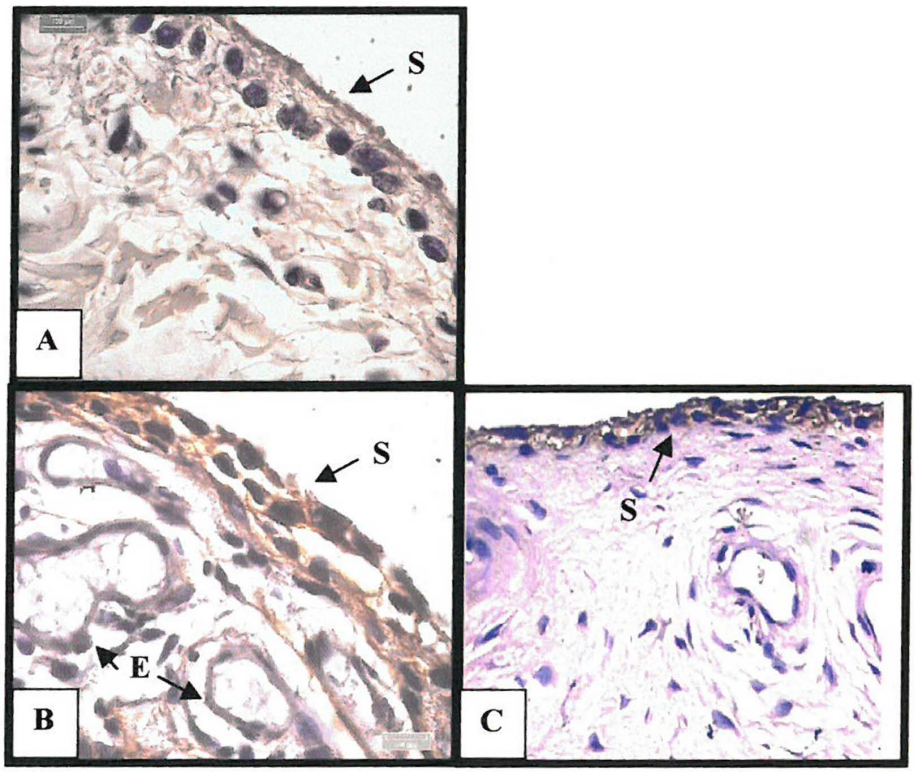


Figure 3.7 Immunovisualisation of kinin B1 receptors in control and rheumatoid synovial tissue

Intense labeling for the kinin B1 receptor is seen in the synovial lining cells (S) and endothelial cells (E) in the rheumatoid synovial tissue (B and C) compared to control (A) synovium. Immunoperoxidase stain. Magnification : x 1000 (A and B.); x 400 (C).

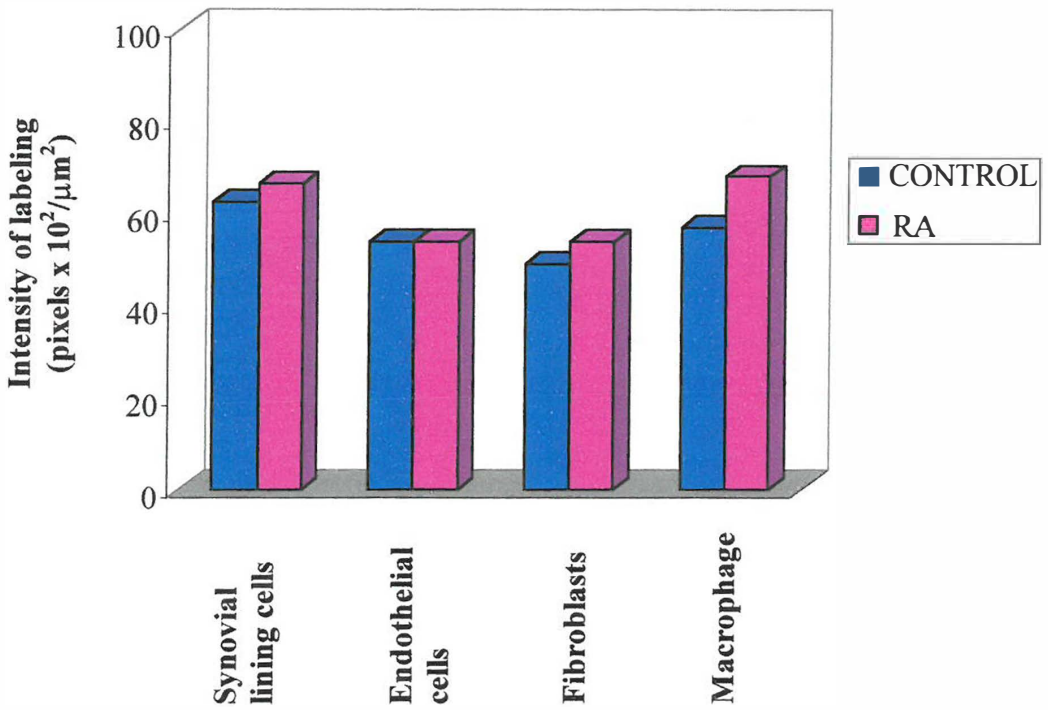


Figure 3.8 Histogram of kinin B1 receptor labeling on synovial tissue

Table 3.3 Intensity of labeling for kinin B1 receptor in synovial tissue (pixels x $10^2/\mu m^2$)

	Synovial lining cells		Endothelial cells		Fibroblasts		Macrophages	
	Control	RA	Control	RA	Control	RA	Control	RA
n	115	78	14	37	41	28	21	28
Mean	62	68	54	54	49	54	57	68
SD	15	15	16	15	13	14	23	12
*t-test	p< 0.01		p > 0.05		p > 0.05		p = 0.07	

RA; rheumatoid arthritis, SD; standard deviation.
* students t-test for unequal variance

3.2.3. Immunolocalisation of the kinin B2 receptor

Kinin B2 receptors have been localized on the collecting ducts and tubules of normal kidneys (Naidoo *et al.* 1996a). During each labeling experiment, normal human kidney tissue was used as the positive controls. For the method control, loss of labeling following preadsorption with an excess of the rabbit anti-human B2 receptor antibody and replacement of the primary antibody with PBS was demonstrated (Fig. 3.9)

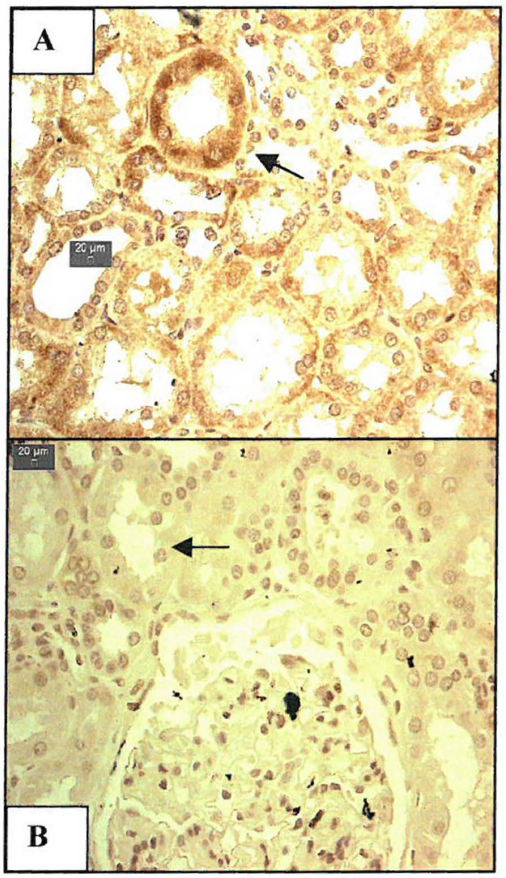


Figure. 3.9 Photomicrographs of control human kidney

Positive labeling for the kinin B2 receptor is seen on the collecting ducts and tubules (➔ A) and absence of labeling in the method control (B). Immunoperoxidase stain. Magnification x 400.

Intense labeling for the kinin B2 receptor was observed in the synovial lining layer, the endothelial cells and in the subintimal fibroblasts and macrophages in both the rheumatoid and control tissue. There were no significant differences (Fig. 3.10). (Table 3.4 and Fig. 3.11).

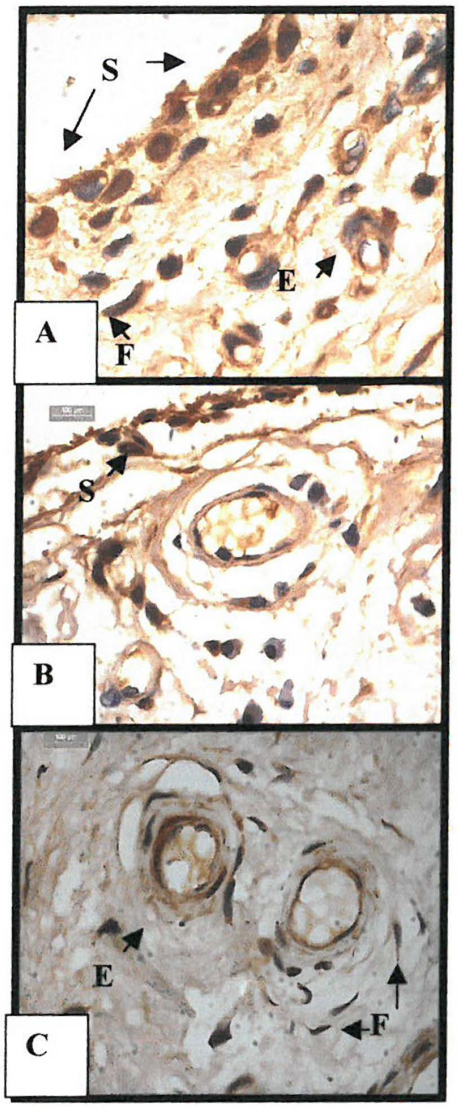


Figure 3.10 Immunovisualisation of kinin B2 receptors in control and rheumatoid synovial tissue

Intense labeling for the kinin B2 receptor is seen in the synovial lining cells (S), endothelial cells (E) and fibroblasts (F) in control (A) and rheumatoid synovial tissue (B and C). Immunoperoxidase stain. Magnification x 1000

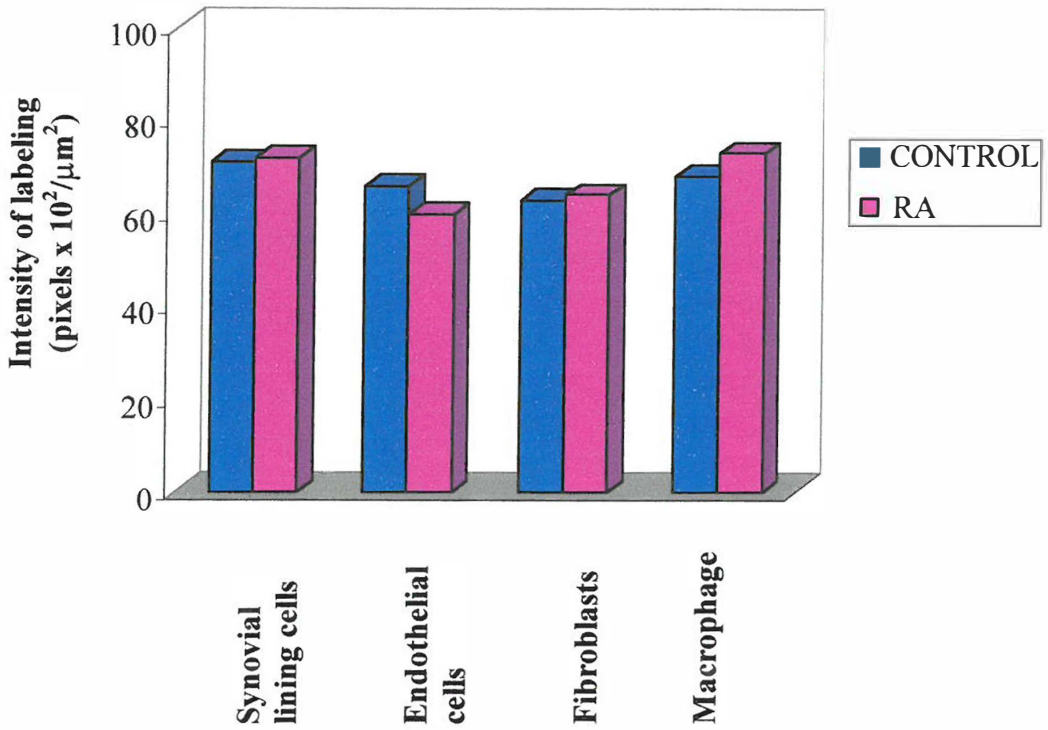


Figure 3.11 Histogram of kinin B2 receptor labeling on synovial tissue

Table 3.4 Intensity of labeling for kinin B2 receptor in synovial tissue

(pixels x $10^2/\mu m^2$)

	Synovial lining cells		Endothelial cells		Fibroblasts		Macrophages	
	Control	RA	Control	RA	Control	RA	Control	RA
N	115	78	14	37	41	28	21	28
Mean	71	72	66	60	63	64	68	73
SD	13	14	12	14	12	12	8	11
*t-test	p > 0.05ns		p > 0.05		p > 0.05		p = 0.08	

RA; rheumatoid arthritis, SD; standard deviation.
* students t-test for unequal variance

3.3 TISSUE KALLIKREIN ACTIVITY: SYNOVIAL FLUID

Tissue kallikrein has been localized in the granules of neutrophils. Since neutrophils degranulate in inflammation, it may be expected that TK would be released from the SF neutrophils in RA. This is supported by the loss of immunoreactivity of TK in SF neutrophils obtained from RA patients (Williams *et al.* 1997). TK has been previously identified in the synovial fluid from patients with arthritis (Worthy *et al.* 1990), and the levels were reported to be higher in RA compared to OA (Selwyn *et al.* 1989), suggesting that there is an increase in the concentration of TK in inflammatory synovitis. However, the correlation of TK levels in SF and disease activity has not been previously reported. In this study the TK activity and the concentration of TK were measured in synovial fluid obtained from 20 patients with RA and the levels correlated with measures of disease activity.

3.3.1 Measurements of TK

An enzymic (amidase) assay was used to measure TK activity. The TK activity was extrapolated from the standard curve obtained in 2.7.1 (Fig. 3.12).

An ELISA was used to determine the concentration of TK in the SF from 20 patients with RA. A standard curve of absorbance versus HUK concentration was plotted with the values expressed as ng/ml (Fig. 3.13). The concentrations of TK in the synovial fluid samples were extrapolated from this graph.

The mean enzymic activity of TK was 1.77 ± 0.85 ng/μg protein (range: 1.37 – 3.03 ng/μg protein), and the mean immunoreactive TK (ELISA) was 20.1 ± 36.5 ng/ml (range: 0.13 – 137.81 ng/ml). There was no direct correlation between the enzymic activity and antigenic TK.

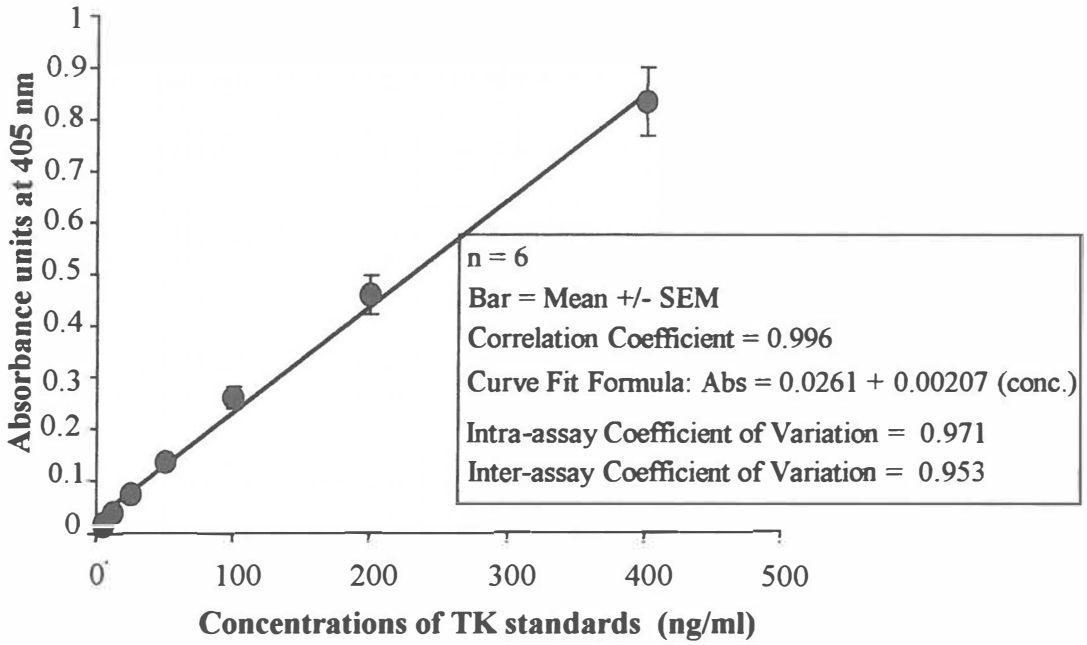


Figure 3.12 TK amidase assay : standard curve

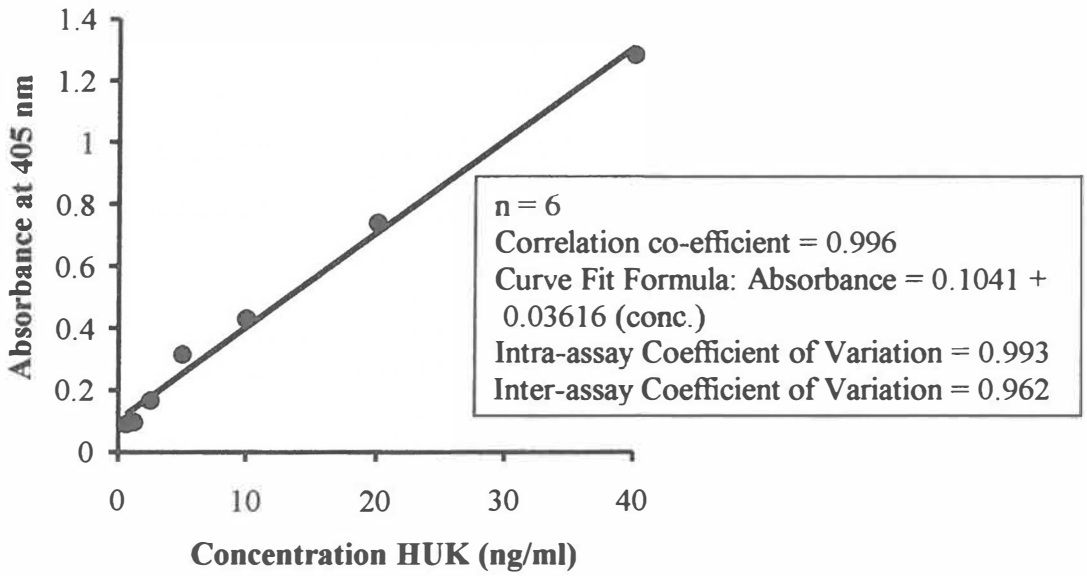


Figure 3.13 Tissue kallikrein ELISA Standard curve

3.3.2 Measurements of disease activity

In the RA patients, disease activity was determined using the following measures:

- Duration of *morning stiffness*: defined as the difference in the time of awakening and the time of maximum mobility during the day.
- *Swollen and tender joint counts*: a twenty-eight joint count (Fuchs *et al.* 1989) was used to determine the number of tender and swollen joints. A binary score of 0/1 was applied. Where there was no pain or tenderness, a score of 0 was given and a score of 1 was given for either a tender or swollen joint count (Appendix 3.5).
- *Pain* on a visual analogue scale. A 10 cm horizontal line was used to score pain from 0 (no pain) to 10 (worst possible pain).
- *Physician's global assessment of disease activity*. This is an objective evaluation of the patient's overall condition at the time of the assessment, and is based on the patient's disease, signs, functional capacity and the physical examination and is independent of the patient's global assessment. A 5- point scale was used (1=very good; 2 = good; 3 = fair; 4 = poor; 5 = very poor).
- *Patient's global assessment of disease activity.* The patient was asked the following question: "Considering all the ways your arthritis affects you, how are you feeling today?" The response was recorded using the 5-point scale as above.
- *Local activity index.* For the affected knee joint a local activity index was calculated using the following parameters :

  increased temperature over the joint

  pain on palpation or passive movement

  presence of effusion

  articular crepitus

  decreased range of movement

muscle atrophy

Each parameter was scored to be either absent (0); mild (1) or severe (2); with a total score ranging from 0 -14.

