INFLAMMATION AND CYTOKINE REGULATION IN STROKE

A thesis submitted to the University of Manchester for the Degree of PhD in the Faculty of Medicine, Dentistry, Nursing and Pharmacy

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Hedley Colin Arthur Emsley
BSc(Hons) MB ChB MRCP(UK)

Neuroinjury
Injury, Repair and Rehabilitation Group,
School of Medicine

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abstract

Stroke has enormous clinical, social and economic impact. Central nervous system and peripheral inflammation is important in the responses to ischaemic stroke, and may also predispose to its development. Inflammation is mediated by molecular (eg cytokines) and cellular (eg leucocytes and microglia) components. The cytokine interleukin-1 (IL-1) mediates cerebral ischaemia, whilst the endogenous IL-1 receptor antagonist (IL-1ra) protects against experimental ischaemic cerebral injury. The roles of IL-1 and other cytokines in patients with acute stroke have yet to be clearly identified. Classic acute phase reactants such as C-reactive protein (CRP) are also modified in stroke and warrant further study. These mediators may be useful in the prediction of events, outcome, and as therapeutic targets.

The aim of this thesis was to test two primary hypotheses, namely (1) peripheral inflammatory responses occur in patients with acute stroke, and cytokines, in particular IL-1, have a role in the regulation of these responses, and (2) recombinant human IL-1ra (rhIL-1ra) treatment is safe, well tolerated, and biologically active in patients with acute stroke.

In the first clinical study, 36 patients with ischaemic stroke within 12 hours of onset of symptoms had serial blood samples taken up to 12 months for analysis of inflammatory markers. Individually matched control subjects were also studied. An early and sustained peripheral inflammatory response to acute ischaemic stroke was seen. Next, enzyme-linked immunosorbent assays were developed for human IL-1β and IL-1ra. In the same patients, plasma IL-1ra concentration was significantly elevated at admission. Whole blood lipopolysaccharide-induced IL-1β, tumour necrosis factor-α and interleukin-6 (IL-6) production was significantly reduced relative to controls. These data suggest early or pre-existing activation of IL-1ra, and that induced cytokine production is reduced after acute ischaemic stroke.

The second clinical study was a randomised phase II study of rhIL-1ra in acute stroke patients. No adverse events were attributed to study treatment in 34 patients randomised. Markers of biological activity, including neutrophil count, CRP and IL-6 concentrations, were lower in rhIL-1ra treated patients. In patients with cortical infarcts clinical outcomes at 3 months in the rhIL-1ra treated group were better than in placebo treated. The findings identify rhIL-1ra as a potential new therapeutic agent for acute stroke.

Declaration

An earlier version of data from work referred to in chapter 3 of this thesis was submitted in support of an MD awarded by the University of Manchester to Dr Carole M Libetta in 2002. Clinical and radiological outcome data from the study referred to in chapters 3 and 5 of this thesis, but not reported in detail here, and interleukin-10 data referred to in chapter 5, are to be submitted in support of applications for the degrees of MD and MSc of the University of Manchester by Drs Craig J Smith and Vijitha Wickramasinghe respectively. No other portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Copyright Statement

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Dedication

This thesis is dedicated to my wife Elisa for her patience and support throughout the period of research and its writing-up, our beautiful daughter Natalia who has brought contentment to our now somewhat busier lives, and to my parents for their support and for their commitment to my education.

Publications and presentations

Original journal articles

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Abbreviations

^{99}mTc HMPAO	*Technetium-99m hexamethylpropyleneamine oxime*
ABPI	*Ankle-brachial pressure index*
ACEI	*Angiotensin converting enzyme inhibitor*
aCL	*Anticardiolipin antibodies*
ADL	*Activities of daily living*
AE	*Adverse event*
AIDS	*Acquired immune deficiency syndrome*
ANOVA	*Analysis of variance*
APC	*Activated protein C*
APR	*Acute phase response*
APS	*Antiphospholipid syndrome*
ATP	*Adenosine triphosphate*
BSA	*Bovine serum albumin*
BBB	*Blood brain barrier*
BI	*Barthel Index*
BP	*Blood pressure*
C4BP	*C4b binding protein*
C. pneumoniae	*Chlamydia pneumoniae*
Ca^{2+}	*Ionised calcium*
Cl^-	*Ionised chloride*
CNS	*Central nervous system*
COX	*Cyclooxygenase*
COX-2	*Cyclooxygenase-2*
CRF	*Case report form*
CRP	*C-reactive protein*
CSF	*Cerebrospinal fluid*
CT	*Computed X-ray tomography*
CV	*Coefficient of variation*
%CV	*Percentage coefficient of variation*
CXC, CC	*Chemokine sub-families*
CXR	*Chest X-ray*
DMC	*Data monitoring committee*
DNA	*Deoxyribonucleic acid*
DWI	*Diffusion weighted imaging*
ECG	*Electrocardiogram*
EDTA	*Ethylenediamine tetraacetic acid*
ELISA	*Enzyme-linked immunosorbent assay*
eNOS	*Endothelial nitric oxide synthase*
ESR	*Erythrocyte sedimentation rate*
FBC	*Full blood count*
FS	*Febrile seizure*
GABA	*γ-aminobutyric acid*
GCP	*Good clinical practice*
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
H. pylori	*Helicobacter pylori*
HIV	*Human immunodeficiency virus*
HMG-CoA	*β-hydroxy-β-methylglutaryl coenzyme A*