- The *modified health assessment questionnaire* (HAQ) (Kirwan and Reeback, 1986) was scored to provide an overall disability index from 0 (no disability) to 3 (complete dependence on others) (Appendix 3.6).
- The *erythrocyte sedimentation rate* (ESR, Westergren method) and C reactive protein were performed as biochemical indices of disease activity.
- A *disease activity score* (DAS) that includes the 28 joint count for tender and swollen joints and the ESR was calculated (Prevoo *et al.* 1995).

3.3.3. Correlation between synovial fluid TK and disease activity

The levels of enzymic activity and concentrations of antigenic TK were correlated with measures of disease activity. There was a significant negative correlation between the SF levels of enzymic TK and the 28 swollen joint count ($r = -0.464$; $p < 0.05$) (Fig. 3.14). There was no correlation between the measures of disease activity and the level of antigenic TK.

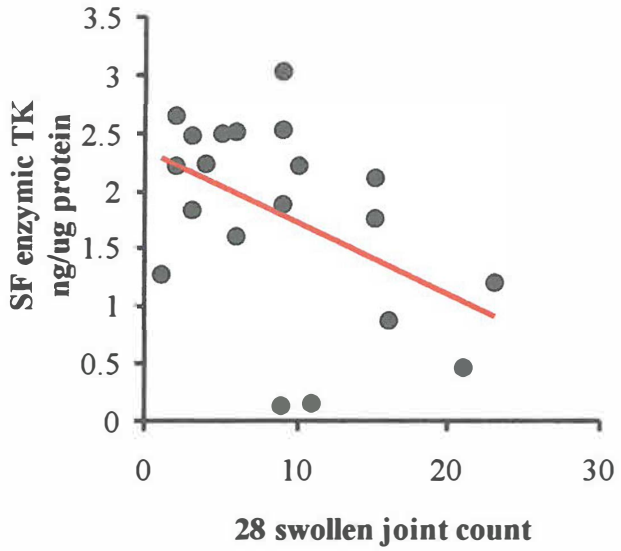


Figure 3.14 Correlation between the SF levels of enzymic TK and the 28 swollen joint count in RA patients.

3.4 KININ GENERATION: SYNOVIAL FLUID

Armstrong *et al* (1957) first reported the presence of a kinin-like substance in SF obtained from RA patients. However, it was only recently that specific and precise techniques for the measurement of kinins were developed. While, the capacity of synovial fluid to generate kinins has been previously demonstrated (Bond *et al.* 1997), kinin levels have not been correlated with clinical and biochemical measures of inflammation. In this study, the basal kinin levels and the capacity to generate kinins in the synovial fluid were measured in the 20 patients with RA. The levels of basal kinins were correlated with levels of generated kinins. Further, the levels of generated kinin were correlated with measures of disease activity (see section 3.3.2). All correlations were performed using Pearson's correlation co-efficient.

3.4.1 Measurement of basal and generated kinins

A competitive ELISA was used to measure basal kinin levels and the capacity to generate kinins in the synovial fluid (see section 2.8). The absorbance values obtained for the standard BK was used to plot a standard curve of absorbance versus log concentration (Fig. 3.15) from which the basal and generated kinin contents of the synovial fluid were determined (Bio Rad microplate manager software).

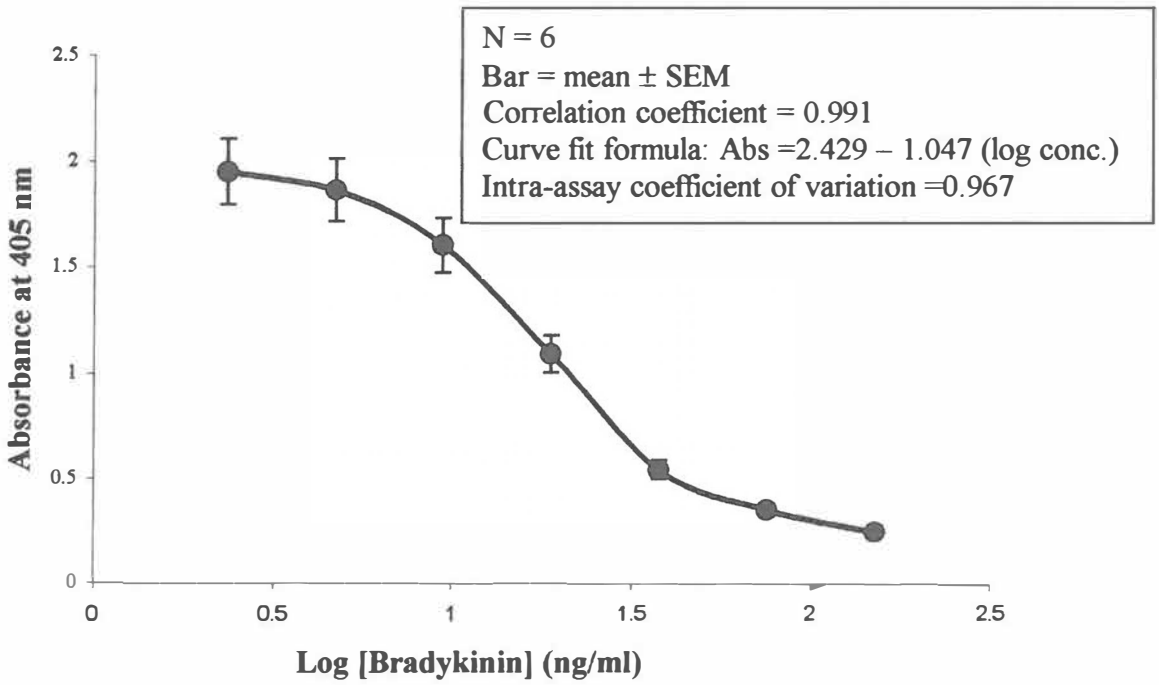


Figure 3.15 Kinin ELISA standard curve

The mean basal kinin level was 5.7 ± 6.1 ng/ml with a range of 0.43 to 26.17 ng/ml. The mean level of generated kinin was 80.6 ± 56.3 ng/ml with a range of 12.5 to 216.02 ng/ml (Table 3.5). There was a significant negative correlation between the basal kinin and generated kinin levels ($r = -0.454$; $p < 0.05$) (Fig 3.16).

Table 3.5 Basal and generated kinin levels in synovial fluid from patients with RA

	Basal kinin ng/ml	Generated kinins ng/ml
n	20	20
Mean	5.7 ± 6.1	80.6 ± 56.3
Range	0.43 to 26.17	12.5 to 216.02
Pearson's correlation	r = -0.454; p < 0.05	

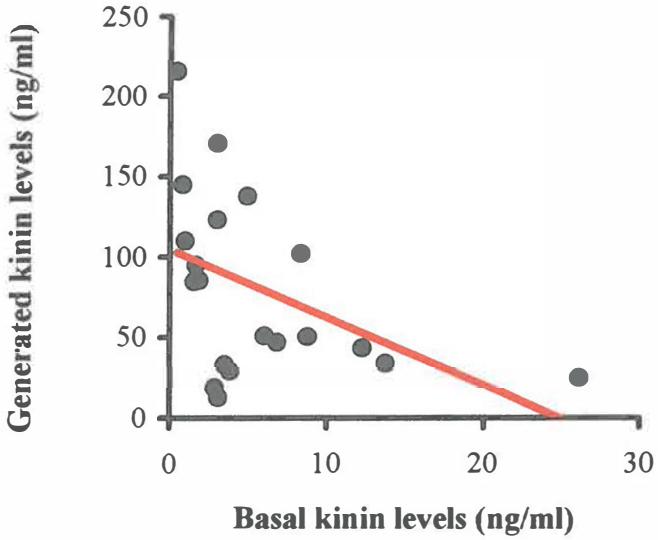


Figure 3.16 Correlation between levels of basal and generated kinins in the SF in RA patients.

3.4.2 Measurement of disease activity

The mean values for the measures of disease activity in the 20 patients in whom basal and generated kinins were measured in the SF, are presented in Table 3.6.

Table 3.6 Measures of disease activity

Index	Mean ± SD	Range
Duration of morning stiffness (min)	97.5 ± 122	0 - 480
Disease activity (VAS) (mm)	48.6 ± 28.6	0 – 98
Pain (VAS) (mm)	54.5 ± 25.4	9 - 90
Physician's global assessment of disease activity (Likert scale)	2.8 ± 0.7	1 - 4
Patients assessment of disease activity (Likert scale)	3.4 ± 0.8	2 - 4
28 tender joint count	5.6 ± 3.8	1 - 12
28 swollen joint count	6.8 ± 3.6	1 - 15
ESR (mm/hr)	50.5 ± 36.2	11 - 124
CRP mg/L	37.5 ± 24.7	5.3 – 73.7
HAQ	1.7 ± 0.8	0.125 - 3
Local activity index	12.6 ± 3.9	6 - 19
Disease activity score	5.1 ± 0.9	3.9 - 7

3.4.3 Correlation between measures of disease activity and basal kinin levels

The basal levels of kinins in the synovial fluid were correlated with the measures of disease activity. There was a significant negative correlation between SF basal kinins and the CRP ($r = -0.537$, $p < 0.05$) (Fig.3.17), and the disease activity score ($r = -0.458$; $p < 0.05$) (Fig. 3.18).

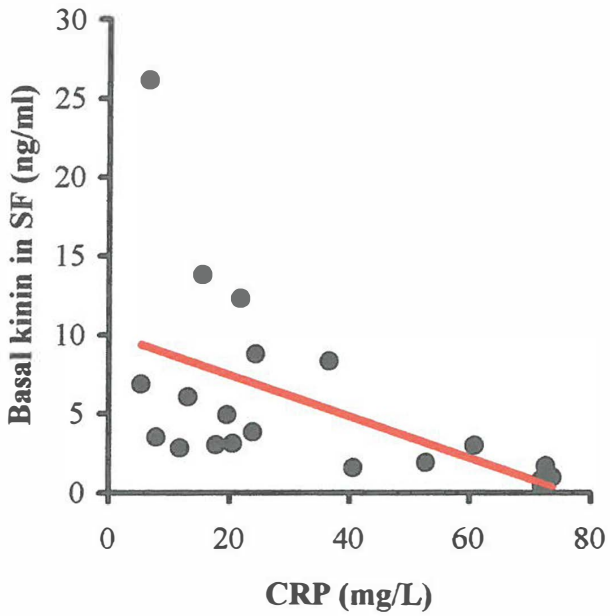


Figure 3.17 Correlation between the CRP and basal kinins in the SF in RA patients

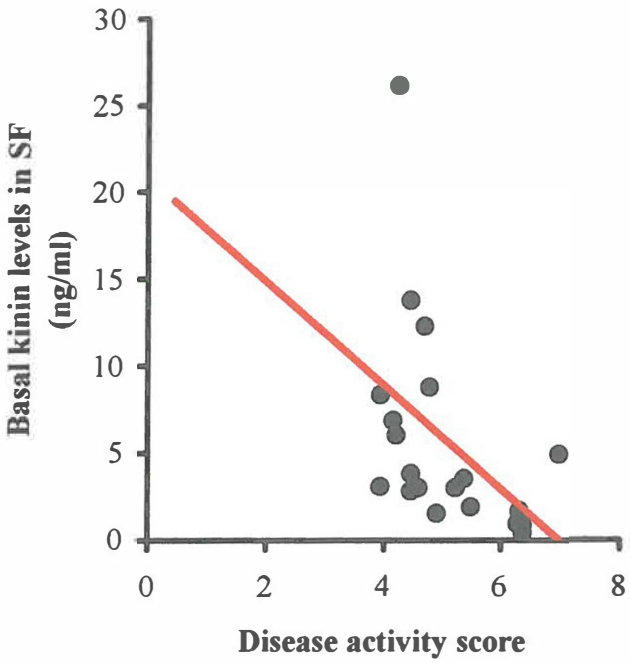


Figure 3.18 Correlation between the disease activity score and basal kinins in the SF in RA patients

3.4.4 Correlation between measures of disease activity and generated kinin levels

The relationship between the measures of disease activity and the levels of generated kinins in the SF was determined using the Pearsons' correlation co-efficient. There was a significant correlation between the generated kinin levels and the 28 tender and swollen joint counts, ESR, CRP, HAQ and the disease activity score (Table 3.7 and Figs. 3.19 – 3.23).

Table 3.7 Correlation between measures of disease activity and generated kinin levels in synovial fluid from the 20 patients with RA

Index	Pearson's correlation r	p value
Tender joint count	0.536	< 0.05
Swollen joint count	0.509	< 0.05
ESR	0.598	< 0.01
CRP	0.725	< 0.01
DAS	0.676	< 0.01

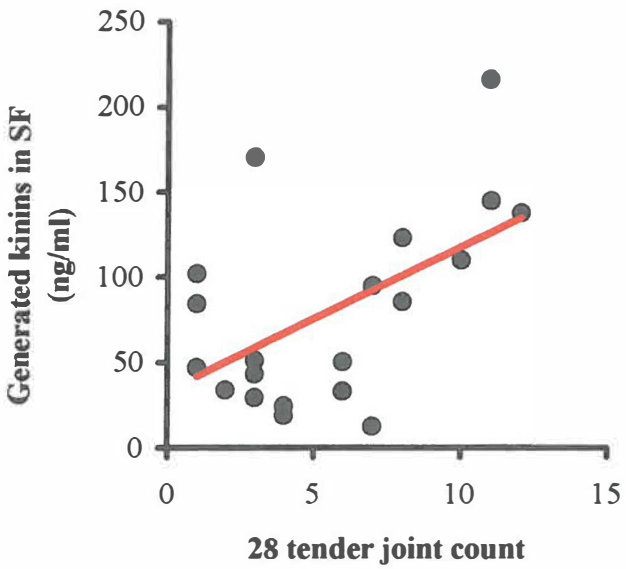


Figure 3.19: Correlation between the 28 tender joint count and the level of SF generated kinins in RA patients.

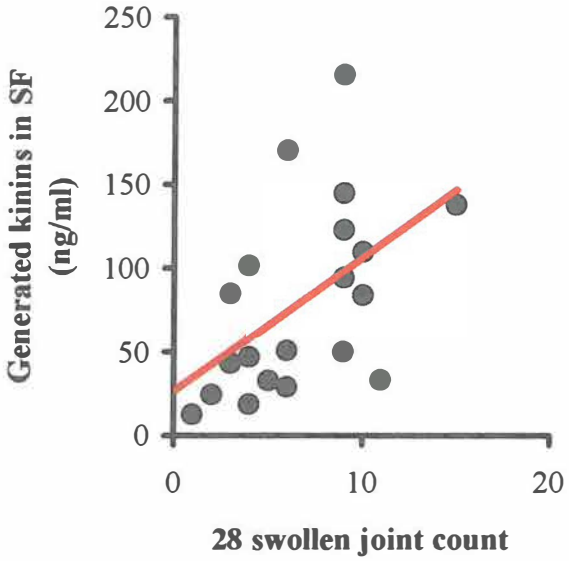


Figure 3.20 Correlation between 28 swollen joint count and the level of SF generated kinins in RA patients.

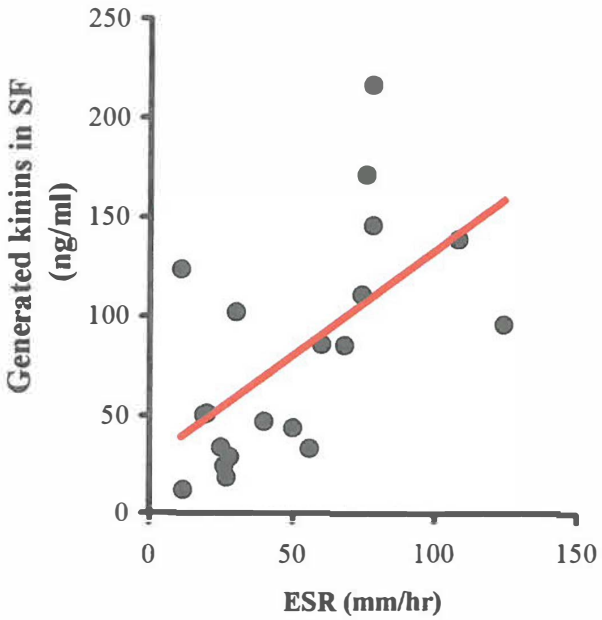


Figure 3.21 Correlation between the ESR and the level of SF generated kinins in RA patients.

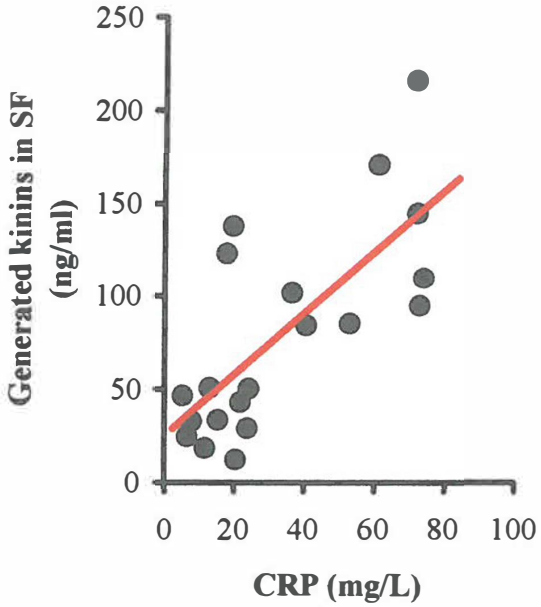


Figure 3.22 Correlation between CRP and SF generated kinins in RA patients.

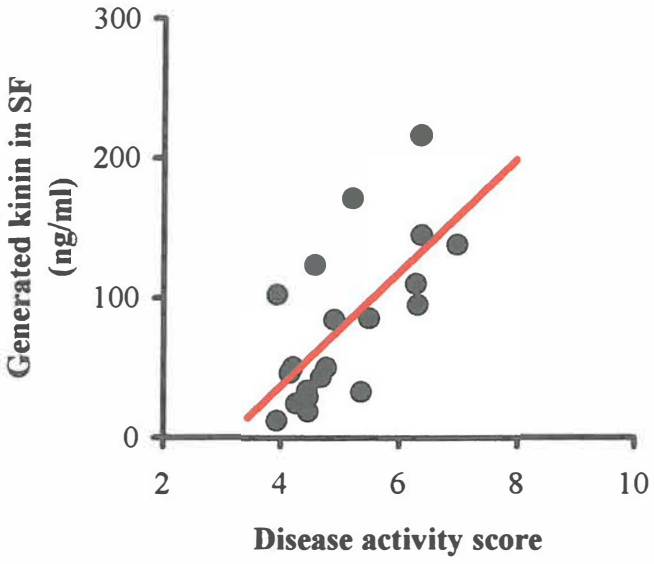


Figure 3.23: Correlation between the disease activity score and SF generated kinins in RA patients.