HPAA	*Hypothalamic-pituitary-adrenal axis*
HPLC	*High performance liquid chromatography*
HRP	*Horse radish peroxidase*
HS	*Hippocampal sclerosis*
hsCRP	*High-sensitivity C-reactive protein*
ICAM-1	*Intercellular adhesion molecule-1*
ICD-9	*International Classification of Diseases-9*
ICE	*Interleukin-1 converting enzyme*
ICH	*International Conference on Harmonisation*
icv	*Intracerebroventricular*
IFN-γ	*Interferon-γ*
Ig	*Immunoglobulin*
IgG	*Immunoglobulin G*
IgM	*Immunoglobulin M*
IL-1	*Interleukin-1*
IL-1α	*Interleukin-1α*
IL-1AcP	*Interleukin-1 accessory protein*
IL-1β	*Interleukin-1β*
IL-1ra	*Interleukin-1 receptor antagonist*
IL-1-RI	*Interleukin-1 receptor type I*
IL-1-RII	*Interleukin-1 receptor type II*
IL-2	*Interleukin-2*
IL-4	*Interleukin-4*
IL-6	*Interleukin-6*
IL-8	*Interleukin-8*
IL-10	*Interleukin-10*
IL-16	*Interleukin-16*
IL-17	*Interleukin-17*
IL-18	*Interleukin-18*
iNOS	*Inducible or immunological nitric oxide synthase*
IQR	*Interquartile range*
iv	*Intravenous*
kDa	*Kilodalton*
LACS	*Lacunar syndrome*
LDL	*Low density lipoprotein*
LFA-1	*Lymphocyte factor-associated antigen-1*
LFT	*Liver function tests*
LPS	*Lipopolysaccharide*
LREC	*Local research ethics committee*
MCA	*Middle cerebral artery*
MCAo	*Middle cerebral artery occlusion*
MCP-1	*Monocyte chemoattractant protein-1*
MedDRA	*Medical Dictionary for Regulatory Activities*
MHRA	*Medicines and Healthcare products Regulatory Agency*
MMP	*Matrix metalloproteinase*
MMP-2	*Matrix metalloproteinase-2*
MMP-9	*Matrix metalloproteinase-9*
MR	*Magnetic resonance*
MRA	*Magnetic resonance angiogram*
MRI	*Magnetic resonance imaging*

mRNA	*Messenger ribonucleic acid*
mRS	*Modified Rankin score*
Na^+	*Ionised sodium*
NF-κB	*Nuclear factor-κB*
nNOS	*Neuronal nitric oxide synthase*
NHS	*National Health Service*
NIBSC	*National Institute for Biological Standards and Control*
NIHSS	*National Institutes of Health Stroke Scale*
NO	*Nitric oxide*
NOS	*Nitric oxide synthase*
OCSP	*Oxfordshire Community Stroke Project*
OD	*Optical density*
OD_{490nm}	*OD at 490 nm*
OPD	*Orthophenylene diamine*
PACS	*Partial anterior circulatory syndrome*
PAI	*Plasminogen activator inhibitor*
PBBS	*Peripheral benzodiazepine binding site*
PBS	*Phosphate buffered saline*
PCA	*Posterior cerebral artery*
PET	*Positron emission tomography*
PICH	*Primary intracerebral haemorrhage*
POCS	*Posterior circulatory syndrome*
PVD	*Peripheral vascular disease*
PWI	*Perfusion weighted imaging*
QC	*Quality control*
RAS	*Renin-angiotensin-aldosterone system*
rhIL-1β	*Recombinant human IL-1β*
rhIL-1ra	*Recombinant human IL-1ra*
RIA	*Radioimmunoassay*
r-metHuIL-1ra	*Recombinant methionyl human IL-1ra*
RPMI	*Roswell Park Memorial Institute*
rt-PA	*Recombinant tissue plasminogen activator*
SAE	*Serious adverse event*
SAH	*Subarachnoid haemorrhage*
SD	*Standard deviation*
sE-Selectin	*Soluble E-Selectin*
sELAM-1	*Soluble endothelial leucocyte adhesion molecule-1*
sICAM-1	*Soluble intercellular adhesion molecule-1*
sIL-1-RI	*Soluble interleukin-1 receptor type I*
sIL-1-RII	*Soluble interleukin-1 receptor type II*
SLE	*Systemic lupus erythematosus*
SNP	*Single nucleotide polymorphism*
SOP	*Standard operating procedure*
SPECT	*Single photon emission computed tomography*
SPSS	*Statistics package for the social sciences*
sTNF-RI	*Soluble tumour necrosis factor receptor type I*
sTNF-RII	*Soluble tumour necrosis factor receptor type II*
sVCAM-1	*Soluble vascular adhesion molecule-1*
TACS	*Total anterior circulatory syndrome*
TGF-α	*Transforming growth factor-α*

TGF-β	*Transforming growth factor-β*
TGF-β_1	*Transforming growth factor-β_1*
TIA	*Transient ischaemic attack*
TIMP-2	*Tissue inhibitor of matrix metalloproteinase-2*
TLE	*Temporal lobe epilepsy*
TNF-α	*Tumour necrosis factor-α*
TOAST	*Trial of ORG-10172 in Acute Stroke Treatment*
t-PA	*Tissue plasminogen activator*
U+Es	*Urea and electrolytes*
UTI	*Urinary tract infection*
VCAM-1	*Vascular adhesion molecule-1*
vWF	*Von Willebrand factor*
VZ	*Varicella zoster*
WBC	*White blood cell*
WHO	*World Health Organisation*
WHO-ART	*WHO-Adverse Reaction Terminology*
ZnPP	*Zinc protoporphyrin*

CHAPTER 1

Introduction

1.1 Introduction to stroke

1.1.1 Definitions and disease burden

"It is impossible to cure a severe attack of apoplexy and no easy matter to cure a mild one" (Hippocrates). This was written more than two millennia ago, when stroke, or apoplexy, was thought to be due to an accumulation of black bile in the arteries of the brain obstructing the passage of animated spirits from the ventricles. Today, stroke is defined by the World Health Organisation (WHO) as a clinical syndrome of rapid onset of focal (or global) cerebral deficit, lasting more than 24 hours or leading to death, with no apparent cause other than a vascular one. Stroke remains the greatest cause of severe disability and the third greatest killer worldwide after ischaemic heart disease and cancer, with two-thirds of stroke deaths occurring in less developed countries (Warlow *et al.*, 2003). By 2020 stroke mortality is expected to have almost doubled. Stroke already accounts for a significant proportion of health service budgets, yet stroke research remains disproportionately underfunded, particularly in the United Kingdom (Rothwell, 2001).

There are three pathological types of stroke, ischaemic (about 80% in white populations), primary intracerebral haemorrhage (PICH) (about 15%), and subarachnoid haemorrhage (SAH) (about 5%) (Warlow *et al.*, 2003). Transient ischaemic attack (TIA) differs from ischaemic stroke in its (arbitrary) duration < 24 h, differential diagnosis (which includes focal seizures to a greater extent than stroke), and ease of diagnosis (relying almost entirely on clinical history). In white populations, approximately 50% of all ischaemic strokes and TIAs are due to atherothrombotic disease of the extracranial or large intracranial arteries. Cardiac embolism accounts for 20%; lacunar infarcts (probably due to occlusion of small, deep, perforating cerebral arteries) account for 25%; with the remainder being due to rarer causes including vasculitides and arterial dissection. These proportions inevitably vary, for instance in the elderly there may be more than one possible cause (eg atrial fibrillation and carotid stenosis), or no definite cause may be found. Risk factors for stroke are similar to conventional risk factors for disease in other parts of the arterial tree – hypertension, age, cigarette smoking,

diabetes mellitus and obesity. Elevated blood pressure appears to be an even more important risk factor than in ischaemic heart disease as there is a steeper continuous relation between blood pressure and stroke risk (Prospective Studies Collaboration, 2002). In contrast to ischaemic heart disease, there appears to be no overall association between plasma cholesterol concentration and stroke (Prospective Studies Collaboration, 1995). Atrial fibrillation is common, especially in the elderly, and carries a high relative risk of stroke. Novel risk factors for stroke have been proposed recently, including inflammation, infection, plasma homocysteine concentrations and various genetic polymorphisms (Lindsberg and Grau, 2003; Homocysteine Studies Collaboration, 2002; Hassan and Markus, 2000).