3.5 CYTOKINE STUDY : SYNOVIAL FLUID

Interleukin 1β and tumour necrosis factor are proinflammatory cytokines, and are thought to play a central role in the immune response observed in RA (Brennan *et al.* 1992a). IL 1β has the ability to recruit cells to the site of inflammation, stimulate the production of other cytokines, augment lymphocyte production, induce endothelial cell proliferation, activate neutrophils to synthesize and release prostaglandins, and induce hepatic production of acute phase proteins. The bone resorption and cartilage destruction in RA is thought to be mediated via IL 1β induced stimulation of prostaglandins and metalloproteinases. Similarly, TNF α is also a potent stimulator of the inflammatory response. The pro-inflammatory actions of TNF α are mediated either directly or through the stimulation of other cytokines and secondary mediators such as prostaglandins, leucotrienes and oxygen free radicals. In RA, TNF α stimulates bone resorption, enzyme release from synovial cells and release of acute phase proteins.

High levels of IL-1β and TNF α have been reported in the synovial fluid obtained from RA patients (Arend and Dayer, 1995; Manicourt *et al.* 1993). In the present study, both IL 1β and TNF α were measured in the synovial fluid of 20 RA patients, and the levels of IL 1β and TNF α correlated with markers of disease activity.

3.5.1 Interleukin 1 β

A quantitative sandwich enzyme immunoassay technique using a commercial ELISA kit (Quantikine HS, R&D Systems) was used to measure IL 1 β levels in the SF. A standard curve was plotted using the absorbance of the standards versus the concentration of the

standards (Fig. 3.24) and the concentrations of IL 1 β in the SF samples was read of this graph in pg/ml.

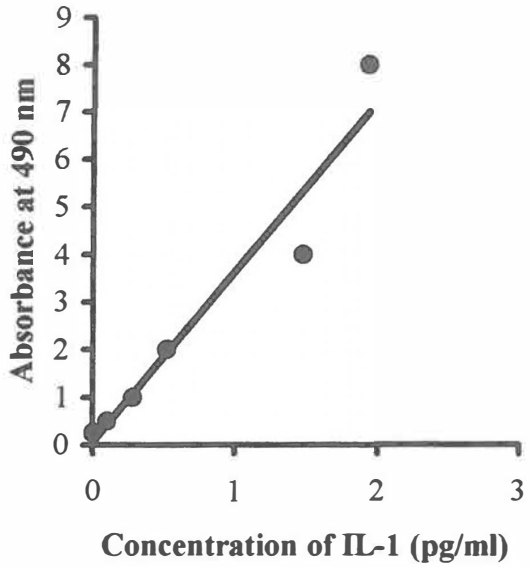


Figure 3.24 IL 1 β Standard curve

The mean IL 1 β level was 8.42 ± 9.67 ρg/ml with a range from 0.27 to 29.04 ρg/ml.

Correlation with disease activity

There was a significant correlation between the SF levels of IL 1β and pain (VAS), physician's global assessment of disease activity, 28 tender joint count and CRP (Table 3.8 and Figs. 3.25 - 3.28). Although there appeared to be a correlation between IL 1β and disease activity score, the relationship was not significant (Fig. 3.29).

Table 3.8 Correlation between measures of disease activity and IL 1β levels in synovial fluid from 20 patients with RA

Measure of disease activity	Pearson's correlation r	p value
28 tender joint count	0.472	< 0.05
Pain (VAS)	0.462	< 0.05
Physician's global assessment of disease activity	0.549	< 0.05
CRP	0.536	< 0.05
DAS	0.412	= 0.07

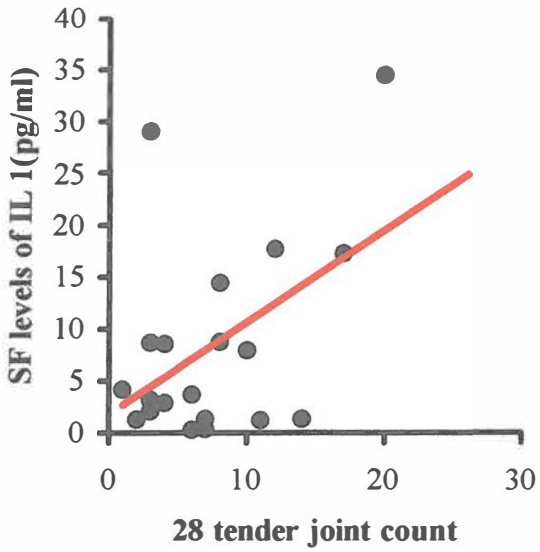


Figure 3.25 Correlation between SF levels of IL 1β and the 28 tender joint count in RA patients.

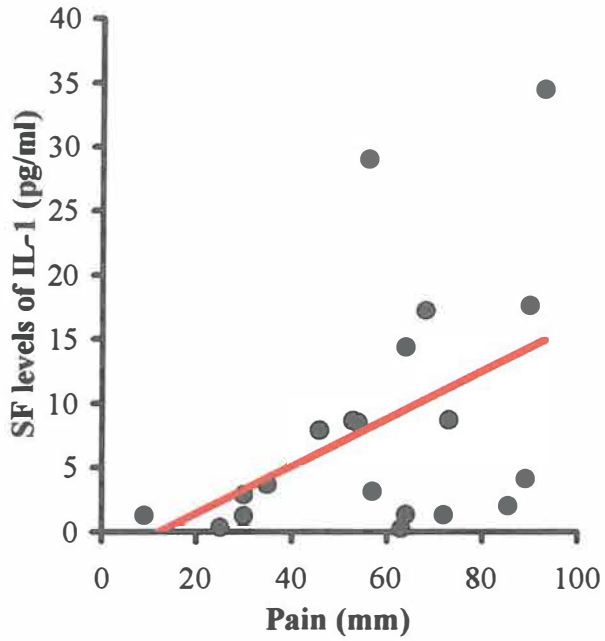


Fig. 3.26 **Correlation between SF levels of IL-1β and pain (VAS) in RA patients**

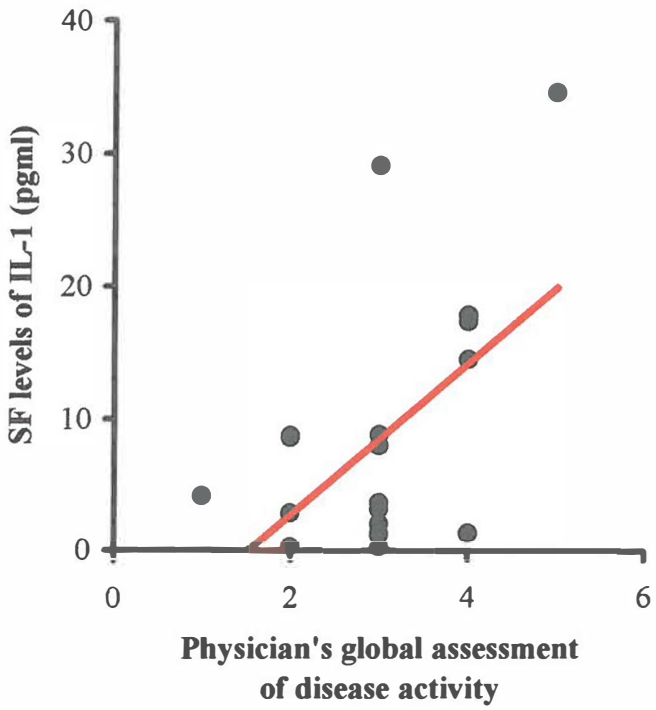


Figure 3.27 **Correlation between SF levels of IL 1β and physician's global assessment of disease activity in RA patients**

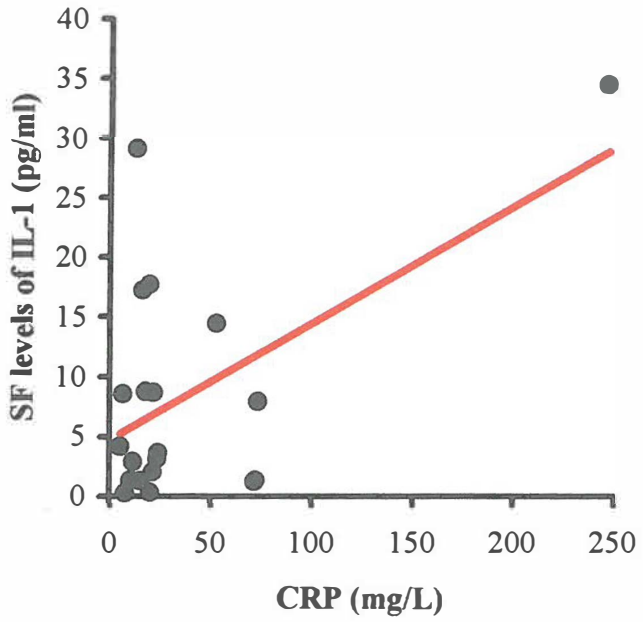


Figure 3.28 **Correlation between SF levels of IL 1β and CRP in RA patients**

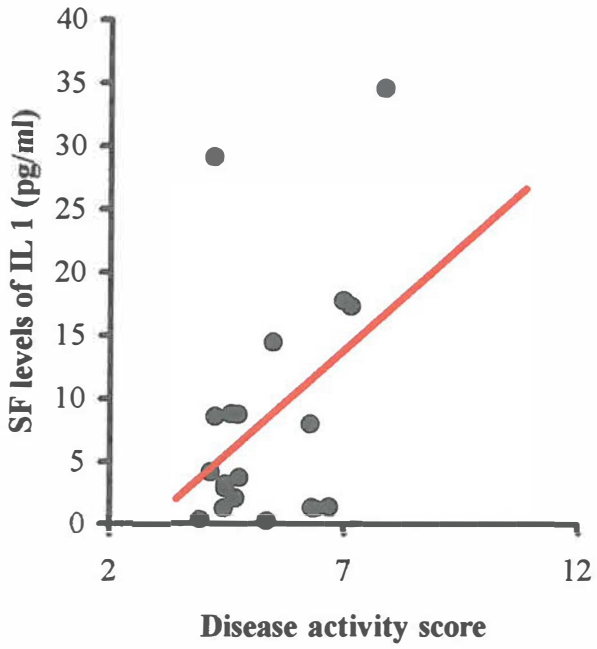


Figure 3.29 **Correlation between the SF levels of IL-1β and the disease activity score in RA patients.**

3.5.2 Tumour necrosis factor α

A quantitative sandwich enzyme immunoassay technique using a commercial ELISA kit (Quantikine HS, R&D Systems) was used to measure TNF α levels in the SF. A standard curve was plotted using the absorbance of the standards versus the concentration of the standards (Fig. 3.30) and the concentrations of the concentration of TNF α in the SF samples was read of this graph in pg/ml.

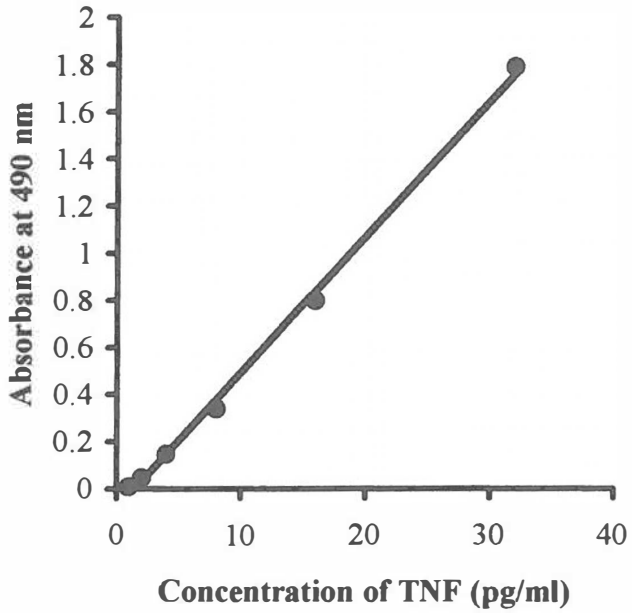


Figure 3.30 TNF α Standard curve

The mean level of TNF α in the synovial fluid from 20 patients with RA was 15.71 ± 10.57 pg/ml with a range of 4.41 to 41.42 pg/ml.

Correlation with disease activity

There was a significant correlation between the levels of synovial fluid TNF α and the 28 tender joint count and CRP (Table 3.9 and Figs. 3.31 & 3.32).

Table 3.9 Correlation between indices of disease activity and TNF α levels in synovial fluid from 20 patients with RA

Index	Pearson's correlation r	p value
Tender joint count	0.458	< 0.05
CRP	0.653	< 0.01

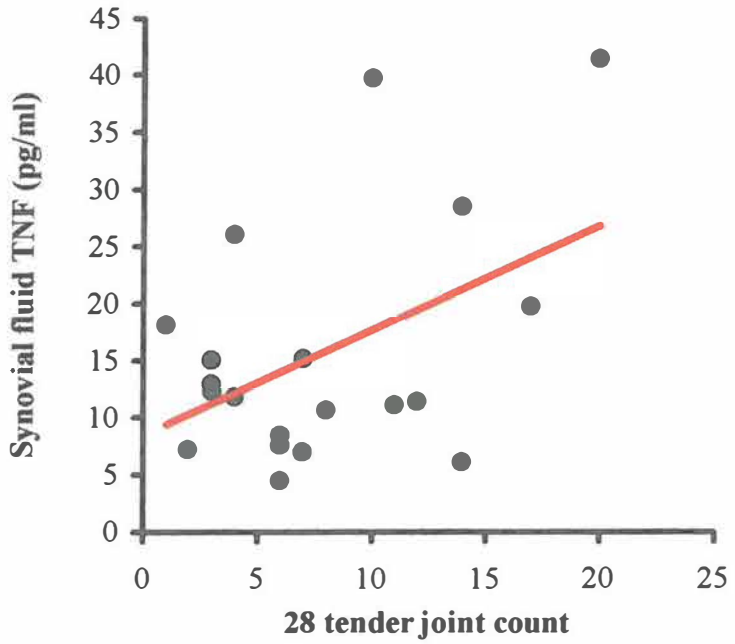


Figure 3.31 Correlation between SF levels of TNF α and the 28 tender joint count in RA patients

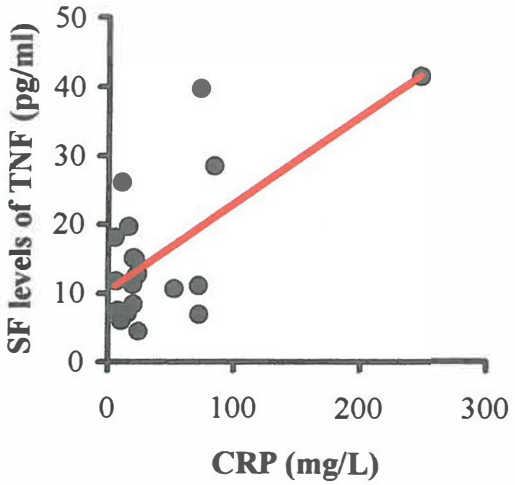


Figure 3.32 Correlation between SF levels of TNF α and CRP in RA patients

3.5.3 Correlation between TNF α and IL-1 β

Synovial fluid levels of both TNF α and IL-1 β were available in 16 patients with RA. There was a significant correlation between the TNF α and IL-1 β levels ($r=0.591$; $p < 0.05$) (Fig. 3.33).

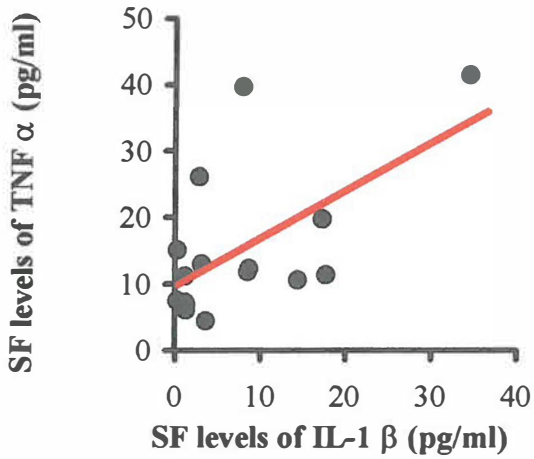


Figure 3.33 Correlation between SF levels of TNF α and IL 1 β in RA patients

3.6 CORRELATION BETWEEN THE KININ AND CYTOKINE CASCADES

To determine the relationship between the kinin and cytokine cascades in RA, the levels of TK and basal and generated kinins in the synovial fluid were compared to the levels of IL - 1β and TNF α.

3.6.1 Correlation between SF levels of IL-1β and enzymic TK

Although there was a trend towards a negative correlation between the SF levels of enzymic TK and IL-1β, this was not consistent (Fig. 3.34).

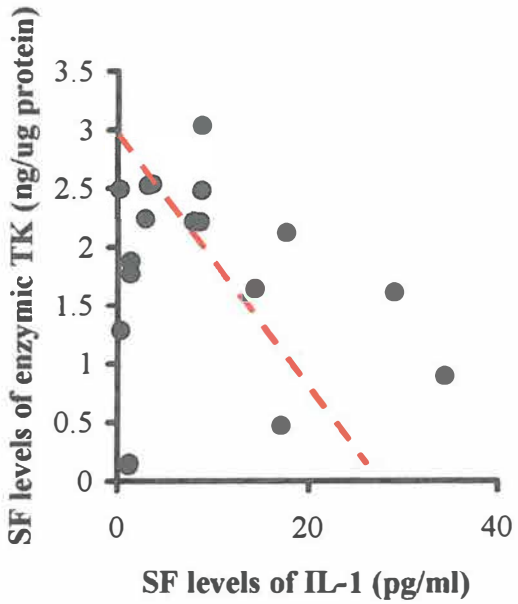


Figure 3.34 Correlation between the SF levels of IL-1β and enzymic TK in RA patients

3.6.2 Correlation between SF levels of IL-1β and generated kinins

There appeared to be no direct correlation between the SF levels of IL-1β and generated kinins (Fig. 3.35).

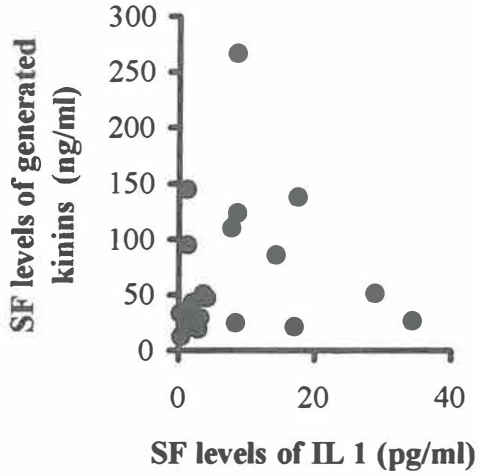


Figure 3.35 Correlation between SF levels of IL-1β and generated kinins in RA patients

However, when the relationship was further analyzed, there was a biphasic correlation, namely a positive correlation with low to moderate levels of IL-1β ($r = 0.51$; $p < 0.05$) and a negative correlation with high levels of IL-1β ($r = -0.50$; $p < 0.05$) (Figs. 3.36 & 3.37).

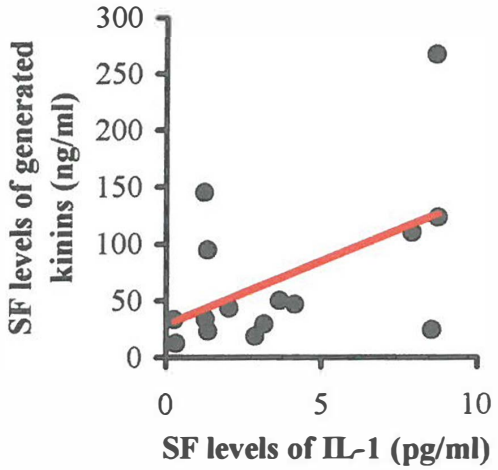


Figure 3.36 Correlation between low to moderate levels of IL-1β and generated kinins in the SF of RA patients.