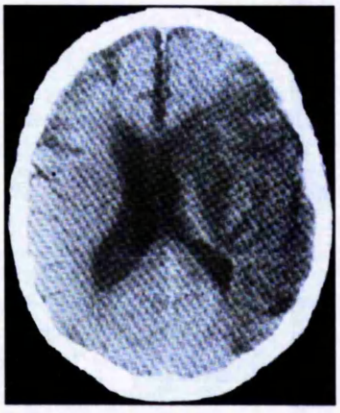
Stroke is a devastating disease. Overall, 30-day mortality has been estimated to be 24% (Intercollegiate Stroke Working Party, 2002), and a large proportion of survivors are left significantly disabled. Early deaths are often secondary to neurogenic complications, while later deaths are more likely to be due to infections and thromboembolic complications. Stroke affects different patients in different ways, depending on the stroke subtype, area(s) of the brain involved, the extent of the brain injury, and various other factors. Brain injury from a stroke can cause a diverse range of deficits including limb weakness, loss of sensation, visual impairment, language and swallowing difficulties, memory and perceptual abnormalities and behavioural disturbance. The severity of the deficit is dependent upon the size and site of the lesion, and the capacity of the brain for recovery. Recovery itself is dependent upon a number of possible mechanisms including resolution of the ischaemic penumbra and neural plasticity.

1.1.2 Diagnosis of acute stroke

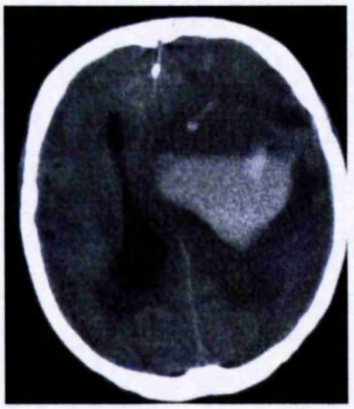
Acute stroke is a medical emergency, and the scope for benefit from treatment diminishes the longer treatment is delayed – "time is brain". Therefore rapid, accurate diagnosis is crucial. Although the clinical diagnosis of stroke (versus not stroke) is reasonably accurate when done by specialists, up to 29% of paramedic, primary care and emergency room clinical diagnoses are incorrect (Harbison *et*

al., 2003). Cerebral infarction cannot be distinguished from haemorrhage on clinical grounds alone, and prompt brain imaging is required.

Computed X-ray tomography (CT) is quick and can be done in almost all patients, however ill. Although CT may not show a definite infarct, a normal scan does not exclude a stroke, and in fact around 50% of infarcts never become visible on CT. Within the first few hours of onset, few infarcts can be seen, but they become visible over the first week as dark hypodense wedge-shaped areas (round if lacunes), with mass effect (figure 1.1a). A proportion of infarcts become transiently invisible through the fogging effect (Becker *et al.*, 1979), subsequently reappearing as cerebromalacia. CT most reliably demonstrates acute haemorrhage within the first week after stroke onset (figure 1.1b). Subsequently, small haemorrhages gradually lose their characteristic white (hyperdense) appearance and may be mistaken for infarcts. Inappropriate management may follow the misdiagnosis of an infarct when CT is performed more than 2 weeks after the stroke. Although magnetic resonance imaging (MRI) within the first few hours of stroke onset may not correctly identify acute haemorrhage, thereafter, a characteristic appearance emerges. Ideally, therefore, in the patient presenting longer than a week after the acute stroke, magnetic resonance (MR) with gradient echo imaging (also known as T2*) should be used.