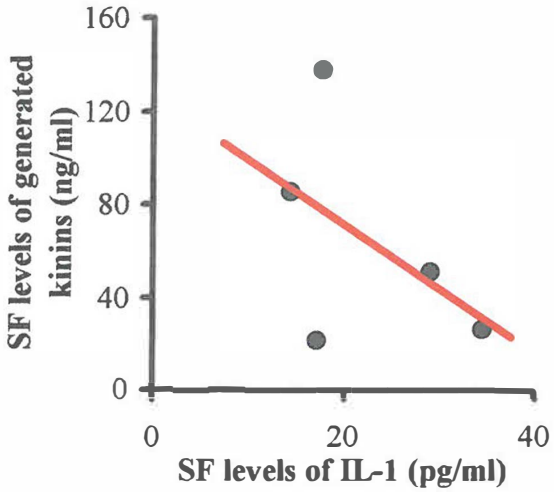


Figure 3.37 Correlation between high levels of IL-1β and generated kinins in the SF of RA patients

3.6.3 Correlation between SF levels of TNF α and the kinin cascade.

There was no correlation between the levels of TNF α and either enzymic TK or generated kinin levels in the SF.

3.7 CIRCULATING AND SYNOVIAL FLUID NEUTROPHILS

The kinin moiety, within the kininogen molecule, and the kinin B2 receptor has been identified on the surface and TK within the granules of normal circulating neutrophils (Figueroa *et al.* 1992; Haasemann *et al.* 1994; Figueroa *et al.* 1989). Since there is an efflux of neutrophils into the SF in the inflamed joints, the SF and circulating neutrophils of RA patients were harvested and studied to determine the status of the kinin cascade on neutrophils in RA.

The air-dried neutrophil slides (see section 2.5.1) were first stained with May Groenwald Giemsa stain to confirm the presence of neutrophils (Fig. 3.38). Thereafter, slides of synovial fluid and circulating neutrophils from 8 patients with RA and circulating neutrophils from 8 healthy volunteers were immunostained to detect (i) the presence or absence of the kinin moiety within the kininogen molecule, (ii) TK and the (iii) kinin B1 and B2 receptors. The slides were viewed under a confocal microscope. For each subject, images were generated from 10 neutrophils, and the intensity of immunofluorescence image quantified and expressed as pixels x $10^2/\mu m^2$ per subject.

The mean intensity of staining for the kinin moiety, TK, kinin B1 and B2 receptors in the control neutrophils was then compared to that present on the circulating and SF neutrophils harvested from RA patients. Further, the intensity of labeling for these antigens was correlated with measures of disease activity (see section 2.3).

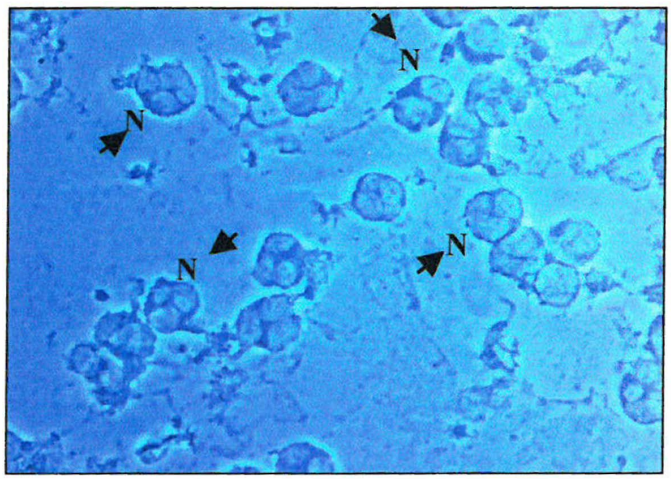


Figure 3.38 **May Groenwald Giemsa stain showing the presence of neutrophils (N). Magnification x 40.**

3.7.1 Immunolocalisation of immunoreactive tissue kallikrein

Tissue kallikrein specifically localizes in the duct cells of the submandibular salivary gland. Human salivary gland was therefore used as positive control tissue for TK labeling during each immunocytochemistry run (Fig. 3.39).

The TK labeling in the circulating neutrophils from healthy volunteers and the circulating and synovial fluid neutrophils from the RA patients is shown in Fig. 3.40. The mean intensity for immunoreactive TK on the circulating neutrophils from the healthy volunteers was 19.5 pixels x $10^2/\mu m^2$ with a median of 18 pixels x $10^2/\mu m^2$ (Fig 3.41 and 3.42 and Table 3. 10). For the circulating and SF neutrophils from the RA patients, the mean intensity for TK was 15.5 pixels x $10^2/\mu m^2$ and 12.3 pixels x $10^2/\mu m^2$ respectively, with corresponding medians of 11 pixels x $10^2/\mu m^2$ and 9.5 pixels x $10^2/\mu m^2$.

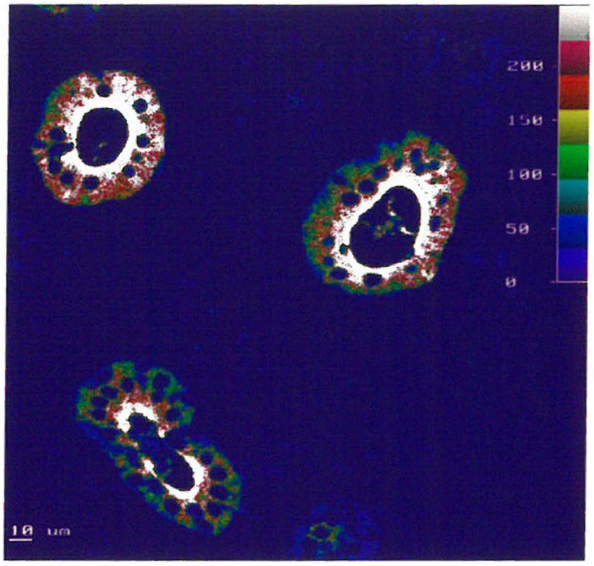


Figure 3.39 Confocal micrograph positive labeling for TK in the duct cells of normal human salivary gland.

The intensity of immunofluorescence is shown on the colour strip: red to white: maximum, green to yellow = moderate, light blue = low and purple to blue = zero

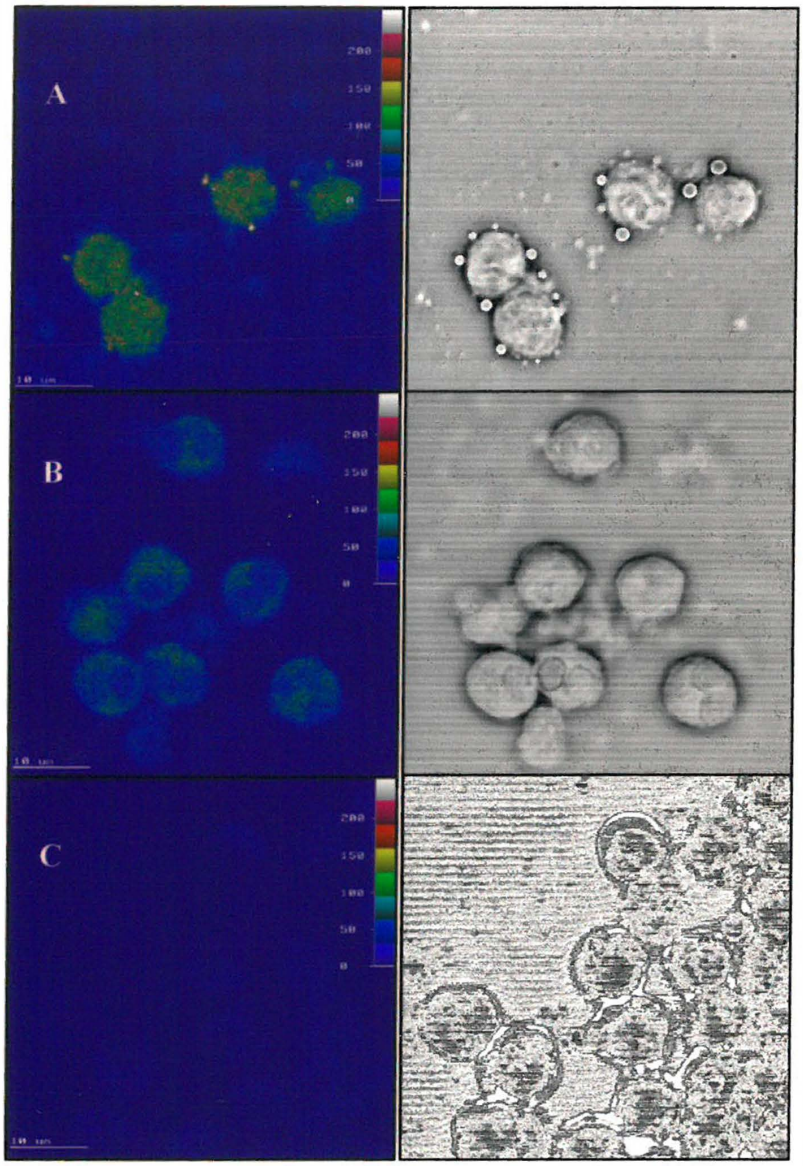
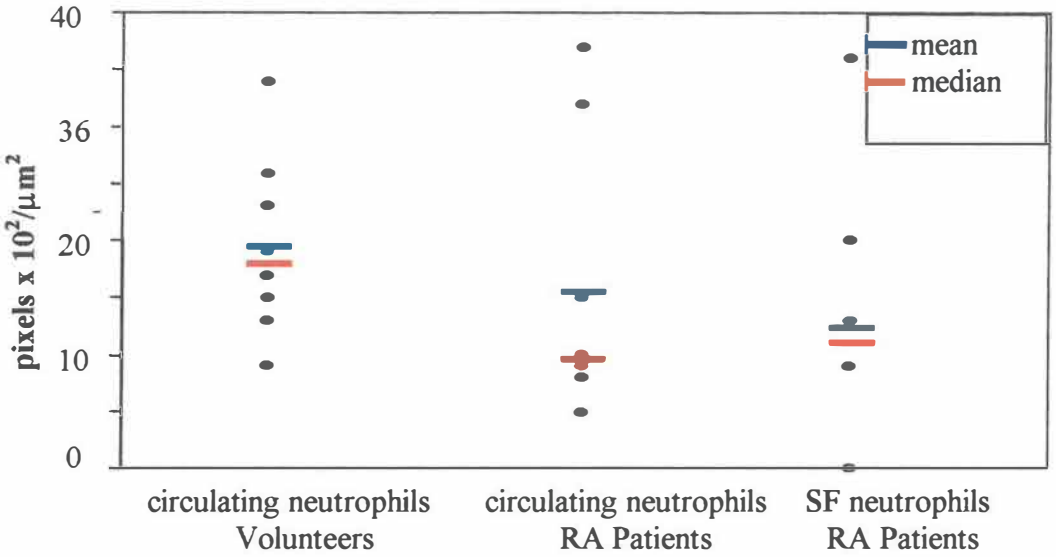


Figure 3.40 Immunovisualisation of tissue kallikrein in neutrophils

Immunolabelling of tissue kallikrein on circulating neutrophils of (A) volunteers, and (B) circulating neutrophils and (C) synovial fluid neutrophils of RA patients. The intensity of immunofluorescence is shown on the colour strip: red to white: maximum, green to yellow = moderate, light blue = low and purple to blue = zero. The corresponding phase contrast images are illustrated in the second panel.



(patients: n=8; neutrophils: n =10 neutrophils per patient.)

Figure 3.41 Intensity of immunolabelling of tissue kallikrein in neutrophils

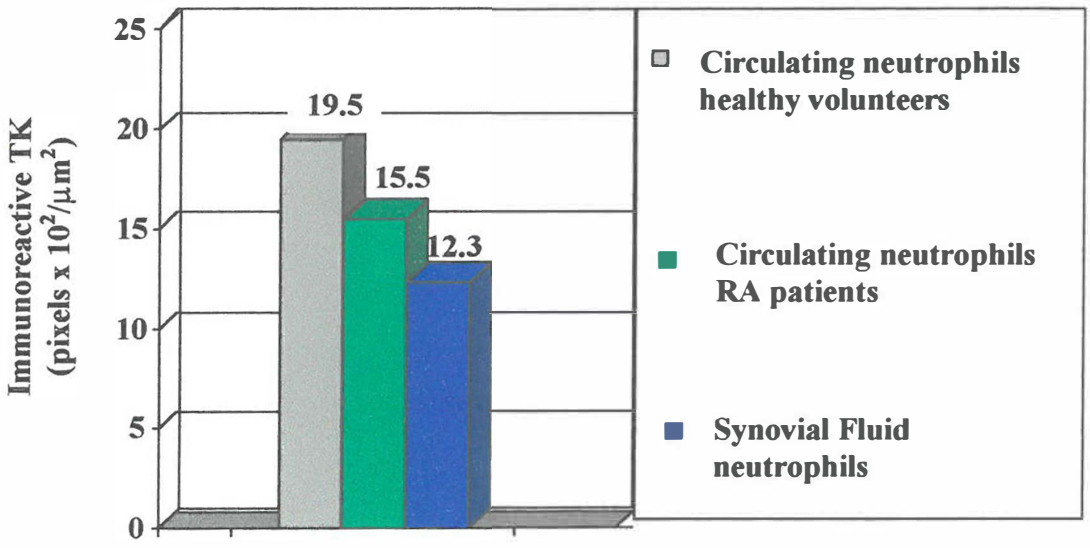


Figure 3.42 Histogram of immunoreactive TK in neutrophils showing mean values for the intensity of labeling of TK (in pixels x $10^2/\mu m^2$ on the neutrophil membrane

When comparing the mean intensity of TK labeling in the circulating neutrophils from healthy volunteers (n=8) and the circulating and synovial fluid neutrophils of the RA patients n=8), there was no statistical difference ($p > 0.05$). However, when the intensity of labeling of the SF neutrophils (n=80) from the RA patients was compared to the circulating neutrophils (n=80) of healthy volunteers, there was a significant loss of TK labeling in the SF neutrophils of the RA patients (1-Way ANOVA, $p < 0.01$). The details for each subject are presented in Table 3.10.

Correlation with indices of disease activity

There was no significant correlation between the immunoreactivity of TK on the circulating neutrophils from the RA patients and the measures of disease activity.

Table 3.10 Values for the intensity of immunolabelling for tissue kallikrein (pixels x $10^2/\mu m^2$)

No	(i) Circulating neutrophils healthy volunteers	(ii) Circulating neutrophils RA patient	(iii) SF neutrophils RA patient
1	13	15	0
2	36	36	13
3	19	8	0
4	26	9	0
5	17	10	9
6	23	8	36
7	15	5	20
8	9	37	20
Mean	**19.5**	**15.5**	**12.3**
Median	**18**	**9.5**	**11**
Kruskal -Wallis	**(i) vs. (ii) : > 0.05**		**(i) vs. (iii) : > 0.05**

3.7.2 Immunolocalisation of the kinin moiety

The kinin moiety has been previously localized on normal human neutrophils (Bhoola *et al.* 1992). Loss of immunolabelling following preadsorption with an excess of the monoclonal mouse antibradykinin antibody and replacement of the primary antibody with PBS served as the negative method control at each labeling run.

On the circulating neutrophils from the healthy volunteers, immunolabelling was observed as a peripheral rim (Fig. 3.43A). The mean intensity of immunolabelling was 20.8 pixels x $10^2/\mu m^2$ with a median of 20 pixels x $10^2/\mu m^2$ (Figs. 3.44 & 3.45 and Table 3.11). In the RA patients, there was a loss of the kinin moiety from both the SF and circulating neutrophils (Fig. 3.38B and C). The mean and median intensities for the kinin moiety on the SF neutrophils were 11 pixels x $10^2/\mu m^2$ and 11.5 pixels x $10^2/\mu m^2$ respectively (Fig.3.44 & 3.45 and Table 3.11). On the circulating neutrophils, the mean intensity was 3.8 pixels x $10^2/\mu m^2$ and the median 1 pixels x $10^2/\mu m^2$.

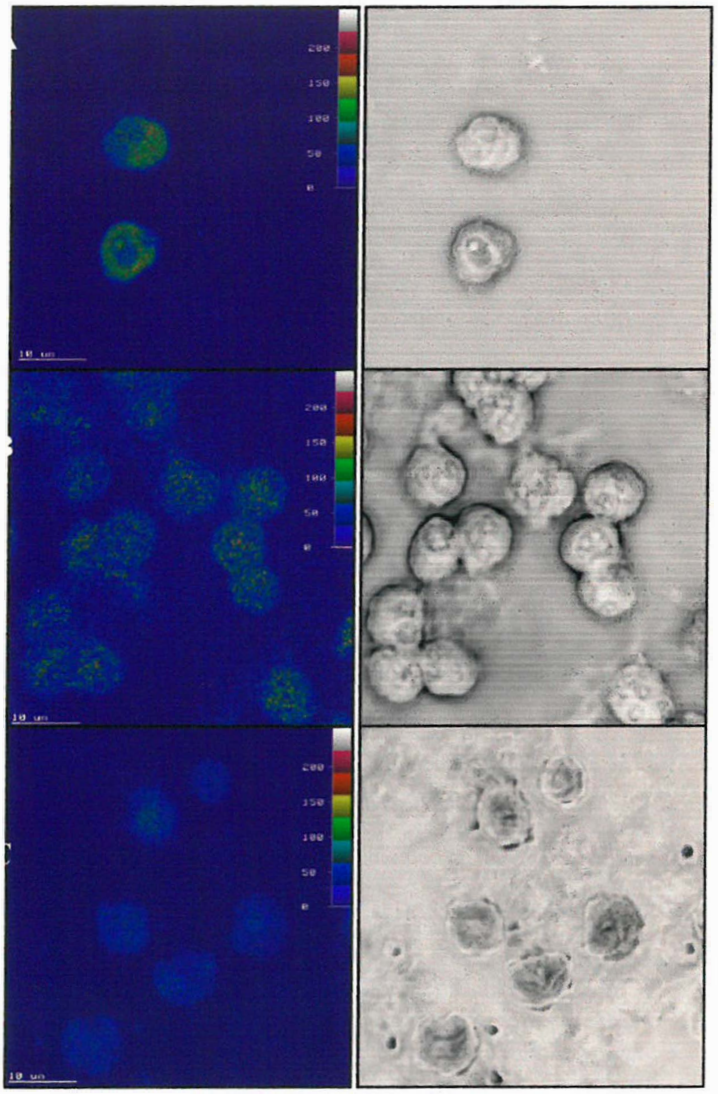
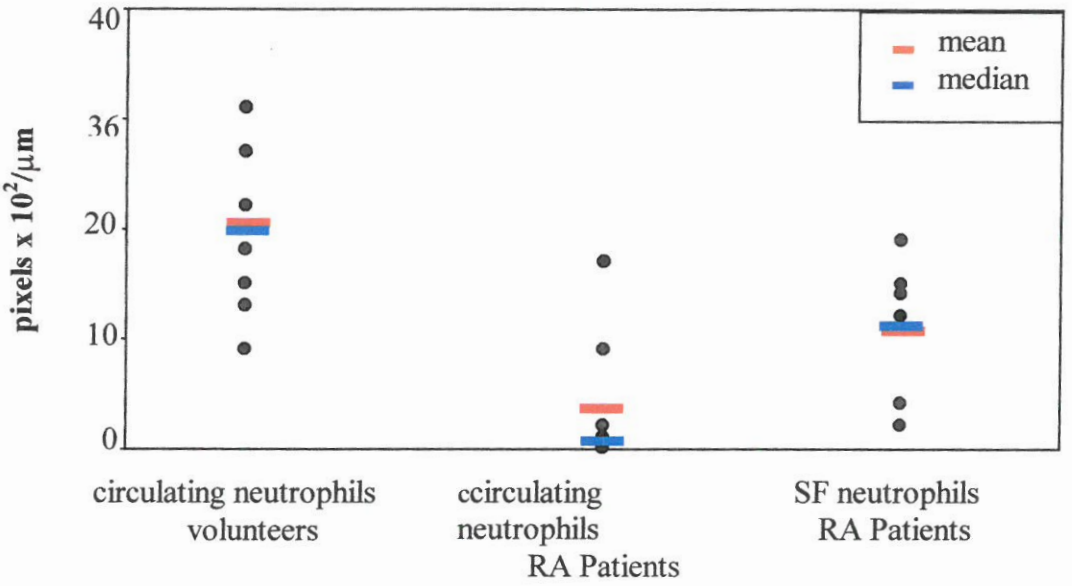


Figure 3.43 Immunovisualisation of the kinin moiety on the neutrophil membrane

Immunolabelling of the kinin moiety on circulating neutrophils of volunteers (A), and the circulating (B) and synovial fluid (C) neutrophils of RA patients. The intensity of immunoflourescence is shown on the colour strip: red to white = maximum, green to yellow = moderate, light blue = low and purple to blue = zero. The corresponding phase contrast images are illustrated in the second panel.