1.1a



1.1b

Figure 1.1 *CT brain scans showing a left total anterior circulatory infarct appearing as a dark, hypodense wedge-shaped area at 5-7 days after onset of acute stroke symptoms (1.1a), and a left cerebral hemisphere primary intracerebral haemorrhage appearing as a hyperdense area with surrounding oedema and significant mass effect (1.1b) (courtesy of Salford Royal Hospitals National Health Service [NHS] Trust).*

MR diffusion-weighted imaging (DWI), which identifies ischaemia or infarction within a few minutes as bright areas, has led to the wider use of MRI in acute stroke, but some infarcts still never become visible. However, MRI cannot be used in certain patient groups including those who are very ill. In addition it has relatively limited availability and requires considerable effort to ensure adequate patient monitoring during the investigation, and is therefore not as universally useful as CT. Recent advances in CT are further underlining its usefulness, for example, perfusion CT surpasses plain CT in depicting the early stages of cerebral hypoperfusion (figure 1.2), can be performed quickly, and is likely to be a useful tool in the assessment of patients with acute stroke (Kŏnig, 2003).

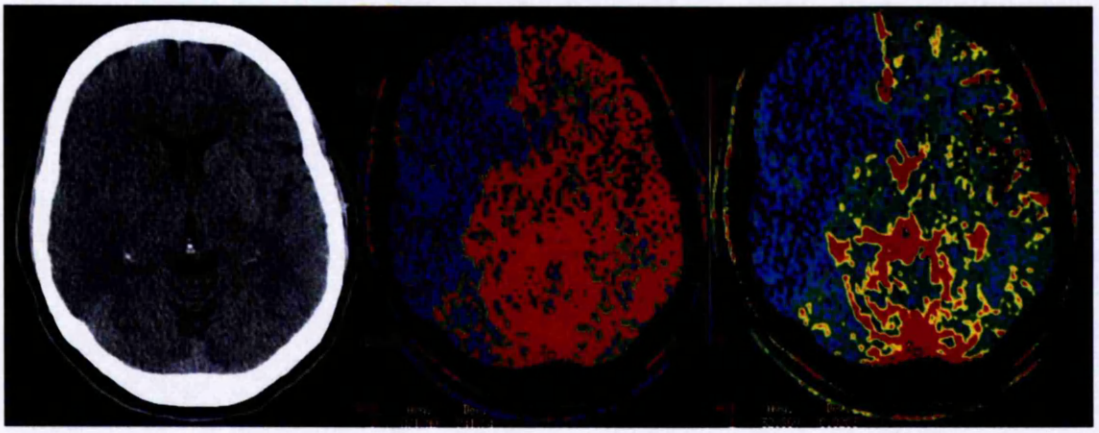


Figure 1.2 *CT brain scan (left) of a 62-year-old female 5 h after onset of symptoms of acute stroke shows signs of early infarction in the right cerebral hemisphere. Corresponding perfusion CT images showing cerebral blood flow (centre) and cerebral blood volume (right) demonstrate the ease with which cerebral ischaemia is detectable using this technique (courtesy of Salford Royal Hospitals NHS Trust).*

1.1.3 Management of acute stroke

Compared with conventional care in a general medical ward, stroke unit care achieves a relative risk reduction of 9% in death or dependency at 1 year after stroke, and a greater number of people in the population will avoid death or dependency through this than as a result of any other intervention (Hankey and Warlow, 1999).

In patients with acute ischaemic stroke, aspirin (150 – 300 mg) should be given immediately after the exclusion of intracranial haemorrhage on brain imaging

and thereafter at a daily dose of 75 – 150 mg (Edinburgh Consensus Statement, 1998). Aspirin lowers the risk of early recurrent stroke and increases the chance of disability-free survival with approximately one less patient dead or dependent per 100 treated (Gubitz *et al.*, 2003). Intravenous thrombolysis with recombinant tissue plasminogen activator (alteplase; 0.9 mg/kg over 1 h) almost certainly improves the chance of disability-free survival in the minority of patients who can receive treatment within 3 h of symptom onset (Wardlaw *et al.*, 2003).

1.1.4 Secondary prevention

The risk of recurrent vascular events following ischaemic stroke and TIA is high, and has been estimated to be 5% per year for stroke (perhaps 10% in the first year) (Warlow *et al.*, 2003). Recent data suggest the early risk of recurrent stroke after a TIA or minor stroke is higher than previously suggested, perhaps 11 – 15% in the first month (Lovett *et al.*, 2003; Coull *et al.*, 2004), emphasizing the need for rapid assessment of these patients and institution of secondary prevention measures. Aspirin lowers the relative risk of stroke by about a fifth, and of myocardial infarction by about a quarter (Antithrombotic Trialists' Collaboration, 2002). Adding modified-release dipyridamole to aspirin may lower stroke risk further (Antithrombotic Trialists' Collaboration, 2002). Clopidogrel may be slightly more effective than aspirin alone, and data concerning clopidogrel and aspirin combination therapy for the prevention of new ischaemic events are awaited (Diener, 2003). Long-term oral anticoagulation decreases stroke risk by about two-thirds compared with control in patients with atrial fibrillation, and is better than aspirin alone (Hart *et al.*, 1999). Reduction of blood pressure by 9 mm Hg systolic and 4 mm Hg diastolic using a combination of perindopril and indapamide achieves a relative-risk reduction of secondary stroke of one-quarter (PROGRESS Collaborative Group, 2001). Although higher plasma cholesterol concentration does not appear to be directly associated with increased stroke risk, lowering the concentration in patients with baseline cholesterol > 3.5 mmol/l with daily simvastatin (40 mg) does reduce the risk (Heart Protection Study Collaborative Group, 2002). The high risk of ischaemic stroke ipsilateral to a recently symptomatic carotid artery stenosis greater than 70% (without near-occlusion) is greatly reduced by carotid

endarterectomy (Rothwell *et al.*, 2003). Lifestyle changes such as stopping smoking, weight reduction, regular exercise, avoiding heavy alcohol consumption, and reduced salt and saturated fat intake are all important in modifying vascular risk but lack formal randomized controlled trial data.

1.2 Mechanisms of ischaemic brain injury

1.2.1 Vascular occlusion, the ischaemic core and penumbra

The pathophysiological event ultimately responsible for the majority of ischaemic strokes is occlusion of carotid or cerebral vessels (see figure 1.3), as a result of atherothrombosis, thromboembolism or cardioembolism. Other mechanisms contribute to small vessel occlusive disease and haemorrhagic stroke. At the heart of a cerebral infarct is the ischaemic 'core', where the virtual absence of blood flow restricts the local delivery of oxygen and glucose. This impedes the energy metabolism necessary to maintain normal trans-membrane ionic gradients and initiates an ischaemic cascade of events leading to cell death. The concept of the ischaemic penumbra, a zone of intermediate blood flow normally surrounding an area of recent cerebral infarction where tissue is functionally impaired but potentially viable, was originally proposed in a baboon model of cerebral ischaemia (Astrup *et al.*, 1981). Criteria for the measurement of penumbra have been proposed which include a physiological description of the state of the tissue, its location and fate, and relation to clinical outcome (Baron, 1999). The notion of a perihaematomal ischaemic penumbra in a subset of patients with primary intracerebral haemorrhage has also been suggested (Schellinger *et al.*, 2003).

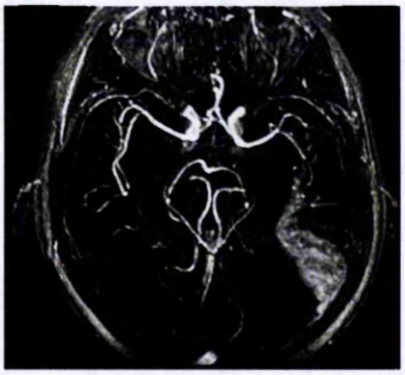


Figure 1.3 *Magnetic resonance angiogram (MRA) showing circle of Willis. A left middle cerebral artery branch occlusion has a cerebral infarct extending from it (courtesy of IW Turnbull, Salford Royal Hospitals NHS Trust).*

1.2.2 Molecular mechanisms of ischaemic brain injury

Cerebral ischaemia triggers a complex cascade of molecular events lasting hours to days (Neumar, 2000). Initially, failure of delivery of energy metabolites leads to depolarisation of neurones and glia, with activation of calcium channels and release of excitatory amino acids such as glutamate, which accumulate in the extracellular space. Following this there is an ion-channel mediated intracellular overload of Ca^{2+}, Na^{+}, Cl^{-} and water which contributes to cerebral oedema. The accumulation of intracellular Ca^{2+} and other stimuli trigger lipolysis, proteolysis, nitric oxide production, endonuclease-mediated deoxyribonucleic acid (DNA) degradation and the activation of kinases and phosphatases that alter protein function and initiate gene expression. Further events contributing to cellular damage and loss include the production of oxygen- and nitrogen-based free radicals, generation of inflammatory mediators, such as cytokines, prostaglandins and nitric oxide, protease activation (such as calpains, caspases and extracellular proteases), mitochondrial and endoplasmic reticulum dysfunction and apoptosis.