(patients: n = 8; neutrophils: n =10 neutrophils per patient.)

Figure 3.44 Intensity of labeling for the kinin moiety on the external surface of neutrophils

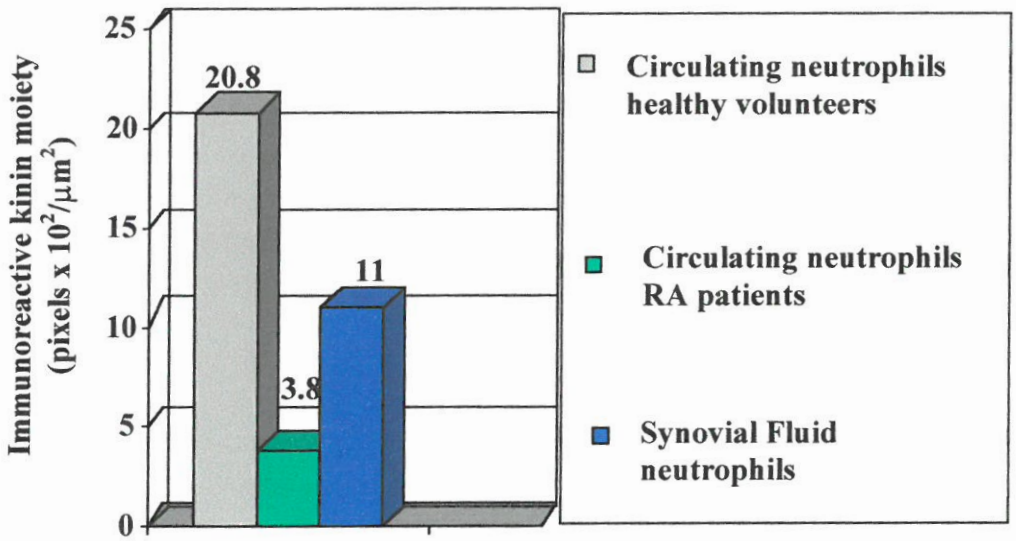


Figure 3.45 Histogram of immunoreactive kinin moiety on neutrophils showing mean values for the intensity of labeling of the kinin moiety (in pixels x $10^2/\mu m^2$on the neutrophil membrane

The Kruskal Wallis test was used to compare the intensity of the kinin moiety on the circulating neutrophils of the control subjects to that on the circulating and SF neutrophils of the RA patients. The loss of the kinin moiety was statistically significant different between the circulating neutrophils from the controls and RA patients ($p < 0.01$), as well as between the circulating neutrophils from the controls and the SF neutrophils from the RA patients ($p < 0.05$). Although there appeared to be a greater loss of intensity on the circulating neutrophils compared to the SF neutrophils in the RA patients, this finding was not statistically significant ($p > 0.05$)

Table 3.11 Intensity of immunolabelling for the kinin moiety (pixels x $10^2/\mu m^2$)

No	(i) Circulating neutrophils healthy volunteers	(ii) Circulating neutrophils RA patient	(iii) SF neutrophils RA patient
1	9	17	4
2	22	9	12
3	27	1	11
4	18	2	19
5	37	0	11
6	13	0	15
7	15	1	14
8	37	0	2
Mean	**20.8**	**3.8**	**11**
Median	**20**	**1**	**11.5**
Kruskal-Wallis	**(i) vs. (ii) : < 0.05**		**(i) vs. (iii) : < 0.01**

There was no correlation between the kinin moiety immunoreactivity and measures of disease activity.

3.7.3 Immunolocalisation of the kinin B1 receptor

The kinin B1 receptors have been demonstrated in the neurons of the substantia gelatinosa in the spinal cord (Raidoo and Bhoola, 1997). Sections of the human spinal cord were therefore used as positive control tissue during the labeling runs (Fig. 3.46).

Kinin B1 receptors were immunolocalised on the circulating neutrophils from healthy volunteers and the circulating and SF neutrophils of RA patients (Fig. 3.47). The mean intensity for kinin B1 receptor on the circulating neutrophils from the healthy volunteers was 8.1 pixels x $10^2/\mu m^2$ with a median of 5.5 pixels x $10^2/\mu m^2$ (Fig 3.48 & 49 and Table 3. 12). For the circulating and SF neutrophils from the RA patients, the mean intensity for the B1 receptor was 14.5 pixels x $10^2/\mu m^2$ and 16.8 pixels x $10^2/\mu m^2$ respectively, with corresponding medians of 13.3 pixels x $10^2/\mu m^2$ and 15.6 pixels x $10^2/\mu m^2$.

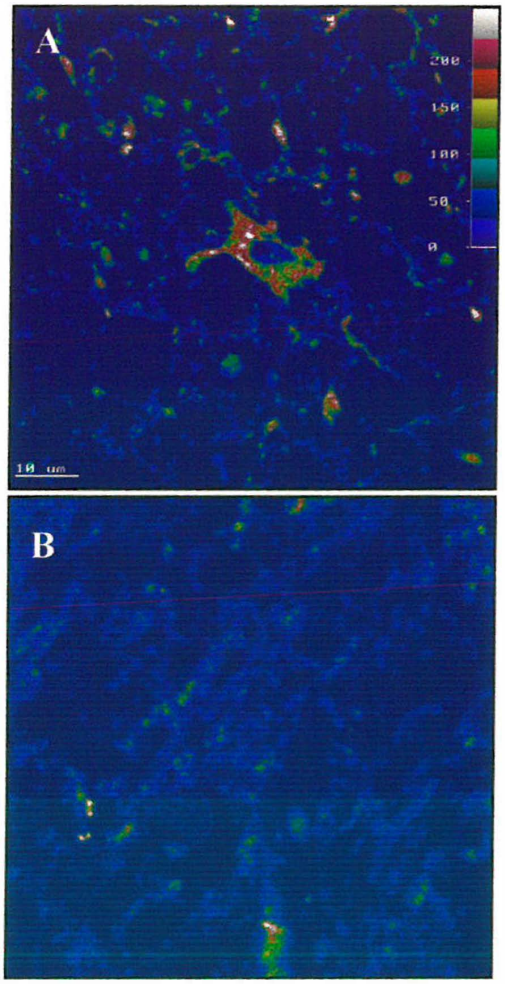


Figure 3.46 Confocal images of kinin B1 receptor labeling on control spinal cord

Positive labeling for B1 kinin receptor is shown on the neurons of the substantia gelatinosa of the spinal cord (A). There is an absence of labeling in the negative method control (B). The intensity of the immunofluorescence is shown on the colour strip: red to white: maximum, green to yellow: moderate, light blue = low and purple to blue = zero.

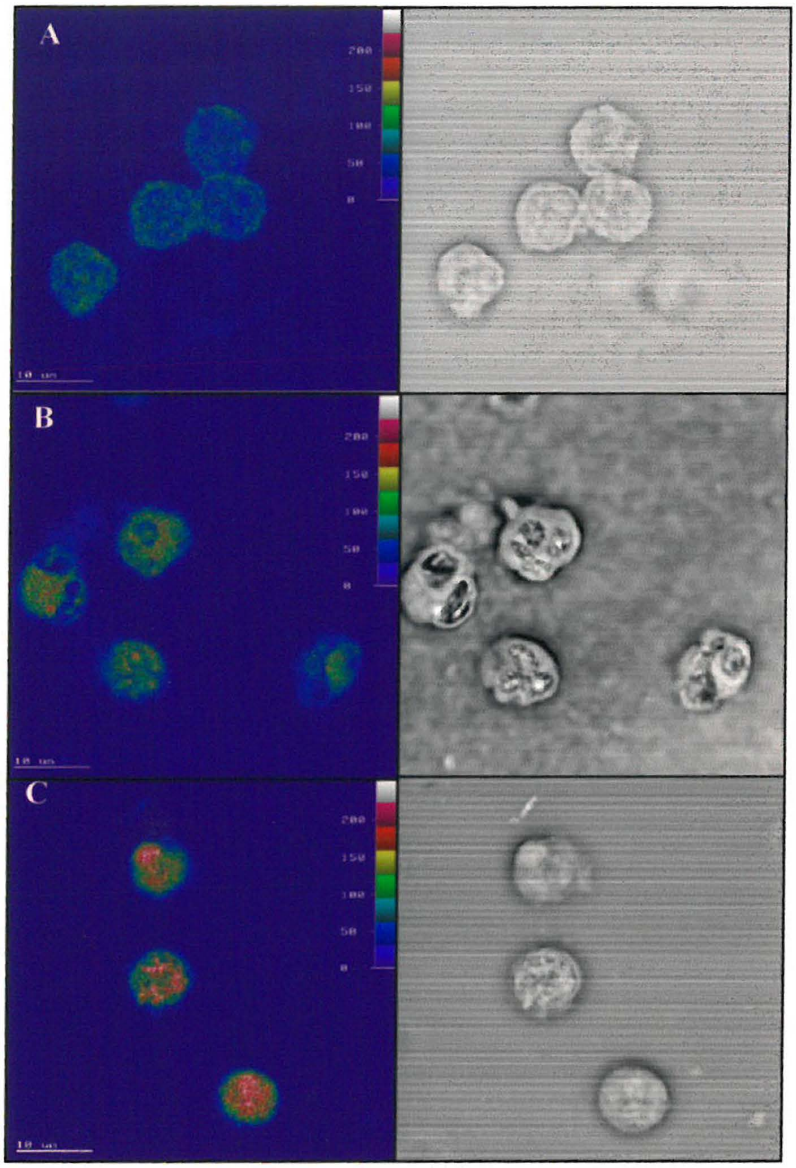
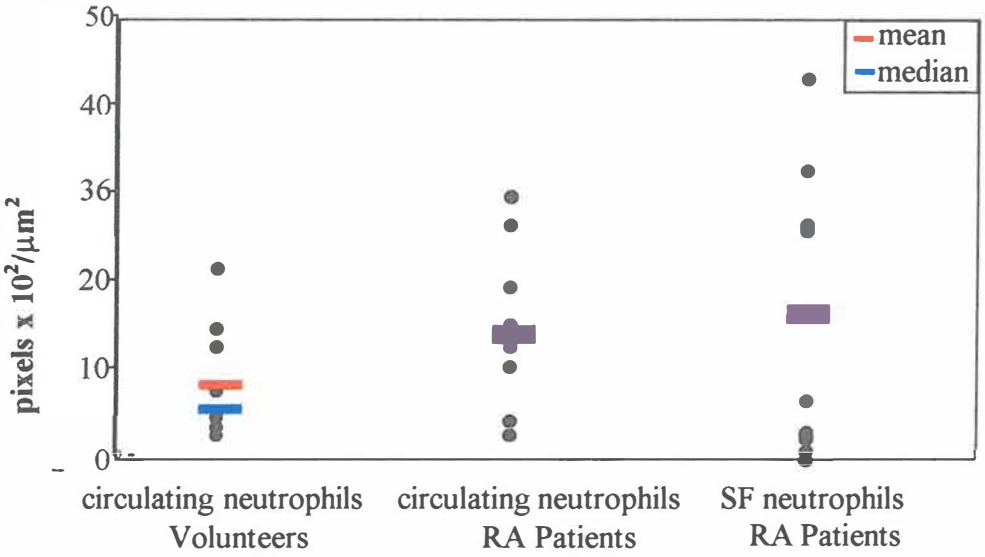


Figure 3.47 Immunovisualisation of the kinin B1 receptors on the neutrophil membrane

Immunolabelling of kinin B1 receptors on circulating neutrophils of volunteers (A), and the circulating (B) and synovial fluid neutrophils (C) of RA patients. The intensity of the immunoflourescence is shown on the colour strip: red to white = maximum, green to yellow = moderate, light blue = low and purple to blue = zero. The corresponding phase contrast images are shown in the second panel.



(patients: n = 8; neutrophils: n =10 neutrophils per patient)

Figure 3.48 Intensity of immunolabelling of kinin B1 receptors on neutrophils

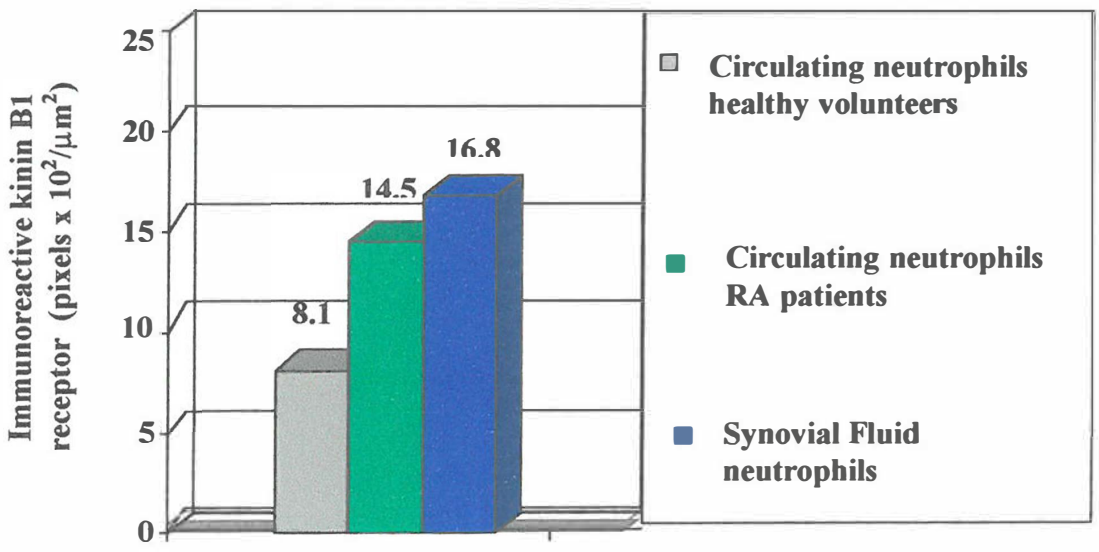


Figure 3.49 Histogram of immunoreactive kinin B1 receptor on neutrophils showing mean values for the intensity of labeling of the kinin B1 receptor (in pixels x $10^2/\mu m^2$) on the neutrophil membrane

Although there was a clear increase in the intensity of labeling of the kinin B1 receptor on the SF neutrophils from RA patients (n=8), when compared to the circulating neutrophils from healthy volunteers (n=8), the mean values did not reach significance (Kruskal Wallis; $p > 0.05$). However, a significant increase in B1 receptor labeling was observed on both the circulating and SF neutrophils of the RA patients (n=80) when compared to circulating neutrophils of the healthy volunteers (n=80) (1 Way ANOVA, $p < 0.01$ and < 0.05 respectively). Details of the intensity of labeling for each of the subjects are shown in Fig. 3.48 and Table 3.12

Table 3.12 Intensity of immunolabelling for the kinin B1 receptor

(pixels x $10^2/\mu m^2$)

No	(i) Circulating neutrophils healthy volunteers	(ii) Circulating neutrophils RA patient	(iii) SF neutrophils RA patient
1	7	12	26
2	4	29.2	0.2
3	2	3.6	5.8
4	2	2	0.7
5	21	25.9	2.3
6	12	14.5	25.3
7	14	18.9	42.4
8	3	9.8	36
Mean	**8.1**	**14.5**	**16.8**
Median	**5.5**	**13.3**	**15.6**
Kruskal-Wallis	**(i) vs. (ii) : > 0.05**		**(i) vs. (iii) : > 0.05**

Correlation with disease activity

There was a positive correlation between the immunoreactivity for the B1 kinin receptor on the circulating neutrophils from the RA patients and the local activity index ($r = 0.783$; $p < 0.05$) (Fig 3.50).

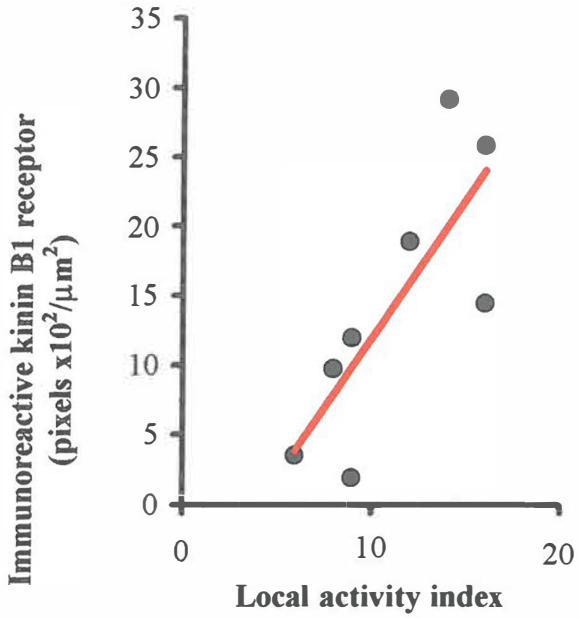


Figure 3.50 Correlation between immunoreactive kinin B1 receptor on circulating neutrophils from RA patients and the local activity index

3.7.4 Immunolocalisation of the kinin B2 receptor

Kinin B2 receptors have been localized on the collecting ducts and tubules of normal kidneys (Naidoo *et al.* 1996a). During each labeling experiment, normal human kidney tissue was used as the positive controls. For the method control, loss of labeling following preadsorption with an excess of the rabbit anti-human B2 receptor antibody and replacement of the primary antibody with PBS was demonstrated (Fig. 3.51).

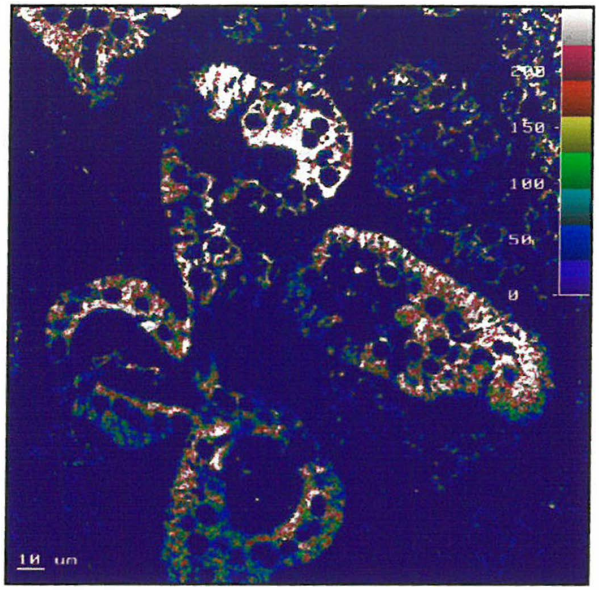


Figure 3.51 Confocal image of control human kidney

Positive labeling for the kinin B2 receptor is present on the collecting ducts and tubules of normal human kidney. The intensity of the immunoflourescence is shown on the colour strip: red to white = maximum; green to yellow = moderate; light blue= low and purple to blue = zero

The immunolabelling for the kinin B2 receptor on the circulating neutrophils from healthy volunteers and the circulating and SF neutrophils from RA patients is shown in Fig. 3.52. The mean intensity for the kinin B2 receptor on the circulating neutrophils from the healthy volunteers was 4.6 pixels x $10^2/\mu m^2$ with a median of 5 pixels x $10^2/\mu m^2$ (Fig 3.53 & 3.54 and Table 3.13). For the circulating and SF neutrophils from the RA patients, the mean intensity for B2 receptor was 22.4 pixels x $10^2/\mu m^2$ and 16.1 pixels x $10^2/\mu m^2$ respectively, with corresponding medians of 15.5 pixels x $10^2/\mu m^2$ and 12 pixels x $10^2/\mu m^2$.