1.3 Inflammation in stroke

Atherosclerosis is the common pathological entity underlying the majority of vascular disease, including cerebral and cardiac ischaemia. The interplay between classic risk factors for atherosclerotic disease such as hypertension, smoking, dyslipidaemia and diabetes mellitus is poorly understood. There is increasing evidence that inflammatory mechanisms are involved in both the development and progression of atherosclerosis and its clinical manifestations (Ross, 1999). Inflammation is certainly important in the pathophysiology of cerebral ischaemia in the setting of stroke (Barone and Feuerstein, 1999). It also appears that in cerebral ischaemia occurring after subarachnoid haemorrhage, head injury or cardiac arrest, inflammatory mechanisms play an important role in pathophysiology (Fassbender *et al.*, 2001; Mussack *et al.*, 2001).

There is now evidence that low-grade inflammation, identified by an elevated C-reactive protein (CRP) level, may be an additional risk factor for the development of ischaemic stroke or TIA (Ridker *et al.*, 1997; Rost *et al.*, 2001).

Even in the absence of significant atherosclerosis, for example in paediatric stroke, inflammatory mechanisms are important (Takeoka and Takahashi, 2002). Preceding systemic infections are also linked to stroke risk (Syrjänen *et al.*, 1988; Grau *et al.*, 1995a), perhaps by eliciting a systemic inflammatory response. Resolving the debate over whether inflammation confers harm, benefit, or perhaps a combination of both, presents a significant challenge (del Zoppo *et al.*, 2001; Feuerstein and Wang, 2001). Clinical investigations in stroke are usually limited to blood or cerebrospinal fluid (CSF) sampling after the event. New approaches such as novel imaging techniques are becoming increasingly important as they facilitate *in vivo* observations of inflammation as a dynamic process in relation to brain tissue damage and neurological outcome.

Inflammation comprises both molecular and cellular components. Experimental studies of ischaemia have shown that a key cellular inflammatory event occurs at the blood-microvascular endothelial cell interface (Hallenbeck, 1996). Locally produced cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) released by microglia, astrocytes, endothelial cells and neurons influence this process (del Zoppo *et al.*, 2000). Within minutes to hours of reduction of cerebral blood flow, leucocyte recruitment, activation and adhesion to the endothelium of the cerebral microvasculature occur. Activated leucocytes obstruct the affected cerebral microvasculature (del Zoppo and Mabuchi, 2003), and transmigration of neutrophils and monocytes/macrophages occurs into the cerebral infarct (Garcia *et al.*, 1994). Reperfusion may also influence the extent of inflammatory injury; furthermore it is possible that inflammation-associated reperfusion injury may limit the efficacy of thrombolytic therapy in acute stroke (Jean *et al.*, 1998). In addition to the central inflammatory response, systemic inflammatory processes also occur, both before and after acute stroke.

1.3.1 Molecular components of inflammation

Inflammatory gene expression

Multiple gene expression elicited by cerebral ischaemia is among the earliest events occurring after stroke (Barone and Feuerstein, 1999). Numerous pro-inflammatory genes are upregulated, including transcription factors, heat shock

proteins, cytokines, chemokines and adhesion molecules. Many such genes are regulated *in vitro* by the transcription factor nuclear factor (NF)-κB, including TNF-α, IL-1β, interleukin-6 (IL-6), nitric oxide synthase (NOS), cyclooxygenase-2 (COX-2) and intercellular adhesion molecule-1 (ICAM-1) (Baeuerle and Henkel, 1994). NF-κB is activated in experimental cerebral ischaemia (Schneider *et al.*, 1999) and may also regulate gene expression in clinical ischaemic stroke, since it is induced in glial cells in human cerebral infarctions (Terai *et al.*, 1996). Recent experimental data suggest that interleukin-1 converting enzyme (ICE) or IL-1 are involved in early NF-κB activation during cerebral ischaemia (Huang *et al.*, 2003).

Cytokines

Cytokines are polypeptides generally associated with inflammation, immune activation, and cell differentiation or death. In the periphery they are produced by activated macrophages, monocytes, lymphocytes, endothelial cells, fibroblasts, platelets and many other cell types, whilst activated microglia are their principal site of production in the central nervous system (CNS). Cytokines act in a self-regulating network, in which there also exist endogenous inhibitors of cytokine activity released by different cells, including soluble cytokine receptors and receptor antagonists. The net effect of any cytokine is dependent on the source and timing of cytokine release, the local milieu in which it acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine. Evidence for the contribution of cytokines to both CNS and peripheral inflammation associated with stroke is derived both from animal models and from clinical studies, although in the latter, data pertaining to the time course and cellular location of cytokine expression are often lacking. Distinguishing between the cytokine response to injury and early expression that might contribute to cell death is therefore difficult. The classic, pro-inflammatory cytokines IL-1 and TNF-α appear to exacerbate cerebral ischaemic injury, whereas the anti-inflammatory molecules interleukin-10 (IL-10) and the naturally occurring selective IL-1 receptor antagonist (IL-1ra) appear to be neuroprotective (Allan and Rothwell, 2001).