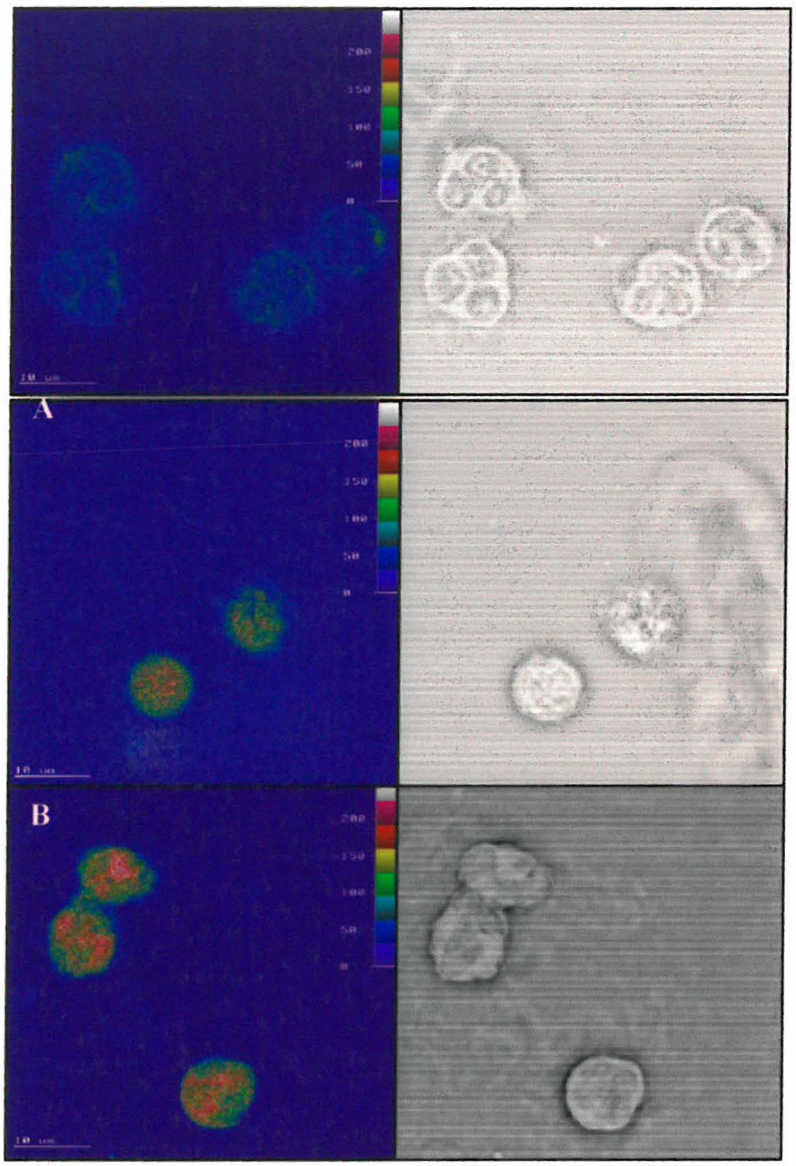
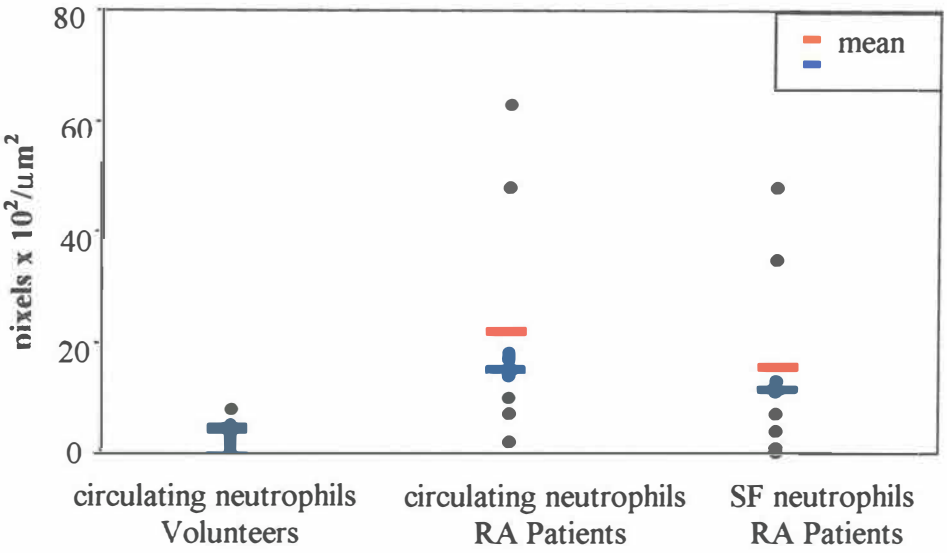


Figure 3.52 Immunovisualisation of the kinin B2 receptor on the neutrophil membrane

Immunolabelling of kinin B2 receptor on circulating neutrophils of (A) volunteers, and (B) circulating neutrophils and (C) synovial fluid neutrophils of RA patients. The intensity of immunofluorescence is shown on the colour strip: red to white: maximum, green to yellow = moderate, light blue = low and purple to blue = zero. The corresponding phase contrast images are illustrated in the second panel.



(patients: n=8; neutrophils: n=10 neutrophils per patient)

Figure 3.53 Immunolabelling for kinin B2 receptor on neutrophils

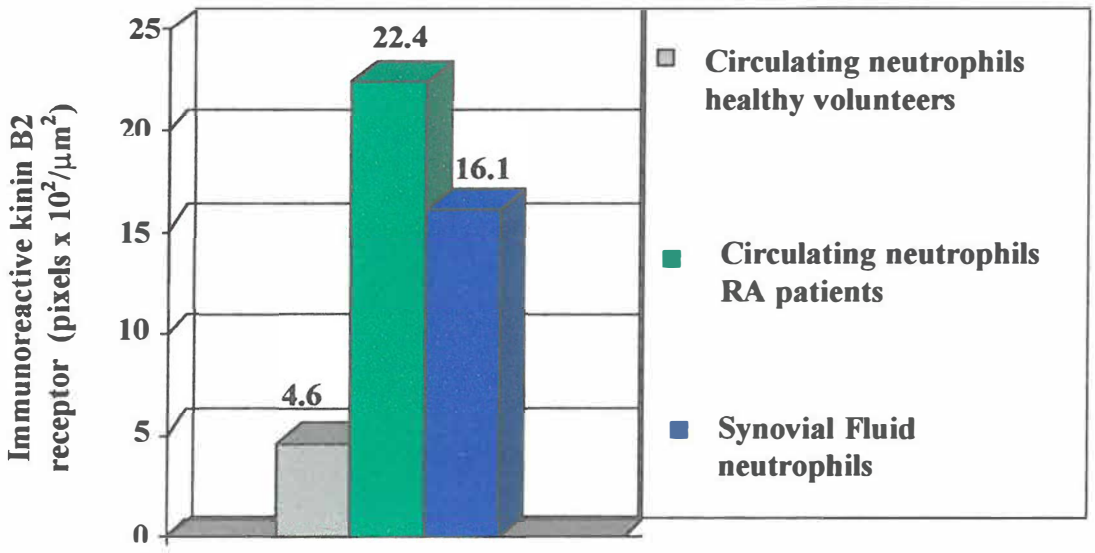


Figure 3.54 Histogram of immunoreactive kinin B2 receptors on neutrophils showing mean values for the intensity of labeling of the kinin B2 receptor (in pixels x $10^2/\mu m^2$) on the neutrophil membrane

Although there was a clear increase of the kinin B2 receptor on the circulating and SF neutrophils from the RA patients compared to circulating neutrophils from healthy volunteers, this was only significant for the circulating neutrophils from the RA patients ($p = 0.05$). Details for each patient are shown in Fig. 3.53 and Table 3.13.

There was no correlation between the measures of disease activity and the intensity of labeling for the kinin B2 receptor on the neutrophils from the RA patients.

Table 3.13 Intensity of immunolabelling for the kinin B2 receptor

(pixels x $10^2/\mu m^2$)

No	(i) Circulating neutrophils healthy volunteers	(ii) Circulating neutrophils RA patients	(iii) SF neutrophils RA patients
1	3	7	7
2	8	14	48
3	1	17	1
4	5	10	11
5	5	2	13
6	2	18	1
7	5	63	37
8	8	48	13
Mean	**4.6**	**22.4**	**16.1**
Median	**5**	**15.5**	**12**
Kruskal-Wallis	**(i) vs. (ii) : < 0.05**		**(i) vs. (iii) : > 0.05**

Appendix 3.1 **Clinical characteristics of the patients with rheumatoid arthritis**

	Lab. no	Name	Age (yrs)	Sex	Duration	Nodules	RF	No. ACR criteria	DMARD	Prednisone dose	Co-morbid diseases
1	191	SM	46	Female	4		Pos	5	No	nil	
2	192	DB	52	Female	6		Pos	6	3	5 mg	HT
3	195	CA	65	Female	14		Pos	6	5	7.5 mg	HT
4	193	SM	50	Male	20	present	Pos	7	3	10 mg	
5	196	MP	53	Male	12		Pos	6	2	5 mg	DM
6	197	KN	40	Female	1.6		Pos	6	2	5 mg	
7	200	IB	47	Female	17		Pos	6	4	7.5 mg	
8	206	MN	56	Female	1.6	present	Neg	6	No	nil	
9	214	MT	49	Female	6		Neg	5	2	nil	Hypothyroid
10	216	CD	60	Female	14		Pos	6	2	10 mg	HT
11	220	MS	50	Female	15		Neg	5	No	nil	
12	222	MM	44	Female	5		Pos	4	2	7.5 mg	HT
13	226	RS	46	Female	15		Neg	5	2	nil	
14	228	LP	46	Female	20		Neg	4	3	3 mg	HT
15	236	MG	50	Female	2.5		Pos	5	No	nil	
16	236	EC	50	Female	15		Pos	6	2	nil	HT
17	237	KM	50	Female	10		Neg	5	1	nil	HT
18	238	PC	40	Female	11		Neg	5	2	4 mg	
19	237	GN	60	Female	7		Neg	4	2	nil	HT
20	247	ZE	45	Female	11		Pos	6	3	nil	

Appendix 3.1 **Clinical characteristics of the patients with rheumatoid arthritis**

	Lab. no	Name	Age (yrs)	Sex	Duration	Nodules	RF	No. ACR criteria	DMARD	Prednisone dose	Co-morbid diseases
21	255	PK	37	Female	4		Pos	6	No	nil	
22	256	AA	65	Female	10	present	Pos	7	1	nil	HT; DM; Hypothyroid
23	262	MG	54	Female	36		Pos	5	3	5 mg	DM
24	263	MS	56	Male	14	present	Pos	7	5	nil	
25	277	RS	72	Female	14		Pos	6	No	nil	
26	276	KG	42	Female	3		Pos	6	No	5 mg	Hypothyroid
27	295	CMA	42	Male	3		Pos	6	2	10 mg	
28	363	CD	43	Male	5		Neg	5	No	nil	
29	364	IJ	56	Female	17		Neg	5	1	nil	
36	370	HBF	41	Female	6		Pos	5	1	5 mg	
37	373	PS	43	Male	20		Neg	5	No	nil	
36	373	ZM	21	Female	2		Pos	5	No	nil	
37	374	AF	48	Female	4		Pos	6	No	7.5 mg	
36	375	AM	40	Female	16		Pos	6	No	nil	
37	379	JZ	52	Female	1		Pos	5	No	nil	HT; DM
36	360	GK	38	Female	1.8		Neg	4	No	nil	
37	361	AM	64	Female	0.8		Pos	6	No	nil	HT ; DM
38	369	VR	55	Female	17		Pos	6	No	nil	HT

Appendix 3.1 Clinical characteristics of the patients with rheumatoid arthritis

	Lab. no	Name	Age (yrs)	Sex	Duration	Nodules	RF	No. ACR criteria	DMARD	Prednisone dose	Co-morbid diseases
39	376	RS	52	Female	3		Pos	5	No	nil	
40	361	MM	52	Male	3		Pos	6	1	nil	
41	362	SB	59	Female	17		Pos	6	2	5 mg	
42	363	AM	37	Female	5		Pos	6	2	nil	
43	365	BG	52	Female	2		Neg	5	No	nil	
44	366	BV	37	Female	6		Pos	6	2	nil	
45	361	PK	37	Female	2.5		Pos	6	1	7.5 mg	
46	375	MN	59	Female	1		Neg	5	No	5 mg	
47	394	MM	63	Male	3		Neg	5	No	nil	
48	395	EN	52	Female	3		Neg	6	1	nil	HT
49	398	AR	64	Female	17		Pos	6	4	nil	
50	408	GR	53	Female	9		Neg	5	5	5 mg	

(Abbreviations: HT, hypertension; DM, diabetes mellitus, neg., negative; pos., positive; mg., milligrams)

Appendix 3.2 Demographic details of the RA patients and healthy volunteers

RHEUMATOID ARTHRITIS					HEALTHY VOLUNTEERS			
Lab No	Name	Age	Sex		Lab No	Name	Age	Sex
191	SM	46	Female		259	PR	46	Female
192	DB	52	Female		390	DS	52	Female
195	CA	65	Female		506	FC	64	Female
193	SM	50	Male		S18	SD	48	Male
196	MP	53	Male		456	PR	52	Male
197	KN	40	Female		463	PP	40	Female
200	IB	47	Female		451	SN	47	Female
206	MN	56	Female		579	NN	58	Female
214	MT	49	Female		S48	MS	48	Female
216	CD	60	Female		448	CM	59	Female
220	MS	50	Female		450	KS	50	Female
222	MM	44	Female		452	MM	45	Female
226	RS	46	Female		385	SN	47	Female
228	LP	46	Female		574	ZM	45	Female
236	MG	50	Female		461	RB	50	Female
236	EC	50	Female		570	MR	49	Female
237	KM	50	Female		578	PK	50	Female
238	PC	40	Female		455	MK	39	Female
237	GN	60	Female		505	HF	58	Female
247	ZE	45	Female		S42	MG	43	Female
255	PK	37	Female		449	PN	37	Female
256	AA	65	Female		445	SS	68	Female
262	MG	54	Female		562	MR	54	Female
263	MS	56	Male		577	SC	54	Female
277	RS	72	Female		444	AM	74	Female
276	KG	42	Female		S36	WV	44	Female
295	CMA	42	Male		251	UGL	42	Male
363	CD	43	Male		453	N	40	Male
364	IJ	56	Female		563	SG	55	Female
370	HBF	41	Female		462	CV	42	Female
373	PS	43	Male		454	GD	43	Female
373	ZM	21	Female		571	NM	22	Female

RHEUMATOID ARTHRITIS					HEALTHY VOLUNTEERS			
374	AF	48	Female		573	CW	49	Female
375	AM	40	Female		460	LP	42	Female
379	JZ	52	Female		567	JR	53	Female
360	GK	38	Female		S15	RN	39	Female
361	AM	64	Female		564	MG	60	Female
369	VR	55	Female		562	SN	55	Female
376	RS	52	Female		561	MM	53	Female
361	MM	52	Male		464	YA	50	Female
362	SB	59	Female		569	BC	59	Female
363	AM	37	Female		386	AN	38	Female
365	BG	52	Female		565	JM	52	Female
366	BV	37	Female		250	JM	36	Female
361	PK	37	Female		S12	PO	36	Female
375	MN	59	Female		575	GK	59	Female
394	MM	63	Male		457	GS	64	Female
395	EN	52	Female		576	B	52	Female
398	AR	64	Female		572	M	64	Female
408	GR	53	Female		566	SN	56	Female

Appendix 3.3 Demographics of patients with RA from whom synovial tissue samples were obtained at arthroscopy

no	Age (yrs)	Swollen joint count	ESR	CRP	Prednisone	DMARD
1	62	7	13	21	No	No
2	51	21	35	74	No	No
3	63	10	1	0	5 mg	MTX
4	32	2	18	n/a	No	No
5	38	5	25	20	No	No
6	27	2	n/a	n/a	No	No

Abbreviations: ESR, erythrocyte sedimentation rate; CRP, C reactive protein; MTX. Methotrexate; n/a, not available

Appendix 3.4 **Demographics of patients from whom synovial tissue samples were obtained at autopsy**

Age	Race	Sex	Cause of death
38	African	Male	Blunt injuries to chest and neck
44	African	Male	Stab chest
45	Indian	Male	Myocardial infarction
18	African	Male	Suicide by hanging
20	African	Male	Suicide by hanging
18	Indian	Male	Gun shot chest
25	African	Female	Stab chest
38	African	Male	Stab chest
48	African	Male	Gun shot chest
25	African	Male	Stab chest

Appendix 3.5 Twenty eight tender and swollen joint count

Joint	Right		Left	
	Pain or tenderness	swelling	Pain or tenderness	swelling
shoulder	☐	☐	☐	☐
elbow	☐	☐	☐	☐
wrist	☐	☐	☐	☐
MCP1	☐	☐	☐	☐
MCP2	☐	☐	☐	☐
MCP3	☐	☐	☐	☐
MCP4	☐	☐	☐	☐
MCP5	☐	☐	☐	☐
PIP2	☐	☐	☐	☐
PIP3	☐	☐	☐	☐
PIP4	☐	☐	☐	☐
PIP5	☐	☐	☐	☐
IP thumb	☐	☐	☐	☐
knee	☐	☐	☐	☐

swollen joint count ☐

tender joint count ☐

Scale: 0 = no pain/tenderness or swelling

1 = pain/tenderness or swelling

Appendix 3.6 Modified Health Assessment Questionnaire

We are interested in learning how your illness affects your ability to function in daily life. Please feel free to add any comments on the back of this page.

PLEASE TICK THE ONE RESPONSE WHICH BEST DESCRIBES YOUR USUAL ABILITIES OVER THE PAST WEEK :

	Without **ANY** difficulty	With **SOME** difficulty	With **MUCH** difficulty	**UNABLE** to do
1. Dressing and Grooming				
Are you able to :				
Dress yourself, including tying shoelaces and doing buttons ?	.....	.....	.....	... .
Shampoo your hair ?	.....	.....	.....	.....
2. Rising				
Are you able to :				
Stand up from an armless straight chair ?	.....	.....	.....	.....
Get in and out of bed ?	.....	.....	.....	.....
3. Eating				
Are you able to :				
Cut your meat ?	.....	.....	.....	.....
Lift a full cup or glass to your mouth ?	.....	.....	.....	.....
Open a new carton of milk (or soap powder) ?	.....	.....	.....	.....

4. Walking

Are you able to :

Walk outdoors on flat ground ?	.....	.....	.....	.....
Climb up five steps ?	.....	.....	.....	.....

PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES :

..... Cane	..... Devices used for dressing (button hook)
..... Walking frame	..... Zipper pull, long handles shoe horn, etc
..... Crutches	..... Built-up or special utensils
..... Wheelchair	.... Special or built-up chair

... ...Other (specify).....

PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON :

.....Dressing and Grooming.....	Eating
.....Rising	..... Walking

PLEASE TICK THE ONE RESPONSE WHICH BEST DESCRIBES YOUR USUAL ABILITIES OVER THE PAST WEEK :

	Without **ANY** difficulty	With **SOME** difficulty	With **MUCH** difficulty	**UNABLE** to do
Wash and dry your entire body ?	.....	.....	.....	.....
Take a bath ?	.....	.....	.....	.....
Get on and off the toilet	.....	.....	.....	.....
Walk outdoors on flat ground ?	.....	.....	.....	.....

5. Hygiene

Are you able to :

	Without **ANY** difficulty	With **SOME** difficulty	With **MUCH** difficulty	**UNABLE** to do
6. Reach				
Are you able to :				
Reach and get down a 5 lb object(e.g. a bag of potatoes) from just above your head ?	.....	.....	.....	.....
Bend down to pick up Clothing from the floor ?	.....	.....	.....	.....
7. Grip				
Are you able to :				
Open car doors ?	.....	.....	.....	.....
Open jars, which have been previously opened ?	.....	.....	.....	.....
Turn taps on and off ?	.....	.....	.....	.....
8. Activities				
Are you able to :				
Run errands and shop ?	.....	.....	.....	.....
Get in and out of a car ?	.....	.....	.....	.....
Do chores such as vacuuming, housework or light gardening ?	.....	.....	.....	.....

PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES :

..... Raised toilet seat ..... Bath rail

..... Bath seat ....Long handled appliances for reach

..... Jar opener (for jars previously opened)

.....Other (Specify)

PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON :

..... Hygiene Gripping and opening things

..... Reach ..... Errands and housework

Appendix 3.7 **Measures of disease activity and synovial fluid basal and generated kinin levels in 20 patients with RA**

	Disability VAS	Pain VAS	Physician's GA	Patient's GA	T28	S28	ESR	CRP	HAQ	LAI	DAS	basal kinin	generated kinin
1	82	83	2	3	3	6	76	60.9	1.75	19	5.22	3.01	170.67
2	38	37	3	3	6	9	19	24.3	1.5	9	4.77	8.78	50.3
3	15	89	1	2	1	4	40	5.37	0.25	6	4.16	6.88	46.8
4	43	80	3	4	1	4	36	36.6	3	18	3.94	8.36	101.79
5	71.5	85.5	3	4	3	3	50	21.8	1.625	17	4.69	12.28	43.29
6	18	16	3	4	1	10	68	40.6	1.625	12	4.91	1.56	84.5
7	80	90	4	2	12	15	108	19.6	2.5	17	6.97	4.95	137.69
8	91	73	3	4	8	9	11	17.8	3	14	4.60	3.03	123.09
9	87	64	4	4	8	3	60	52.8	2.25	15	5.49	1.9	85.3
10	14	9	3	4	2	11	25	15.5	0.125	8	4.45	13.77	37.63
11	54	54	2	4	4	2	26	6.59	2.125	14	4.26	26.17	24.53
12	22	25	2	2	7	1	12	20.5	1.625	9	3.94	3.1	12.5
13	98	72	3	4	7	9	124	72.7	2.875	12	6.37	1.68	94.84
14	56	56	3	4	3	6	20	13.1	1	15	4.21	6.05	50.88
15	36	63	3	3	6	5	56	7.89	0.75	16	5.36	3.52	37.01
16	52	57	3	3	3	6	28	23.8	1.125	9	4.47	3.83	29.08
17	48	36	3	4	11	9	78	72	2.5	12	6.37	0.83	144.65
18	41	46	3	3	10	10	74	73.7	1.75	8	6.28	0.97	109.94
19	36	36	2	3	4	4	27	11.8	0.5	10	4.47	2.85	18.67
20	0	37	3	4	11	9	78	72	2.5	12	6.37	0.43	216.02

Abbreviations: RA, rheumatoid arthritis; VAS, visual analogue scale; GA, global assessment; T28, twenty eight tender joint count, S28, twenty eight swollen joint count; ESR, erythrocyte sedimentation rate, CRP, C reactive protein; HAQ, health assessment questionnaire, LAI, local activity index; DAS disease activity score.

CHAPTER 4

DISCUSSION

4.1 PATHOPHYSIOLOGY OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis is an autoimmune disease of unknown aetiology characterized by a chronic synovitis, which manifests as joint pain and swelling, and often progresses to bone and joint destruction. If effective therapy is not instituted, the progressive joint destruction results in a significant loss of function and disability and an increased mortality.

The exact aetiology of RA is unknown and the current hypothesis is that the inflammation and tissue destruction result from complex cell-cell interactions. The initial step is thought to be the interaction between the antigen presenting cells and CD4+ T cells in a genetically susceptible host. This results in macrophage activation and pro-inflammatory cytokine release and kinin peptide formation. These mediators stimulate synovial fibroblasts and chondrocytes to secrete enzymes that degrade proteoglycans and collagen, leading to tissue destruction.

The disease is characterized by five stages. In the first stage, the immune system is triggered by exposure to an unknown antigen. In stage 2, the B and T cells are activated, in stage 3, synovial proliferation occurs, and a large number of PMN cells accumulate in the synovial fluid. The fourth stage is characterized by the presence of an invasive pannus associated with bony erosions and degradation of the cartilage. In stage 5, the pannus invades the cartilage and there is joint space narrowing.

Clinically, RA presents predominantly as a symmetrical polyarthritis with variable extra-articular systemic involvement. The activity of the disease is reflected clinically by the duration of morning stiffness, presence of pain, and the number of swollen and tender joints, and biochemically by a raised erythrocyte sedimentation rate (ESR) and C reactive

protein (CRP). With severe or chronic inflammation, radiographic evidence of bone and joint destruction becomes apparent. Early radiographic changes include periarticular osteoporosis and bony erosions. With advanced disease, there is joint space narrowing and extensive bony destruction.

The histopathological changes in the synovium include hyperplasia with an increase in the thickness of the synovial lining layer, infiltration of the sublining layer with mononuclear cells, T and B lymphocytes, macrophages and plasma cells and proliferation of new blood vessels within the synovium (angiogenesis). A variety of inflammatory cells is implicated in the pathogenesis of the inflammatory process. These include the B and T lymphocytes, macrophages, neutrophils and synovial fibroblasts. A complex interaction of these cells with inflammatory mediators such as the proinflammatory cytokines, kinins, and metalloproteinases is responsible for the pathological findings in RA. In addition, there is an exudation of fluid into the joint space. Unlike the normal joint, the synovial fluid in RA contains a large number of inflammatory cells, including neutrophils, T cells, macrophages and plasma cells, which provide a continuing local immune response in the joint.

The pro-inflammatory cytokines, IL-1β and TNFα are important mediators of inflammation, tissue injury and immunologic reactions. Several studies have demonstrated elevated levels of IL-1β in the synovial fluid and serum of patients with RA (Odeh, 1997), and a correlation between the degree of inflammation and IL-1β levels (Kahle, *et al.* 1992). Interleukin-1β is a potent mediator of bone resorption and cartilage destruction (Pettipher *et al.* 1985) and stimulates the production of other pro-inflammatory and angiogenic cytokines. The systemic features of RA, such as malaise, fatigue, fever, anaemia and elevation of acute phase proteins (APP) are also due to IL-1β (Dinarello, 1984). The role

of IL-1β in RA is supported by the decrease in the number of tender joints and APPs in clinical trials of IL-1 receptor antagonist in patients with RA (Bresnihan and Cunnane, 1998). Abundant evidence exists for a significant role for TNFα in the pathogenesis of RA (Brennan *et al.* 1992a). Tumour necrosis factor α also stimulates bone resorption, cartilage destruction and release of other cytokines (Odeh, 1997). High concentrations of TNFα are present in the serum and synovial fluid of RA patients and in the synovial lining cells and macrophages (Tetta *et al.* 1990, Manicourt *et al.* 1993). In addition, treatment with monoclonal antibodies to TNFα results in significant clinical and biochemical improvement (Keystone, 1999).

Considerable evidence also exists for the role of kinins as primary mediators in RA. Kinins are vasoactive peptides that induce the cardinal manifestations of inflammation, namely pain, vasodilatation and oedema. In addition, they stimulate the release of IL-1β and TNFα (Tiffany and Burch, 1989), several other second-generation mediators of inflammation (Bhoola *et al.* 1992), and enhance bone resorption via prostaglandin formation. High levels of kinins and kallikreins have been reported in the synovial fluid of RA patients (Hargreaves *et al.* 1988;Volpe Junior *et al.* 1996; Selwyn *et al.* 1989). Kinin receptors have been localized on synovial fibroblasts (Bathon *et al.* 1992a) and the kinin receptor antagonist has been shown to suppress joint swelling in experimental models of arthritis (Cruwys *et al.* 1994; Sharma and Wirth, 1996).

The principal aims of treatment in RA are to control inflammation and to prevent joint destruction. Current treatment with first and second line drugs is inadequate in that they only partially control established RA, and the side effect profile limits their use especially for prolonged periods. The traditional disease modifying antirheumatic drugs have been

shown to provide symptomatic and clinical improvement but do not significantly alter long term outcome. The development of targeted biological agents such as TNF α receptor blockade is a recent advance. However, this therapeutic measure has been found not to be universally effective. In view of the overall poor prognosis of RA with current therapeutic regimens, there is a need for new approaches to the treatment and a better understanding of its pathogenesis.

4.2 TISSUE KALLIKREIN IN SYNOVIAL TISSUE.

Considerable experimental evidence has linked the kallikrein-kinin (KK) cascade to inflammation. The presence of the KK cascade in synovial tissue was first suggested by the ability of bradykinin to increase intracellular cyclic AMP release from cultured synovial fibroblasts (Fahey *et al.* 1977), and to increase vascular permeability of canine synovial tissue (Grennan *et al.* 1977). Later, TK was implicated in neutrophil diapedesis (Figueroa *et al.* 1989), and in the processing *in vitro* of a variety of promolecules that include matrix metalloproproteinases (Tschesche *et al.* 1989), procollagenase (Eeckhout and Vaes, 1977) and progelatinase (Menashi *et al.* 1994). The potential activation of these proproteinases by TK, suggests that TK either directly or through the formation of kinins may promote endothelial cell migration, leucocyte aggregation and tissue remodeling. A pathogenic role for TK in inflammatory synovitis is supported by the identification of TK in both the synovial fluid (Worthy *et al.* 1990; Bhoola and Dieppe, 1991) and the granules of synovial fluid neutrophils of RA patients (Figueroa *et al*; 1989, Rahman *et al.* 1994). Reduced TK immunoreactivity has been shown on the SF neutrophils in RA (Williams *et al.* 1997), suggesting that TK is released from the granules of neutrophils during inflammation.

To strengthen further the view that TK is linked to joint inflammation, one aim of this study was to examine the presence of TK in control and inflamed synovial tissue by immunolabelling experiments using recombinant TK as the primary antigen. TK was expressed on the synovial lining cells, subintimal macrophages and the endothelial cells of blood vessels in both the control and inflamed synovium (Cassim *et al.* unpublished). There was a significant increase in the intensity of labeling in the endothelial cells of rheumatoid tissue compared to controls. This finding suggested that TK might play a role in the pathological changes associated with RA. The localization of TK in the endothelial cells suggests that it may modulate endothelial function, thereby contributing to the increase protein and fluid leakage into the tissues and synovial space, leucocyte aggregation and angiogenesis which are dominant features in inflamed synovium. This concept is supported by the intense expression of TK in angiogenic endothelial cells (Plendl *et al.* 2000). In addition, there are a large number of activated macrophages in the rheumatoid synovium (Burmester *et al.* 1997). These macrophages produce prostaglandins and proinflammatory cytokines (Seitz and Hunstein, 1985; Firestein *et al.* 1990). Kinins are potent stimulators of IL-1β and TNFα release from macrophages (Tiffany and Burch, 1989) and conversely IL-1β and TNFα induce the effects of kinins (Bathon *et al.* 1989). The presence of TK in macrophages thus provides further evidence for role of kinins in regulating the production and release of cytokines from the macrophages. Tissue kallikrein has also been identified normal and in inflamed colon and specifically in macrophages forming granulomas in experimental colitis (Stadnicki *et al.* 1998). However, he TK concentrations were markedly reduced in the inflamed caecum compared with normal caecum. The authors postulate that the pro-inflammatory effects of TK in the intestine are due to macrophage production and secretion. A similar mechanism may apply in the inflamed synovium. Kinins cleaved from the kininogen molecule via the enzymatic

action of kallikreins exert their vasoactive properties by activation of specific kinin receptors. Alternatively TK has been shown to activate the kinin receptors directly, independent of BK release (Hecquet *et al.* 2000).

4.3 KININ B1 RECEPTORS IN SYNOVIAL TISSUE.

The actions of the vasoactive peptides, BK and kallidin are mediated via the kinin B1 and B2 receptors. The B1 receptors are not present normally, and appear to be induced in experimental inflammation, by noxious stimuli and cytokines (Bhoola *et al.* 1992). Minute amounts of kinin B1 receptor binding sites were identified in healthy porcine vascular tissues by autoradiography. In contrast, there was a three-fold increase in the number of binding sites in inflamed or septic tissue (Schremmer-Danniger *et al.* 1996, 1998). The role of B1 receptors in inflammation is further suggested by the upregulation of B1 expression by the pro-inflammatory cytokines, IL-1β, IL-8 and TNF α (Haddad *et al.* 2000; Bastian *et al.* 1998).

In rats, intra-articular injection of the B1 agonist, desArg^9 BK into normal knees induced significant plasma extravasation that was inhibited by desArg^9 (Leu^8) BK, a B1 receptor antagonist (Cruwys *et al.* 1994). In another study, IL-1β and TNF α increased B1 agonist induced paw oedema (Campos *et al.* 1998). However, reports on the role of B1 antagonism as a therapeutic modality are conflicting. In a porcine model of LPS induced endotoxic shock, treatment with a B1 antagonist had no beneficial effect (Siebeck *et al.* 1996), and the authors suggest that the upregulation of B1 receptors during LPS shock may be an important mechanism of host defense. Further, in the peptidoglycan-polysaccharide induced model of arthritis in the Lewis rat, administration of a B2 receptor antagonist blocked the systemic effects of inflammation. However, inhibition of the B1 receptor

prevented an anti-inflammatory response (Uknis *et al.* 2001). In contrast, B1 antagonism has been shown to reduce B1 agonist induced paw oedema (Campos *et al.* 1998).

Although B1 receptors have been identified on animal models of inflammation and sepsis, they have not been previously reported on human synovial tissue. Immunolabelling experiments were performed to localize the expression of B1 receptors in control and inflamed synovial tissue. In control synovial tissue, labeling of B1 receptors was visualized in the synovial lining cells, macrophages and endothelial cells (Cassim *et al.* unpublished). In the inflamed synovial tissue, there was a significant increase in the intensity of labeling in the synovial lining cells compared to control tissue. In contrast, no immunolabelling for B1 receptors was seen in synovial tissue obtained at arthroplasty, namely from patients with long standing chronic arthritis (Cassim *et al.* 1997). These findings suggest that B1 receptor expression is upregulated during acute inflammation. The increased expression of IL-1β and TNF α in inflammation may contribute to the enhanced expression of the kinin B1 receptors. The role of the kinin B1 receptor in inflammation remains speculative. Preliminary studies in animal models suggest a therapeutic effect of B1 antagonists.

4.4 KININ B2 RECEPTORS IN SYNOVIAL TISSUE

The B2 receptor is ubiquitous, and mediates most of the actions of BK and kallidin (Bhoola *et al.* 1992). B2 receptors have been localized on the plasma membrane of normal human neutrophils (Haasemann *et al.* 1994). In the normal human kidney, B2 receptors are thought to be responsible for most of the physiological effects of BK (Naicker *et al.* 1999). In contrast, in RA, an upregulation of the B2 receptor on circulating and synovial

fluid neutrophils was noted (Cassim *et al.* 1996). Additionally, a higher density of B2 receptors was observed in RA synovial tissue compared to OA (Bathon et al. 1992a), and IL-β enhanced the number of B2 receptor binding sites suggesting a functional role for B2 receptors in inflammatory synovitis. This is supported by the reports that the B2 receptor antagonist, HOE 140, reduced BK induced plasma extravasation in rat arthritic joints (Cruwys *et al.* 1994), and suppressed knee swelling in adjuvant-induced arthritis in rats (Sharma and Wirth, 1996).

In the current study, kinin B2 receptor expression was quantified in the synovial lining cells, fibroblasts, macrophages and the endothelial cells. The kinin B2 receptor was present in both the control and inflamed synovium and was expressed on the synovial lining cells, fibroblasts, macrophages and endothelial cells in both control and inflamed tissue (Cassim *et al.* unpublished). In RA, there is an increase in the thickness of the synovial lining layer, proliferation of fibroblasts, macrophage infiltration and new vessel formation. Therefore, there is likely to be an increase in the density of kinin B2 receptors compared to controls. Most of the vasoactive properties of kinins are mediated via the B2 receptors and their increased expression in the inflamed synovium supports a role for kinins as mediators of inflammation. Activation of the receptors in the synovial lining layer may contribute to the invasion of adjacent bone and cartilage and subsequent tissue destruction. As a G-protein coupled receptor, the kinin B2 receptor promotes several signal transduction events at the cellular level (Bhoola *et al.* 1992). These include activation of phospholipase A2 (PLA2) and stimulation of adenyl cyclase. Following activation of PLA_2 arachidonic acid is cleaved from membrane phospholipids and metabolized to produce prostaglandins and leucotrienes. The prostaglandins increase vascular permeability and induce osteoclastic bone resorption. The leucotrienes attract

leucocytes to the site of inflammation, promote adherence of leucocytes to endothelial cells and activate the secretion of reactive oxygen species and degradative enzymes from neutrophils. Thus, kinins may promote the inflammatory process either directly or via second messenger systems.

4.5 TISSUE KALLIKREIN IN THE SYNOVIAL FLUID

The kallikreins, tissue kallikrein (TK) and plasma kallikrein (PK), by their enzymatic action cleave the vasoactive peptides, kinins, from kininogens. Augmented activity of both TK and PK has been reported in the synovial fluid compared to plasma in patients with inflammatory arthritis (Volpe-Junior *et al* 1996). However, of the 10 patients studied only three had RA

Plasma kallikrein cleaves bradykinin from HK and from LK in the presence of neutrophil elastase (Sato and Nagasawa, 1988). Both active and total PK have been identified in the synovial fluid obtained from patients with arthritis (Rahman et al. 1995). Most of the PK in the synovial fluid appears to be in the proenzyme form and approximately half of the active form is bound to the protease inhibitor α_2 macroglobulin (Nagase et al. 1982). Other inhibitors include C1 esterase, α_1 antitrypsin and antithrombin III. Once the PK-inhibitor complexes are formed, they are rapidly cleared by the circulation. The PK found in synovial fluid may be due to transudation from plasma, an increased activation of plasma pre-kallikrein, or increased entry on migrating neutrophils.

Tissue kallikrein cleaves the decapeptide lys-bradykinin (kallidin) from both HK and LK. Kallidin, like BK appears to enhance bone resorption via prostaglandin formation (Lerner *et al.* 1987; Gustafson *et al.* 1998). Tissue kallikrein has previously been identified in

synovial fluid from arthritic joints (Worthy *et al.* 1990; Bhoola and Dieppe, 1991), and active enzyme levels were reported to be higher in patients with RA compared to OA (Selwyn *et al.* 1989). However, the relationship between TK activity and measures of disease activity was not studied. The presence of TK in the synovial fluid from inflamed joints suggests that kinins are released into the joint, where they may exhibit their vasoactive properties. Since neutrophils are abundant in the synovial fluid, the kininogen substrate is readily available to be cleaved by TK. In addition, TK may directly activate the kinin receptors present on the adjacent synovial tissue and neutrophils (Hecquet *et al.* 2000)

This is the first study to correlate comprehensively synovial fluid levels of TK with clinical and biochemical measures of disease activity. A negative correlation was observed between the active enzyme levels and the 28 swollen joint count in the RA patients. The swollen joint count is a validated method to assess disease activity, and is more reliable than the tender joint count as tenderness does not always correlate with inflammation. In the 28 swollen joint count joint, 14-paired joints, which are commonly involved in RA, were examined. The decrease in TK levels with an increasing swollen joint count probably reflected sequestration or complexing of TK by kallistatin type inhibitors in patients with active disease. This view is supported by the reports of high levels of α1 anti-trypsin, the normal endogenous inhibitor of TK, in the synovial fluid obtained from patients with RA (Brackertz *et al.* 1975; Robinson *et al.* 1981; Rahman *et al.* 1994)). It is postulated the kinins released from the kininogen molecule by both kallikreins activate the kinin receptors and other second messenger systems, thereby increasing vascular permeability and exudation of fluid into the synovial joint.

4.6 BASAL AND GENERATED KININ LEVELS IN SYNOVIAL FLUID.

Kinins are thought to play an important role in the pathogenesis of inflammation. They are potent vasoactive peptides that stimulate nociceptive C fibres, extravasation of vascular fluid, release of neurotransmitters and other second messengers including cytokines, prostaglandins and leucotrienes.

A pain producing substance, resembling BK was first isolated in synovial fluid of patients with RA (Armstrong *et al.* 1957), and subsequently in acute gouty arthritis (Eisen, 1966, Melmon *et al.* 1967). The ability of a serine protease inhibitor of PK to block the formation of BK *in vitro* in a rat model and to produce analgesia provided further evidence for the role of kinins in inflammation and pain (Hargreaves *et al.* 1988). This was supported by a recent study in which a new specific PK inhibitor significantly reduced joint swelling in peptidoglycan-induced arthritis in the Lewis rat (De La Cadena *et al.* 1995). In addition, both BK and kallidin enhance bone resorption via prostaglandin formation (Lerner *et al.* 1987; Gustafson *et al.* 1998). These studies support the role of kinins as mediators of the inflammatory response and joint damage in inflammatory arthritis and raise the possibility of new and novel treatment for inflammatory arthritis.

Measurement of basal kinins in the synovial fluid is difficult because of their short half-life and rapid degradation by kininases. To overcome this problem an assay to measure the capacity of synovial fluid to generate kinins was developed. Although the capacity of synovial fluid to generate kinins has been demonstrated (Bond *et al.* 1997), correlation with disease activity has not been reported previously.

In the 20 RA patients studied, the mean basal kinin level in the synovial fluid was 5.6 ng/ml (range: 0.43 – 27.17 ng/ml), and the mean level of generated kinins was 80.6 ng/ml (range: 12.5 – 216.02 ng/ml). The low levels of basal kinins in the synovial fluid confirm previous reports that *in vivo* kinin concentrations are very low in biological fluids (Bond *et al.* 1997). This is presumably due to the presence of kininases in the synovial fluid. Further, greater kininase activity has been demonstrated in the inflamed joint compared to that in the non-inflamed joint (Chercuiffe *et al*; 1987; Sheikh and Kaplan, 1987). The negative correlation between the basal and generated kinin levels suggests that there is a greater kininase activity in synovial fluid with high generating capacity in order to maintain low kinin levels in *vivo*. In addition, we have shown a negative correlation between the SF levels of basal kinins and the C reactive protein (CRP) and disease activity score.

Since kinins are vasoactive peptides that evoke the cardinal signs of inflammation, it is expected that the synovial fluid of patients with greater disease activity will have a higher kinin generating capacity. This is the first report of correlation between the generated kinin levels in the synovial fluid and clinical and biochemical measures of disease activity. The 28 tender and swollen joint counts were used to assess disease activity clinically since they are easier to use than the traditional joint counts using a larger number of joints and have been previously validated (Fuchs *et al.* 1989). There was a significant correlation between the level of generated kinins and the 28 swollen and tender joint count reflecting an increase in the kinin generating capacity of the synovial fluid with increased disease activity.

The kinin generating capacity is a complex and dynamic cascade involving the bioregulation of all the components of the kallikrein-kinin system, and therefore

correlation with disease activity may not be restricted as with a single component of the cascade. Thus, the correlation between disease activity and kinin generating capacity may be due to increased kallikrein, increased kininogen or decreased kallistatin.

The association between kinin generating capacity and inflammation was further strengthened by the correlation with the biochemical indices of the acute phase response, namely the ESR and CRP. The ESR is an indirect measure of the acute phase response while the CRP is the most accurate measure of the acute phase response that accompanies tissue inflammation. Both the ESR and CRP predominantly reflect the hepatic production of APPs, however APPs may also be produced locally at the site of inflammation (Van Leeuwen and Van Rijswijk, 1992). The ESR and CPR are widely used to assess disease activity and response to therapy. The relationship between the kallikrein-kinin cascade and the acute phase response has not been investigated previously. We postulate that kinins may stimulate the production of either hepatic or local production of APPs directly, or via the stimulation of IL-1β. Since evaluation of disease activity by single variables leads to methodological problems, several indices consisting of more than one variable have been developed. We used the previously validated modified disease activity score that includes the ESR, CRP and the 28 swollen and tender joint counts (Prevoo *et al.* 1995). The significant correlation between the SF levels of generated kinins and the disease activity score confirms that generated kinin levels are a true reflection of the degree of inflammation. These findings have important implications for not only the pathogenesis of inflammatory synovitis, but also may have therapeutic implications. Current therapeutic regimens for RA are not directed against the kallikrein-kinin cascade and this may explain the lack of a universal response even when biologic agents are used. Initial studies have reported the efficacy of kinin receptor antagonists in animal models of arthritis. The

further development of kinin receptor antagonists may introduce a novel therapeutic modality for inflammatory arthritis.

4.7 SYNOVIAL FLUID CYTOKINES

Cytokines are low molecular weight proteins responsible for direct cell-to-cell communication and play a pivotal role in inflammation. They are not commonly detected in the circulation, but act in a local or immediate microenvironment where they act on adjacent cells in a paracrine fashion or on the same cells that produce them in an autocrine fashion. While IL-1β, IL-6, IL-8 and TNF α are pro-inflammatory cytokines, IL-4, IL-10 and TGFβ appear to have anti-inflammatory properties and modulate the inflammatory response. In addition, there are natural inhibitors and binding proteins that neutralize the activity of IL-1β and TNF α (Weckmann and Alcocer-Varela, 1996) which down regulate the inflammatory response. In the RA joint there appears to be disequilibrium between the cytokine production and anti-cytokine homeostatic response resulting in an inflammatory reaction (Maini, 1996).

4.7.1 Interleukin 1β

Interleukin 1β is secreted by macrophages, fibroblasts, endothelial cells, synovial lining cells and T and B-lymphocytes during antigen presentation and has both systemic and local effects (Dinarello, 1994). These include the ability to recruit inflammatory cells to the site of inflammation, stimulate the production of other proinflammatory (IL-6 and TNFα) and angiogenic cytokines (IL-8), mediate bone resorption and cartilage destruction and induce the hepatic synthesis of acute phase proteins, all of which are cardinal features of

inflammatory synovitis. IL-1β can also induce the proliferation of synovial fibroblasts and other mediators of inflammation such as prostaglandins and proteases.

Increased levels of IL-1β have been demonstrated in the serum and synovial fluid from patients with inflammatory arthritis (DeMarco and Zurier, 1997). Increased expression of the pro-IL-1β gene in synovial cells of OA and RA patients further supports a role for IL-1β in the initiation and/or progression of arthritis (DeMarco and Zurier, 1997). Of note, is that a wide variation in the expression and production of IL-1β has been reported in patients with RA (Ulfgren *et al.* 2000). This may explain the variation in response different modes of therapy.

The concentration of IL-1β in the SF from 20 patients with RA was measured using an ELISA method. The mean concentration of IL-1β was 8.42 ± 9.67 pg/ml with a range of 0.27 – 29.04 pg/ml. The correlation between IL-1β concentrations and measures of disease activity was then determined using the Pearson's correlation coefficient. There was a positive correlation between IL-1β levels and clinical measures of disease activity, namely the 28 tender joint count, pain as measured on the VAS and the physician's global assessment of disease activity. The 28 tender joint count and the physician's global assessment of disease activity reflect the degree of inflammation. Their correlation with IL-1β is therefore not surprising, as IL-1β has been shown to induce inflammation. Pain is a consequence of the inflammatory process, and therefore may correlate with IL-1β levels. An alternative explanation for the correlation of pain and IL-1β may be that the pain is mediated via an IL-1β stimulation of kinin release. Kinins are potent pain producing substances. Intramuscular injections of BK has been shown to produce significantly increased pain compared to an infusion of saline (Babenko *et al.* 2000). This algesic effect

may be due to kinin-induced release of thromboxanes and prostaglandin or to the direct sensitization of afferent neurons. In addition, IL-1β has been shown to increase the kinin-induced release of prostaglandins from human synovial cells (O' Neill and Lewis, 1989). The prostaglandins are potent mediators of inflammation, pain and bone resorption, and prostaglandin inhibitors are widely used as analgesic and anti-inflammatory agents.

4.7.2 Tumour necrosis factor α

TNFα is thought to be the key mediator in the cytokine network and to induce the synthesis and release of a number of other proinflammatory cytokines (Brennan *et al.* 1992a). Other cellular actions of TNFα include modulation of PMN function (Kowanko *et al.* 1990), a prostaglandin-dependent stimulation of bone resorption (Lorenzo *et al.* 1991), inhibition of collagen synthesis, and cartilage destruction (Canalis, 1987). High levels of TNFα have been reported in the synovial fluid from patients with RA (Cope *et al.* 1992) and a correlation between the TNFα level in the SF and the SF leucocyte count and ESR has been documented (Saxne *et al.* 1998). Monoclonal antibodies against TNF have been shown to be effective in controlling disease activity in RA (Keystone, 1999), however their effect is not universal. This may be due to the presence of other pro-inflammatory mediators in the synovial fluid.

The synovial fluid concentration of TNFα was measured in 20 patients with RA, using the ELISA method. The mean TNFα level was 15.71 ± 10.57 pg/ml with a range of 4.41 – 41.42 pg/ml. There was a correlation between the TNFα concentration and the 28 tender joint count and CRP. However, there was no correlation between the TNFα level and other markers of disease activity, namely the 28 swollen joint count, pain, physician's

global assessment of disease activity, ESR and the disease activity score. This was surprising since, treatment with TNFα antagonists has been associated with a significant reduction in these parameters ((Weinblatt *et al.* 1999). One explanation may be that the measurement of the concentration of TNFα was influenced by the presence of peptidases or the induction of anti-inflammatory cytokines. High levels of prostaglandin E_2, a potent inflammatory mediator has been shown to down regulate TNFα production (Maini, 1996). In addition, TNFα binding proteins, soluble fragments of the TNFα receptor released during proteolytic cleavage of the receptor molecules, act as natural inhibitors of TNFα. The anti-inflammatory cytokines, IL-4, 10 and interferon γ, which may also be present in the synovial fluid, down regulate the production of TNFα.

4.7.3 Correlation between synovial fluid concentrations of IL-1β and TNFα.

IL-1β and TNFα are both proinflammatory cytokines. TNFα has the ability to increase the production of IL-1 (Brennan *et al.* 1992a), and conversely, IL-1 stimulates TNFα synthesis. Therefore the relationship between synovial fluid concentrations of IL-1β and TNFα in 16 patients with RA was studied. There was a significant correlation between the IL-1β and TNFα levels. This confirms the relationship between IL-1β and TNFα and suggests a synergistic role for these cytokines in inflammatory synovitis.

4.8 FUNCTIONAL INTERACTION BETWEEN THE CYTOKINE AND KININ CASCADES

Sufficient evidence exists for a role for both the cytokine and kinin cascades in inflammation. In this study, we report the expression of components of the kinin cascade in inflamed synovium and the correlation between the kinin generating capacity and

disease activity. In addition, we have also shown a correlation between IL-1β and TNFα with disease activity. It was therefore postulated that there is a functional inter-relationship between the cytokine and kinin cascades. Bradykinin is a potent stimulator of IL-1 and TNF release from the macrophages (Tiffany and Burch, 1989), and conversely IL-1 has been shown to up regulate the number of kinin receptors on human synovial tissue (Bathon *et al.* 1992a). Interleukin-1 has also been shown to potentate BO and TNFα-induced release of prostaglandins (O'Neill and Lewis, 1989). We therefore attempted to determine the relationship between the synovial fluid TK and generated kinin concentrations and the concentrations of IL-1β and TNFα.

Although there was a trend towards a negative correlation between the SF levels of enzymic TK and IL-1β α, this was not significant. One obvious explanation is the wide variance in the IL-1β concentrations. However, in this study a negative correlation between the concentration of enzymic TK and disease activity and a positive correlation between the concentrations of IL-1β and disease activity was shown, it therefore follows that the concentration of enzymic TK would decrease as that of IL-1β increases. Despite expectations, no correlation was noted between the concentration of enzymic TK and that of TNFα. Larger studies are required to confirm the hypothesis of a causal relationship between the cytokines and kinins.

The basal and generated kinin levels in the SF were then compared to the cytokine concentrations. There was no correlation between basal kinin levels and either IL-1β or TNFα. When the concentration of SF generated kinins were correlated with IL-1β in the 20 RA patients studied, there appeared to be no direct correlation between the two values. However, on further analysis, a positive correlation was found between low to moderate

levels of SF IL-1β and the SF generated kinin concentrations. In contrast, there was a negative correlation between the higher values of SF IL-1β and the SF generated kinin concentrations. This may be explained by the previously reported wide variation in expression and production of IL-1β by synovial tissue (Ulfgren *et al.* 2000). A number of factors influence the concentration of IL-1β in biological fluids. These include the capacity to produce cytokines, the short half-life of cytokines in biological fluids and the presence of natural inhibitors such as the IL-1 receptor antagonist and anti-inflammatory cytokines namely, IL-10 and transforming growth factor β (TGF β). In addition, an increase in intracellular c AMP and prostaglandin E_2 (PGE_2) suppress IL-1β production by activated macrophages. Kinins have been shown to stimulate the release of IL-1β from macrophages (Tiffany and Burch, 1989). However, kinins, via activation of the G-protein coupled receptors promote the activation of phospholipase A2 resulting in the formation of prostaglandins and stimulate adenyl cyclase thereby increasing cAMP, and may therefore subsequently down regulated the production of IL-1β. This suggests that there is a complex relationship between the kinin system and IL-1β, which may explain the lack of a direct correlation between the levels of generated kinins and IL-1β in the synovial fluid. In addition, there may be a biphasic relationship between IL-1β and the kinin system, in that at higher levels IL-1β suppresses kinin generation in an attempt to down regulate the inflammatory process. No correlation was found between the concentrations of TNFα and generated kinin concentrations in the synovial fluid.

4.9 TISSUE KALLIKREIN IN NEUTROPHILS

Tissue kallikrein has previously been localized in the granules of normal human neutrophils (Figueroa *et al.* 1989). Since neutrophils are abundant in the SF, this study was

undertaken to determine the changes in TK expression in the circulating and SF neutrophils from RA patients. There was no significant difference between the mean values for the intensity of TK labeling in the circulating and SF neutrophils from RA patients compared to that in circulating neutrophils from healthy volunteers. To overcome this problem, we compared the intensity of labeling for TK on each neutrophil, there was a significant loss of TK labeling in the SF neutrophils from the RA patients. This finding confirms a previous report (Williams *et al.* 1997), and suggests that TK is released from the neutrophil granules during inflammation and is therefore able to cleave the kinin moiety from the kininogen molecule. TK has also been implicated in neutrophil diapedesis (Figueroa *et al.* 1989), and may contribute to the efflux of neutrophils into the SF in RA thus potentiating the inflammatory response. In addition, the TK released may also directly activate the kinin receptors (Ulfgren *et al.* 2000).

4.10 KININ MOIETY ON THE SURFACE OF NEUTROPHILS

The kininogen molecule has been previously localized on the surface on normal human neutrophils (Figueroa *et al.* 1992). The kinin moiety is interleafed between the two polypeptide domains (3 and 5) of the kininogen molecule, and is cleaved from the kininogen molecule by kallikreins. Loss of the kinin moiety from neutrophils has been previously reported in sepsis (Naidoo *et al.* 1996b) and in RA (Cassim *et al.* 1996). The release of kinins into the SF in RA patients is supported by the capacity of the SF to generate kinins (Bond et al. 1997). In this study, we compared the intensity of labeling for the kinin moiety on circulating and SF neutrophils from RA patients to that on circulating neutrophils from healthy volunteers. There was a significant loss of labeling for the kinin moiety on the circulating and SF neutrophils from RA patients, confirming that the kinin moiety is released during inflammation from circulating and SF neutrophils from RA

patients. This finding suggests that kinins may be implicated in both the local and systemic features of RA.

4.11 KININ B1 RECEPTORS ON NEUTROPHILS

The kinin B1 receptors are not present normally but have been induced in experimental models of inflammation and sepsis. There are no previous studies looking at kinin B1 receptor expression on neutrophils. This study is the first report showing the presence of kinin B1 receptors on circulating neutrophils from normal volunteers and on circulating and SF neutrophils obtained from RA patients. There was a lower intensity of labeling for the B1 receptors on the circulating neutrophils from the healthy volunteers, with a mean of 8.1 pixels x $10^2/\mu m^2$. On the circulating and SF neutrophils from the RA patients, there was an upregulation of the B1 receptors with a mean intensity of 14.5 pixels x $10^2/\mu m^2$ and 16.8 pixels x $10^2/\mu m^2$ respectively. This suggests that the B1 receptor is induced in inflammation. The correlation between the intensity of B1 labeling and the local activity index supports a role for B1 receptors at the site of inflammation. The local activity index was used as a measure of inflammation of the knee joint from which the SF neutrophils were obtained. There was no correlation between the intensity of B1 labeling and the ESR, CRP, 28 tender and swollen joints, patients and physician's assessment of disease activity and the disease activity score. It can be argued that these measures reflect global disease activity, whereas the local activity index reflects the degree of local inflammation. The exact role for the B1 receptor in inflammation is unknown. While in some models of experimental inflammation or sepsis, the upregulation of B1 receptors may be a defense mechanism in the inflamed joint B1 receptors may play a pathogenetic role. With the development of specific B1 receptor antagonists, the role of B1 receptors may be further elucidated.

4.12 KININ B2 RECEPTORS ON NEUTROPHILS

The vasoactive properties of kinins are mediated primarily via the activation of kinin B2 receptors. Since Kinin B2 receptors have been previously identified on the surface of normal neutrophils and there is an efflux of neutrophils into the inflamed, joint we compared the intensity of labeling for B2 receptors on circulating neutrophils from healthy volunteers to that on circulating and SF neutrophils obtained from RA patients. There was a clear increase in the intensity of labeling in the neutrophils from RA patients compared to those from healthy volunteers. This suggests that kinins may be responsible for the systemic and local features of RA.

B2 receptor activation is thought to increase vascular permeability. This may account for the efflux of neutrophils in the inflamed joint. Further B2 receptor antagonists have been shown to inhibit PMN elastase release, reduce LPS induced increase in vascular permeability, inhibit BK-induced release of arachidonic acid, IL-6 and IL-8 and to have an analgesic effect. The further development of more specific B2 receptor antagonists may be improve not only the understanding of the pathogenesis of inflammatory arthritis but also provide a novel therapeutic modality.

4.13 CONCLUSIONS

This is the first comprehensive study of the kallikrein–kinin cascade in synovial tissue, synovial fluid and neutrophils in RA, and of the relationship between the KK and cytokine cascades and clinical markers of disease activity.

i) Tissue kallikrein and the kinin B1 and B2 receptors have been localized in control and rheumatoid synovial tissue.

ii) A correlation between tissue kallikrein activity and the kinin generating capacity in synovial obtained from RA patients and validated markers of disease activity was demonstrated.

iii) A loss of labeling of the immunoreactive tissue kallikrein and the kinin moiety, and the upregulation of the kinin B1 and B2 receptors was observed in the neutrophils obtained from RA patients.

iv) Although no direct correlation was demonstrated between the kallikrein-kinin cascades, our results suggest a complex and dynamic relationship between these systems.

These findings provide convincing evidence for the role for the kallikrein-kinin system in the pathogenesis of rheumatoid arthritis and have important implications for the development of novel therapeutic modalities.

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