Interleukin-1

Interleukin-1 is considered separately in section 1.8.

Interleukin-6

A role for IL-6 in the response to focal ischaemic brain damage is supported by induction of its mRNA (Wang *et al.*, 1995), and a dramatic increase in IL-6 bioactivity in the ischaemic hemisphere (Loddick *et al.*, 1998) after middle cerebral artery occlusion (MCAo) in the rat. Intracerebroventricular injection of recombinant IL-6 significantly reduces ischaemic brain damage after MCAo, suggesting that this cytokine is an important endogenous inhibitor of neuronal death during cerebral ischaemia (Loddick *et al.*, 1998).

In a small study of patients with acute cerebral ischaemia, circulating IL-6 concentrations increased significantly, reaching a plateau between 10 h and 3 days, before returning to baseline by 7 days (Fassbender *et al.*, 1994a). Elevated IL-6 concentrations showed a modest, but significant positive correlation with volume of computed tomography brain lesion as well as poor functional and neurological outcome. Other reports support the positive correlation with circulating IL-6 concentration and infarct volume (Vila *et al.*, 2000a; Perini *et al.*, 2001). There have been various other reports of elevated plasma IL-6 concentrations in patients who have had acute strokes (Beamer *et al.*, 1995; Tarkowski *et al.*, 1995; Kim *et al.*, 1996; Fassbender *et al.*, 1997; Carlstedt *et al.*, 1997; Ferrarese *et al.*, 1999; Vila *et al.*, 2000b). Stimulated blood cells from stroke patients show a significant increase in IL-6 release from days 1 to 2 until 1 month (Ferrarese *et al.*, 1999). It has been suggested that inter-individual variation in IL-6 levels in acute stroke patients depend on the IL-6 promoter haplotype (Acalovschi *et al.*, 2003). CSF IL-6 concentrations are significantly higher than serum concentrations, peaking on days 2 and 3, with initial CSF IL-6 concentrations significantly correlating with MRI infarct volume at 2-3 months (Tarkowski *et al.*, 1995). These data suggest intrathecal production of IL-6 in patients with stroke, but it remains unclear whether these findings reflect overproduction of IL-6 associated with greater tissue injury or a directly damaging effect of IL-6 itself. By contrast, IL-6 may have anti-inflammatory (Tilg *et al.*, 1994) and neuroprotective (Loddick *et al.*, 1998) effects as well as

pro-inflammatory effects. Raised circulating IL-6 levels have also been proposed as part of the inflammatory signature of advanced atherosclerosis (Erren *et al.*, 1999).

Tumour necrosis factor-α

Experimental evidence is accumulating for a role for TNF-α in ischaemic brain injury (Hallenbeck, 2002). Induction of TNF-α mRNA occurs in ischaemic cortex after both permanent MCAo (Liu *et al.*, 1994; Buttini *et al.*, 1996) and transient MCAo (Wang *et al.*, 1994) in rats, in addition to the presence of neuronal TNF-α protein following permanent MCAo (Liu *et al.*, 1994; Buttini *et al.*, 1996). Focal experimental cerebral ischaemic injury is reduced by inhibition of TNF-α activity using soluble TNF-receptor I (sTNF-RI) (Dawson *et al.*, 1996; Barone *et al.*, 1997) or anti-TNF-α monoclonal antibody (Barone *et al.*, 1997), whilst it is exacerbated by administration of TNF-α (Barone *et al.*, 1997). TNF-α may also have a neuroprotective role as mice genetically deficient in TNF-receptors show enhanced ischaemic injury (Bruce *et al.*, 1996).

TNF-α is upregulated in the post-mortem brain tissue of patients with acute cerebral infarction (Tomimoto *et al.*, 1996), and appears sequentially in infarction core and peri-infarct areas prior to expression in contralateral hemisphere and brain areas remote from the infarction (Sairanen *et al.*, 2001). TNF-α expression is intense and prolonged in microglia, its probable principal source in the brain (Dziewulska and Mossakowski, 2003). CSF TNF-α concentrations are elevated in patients with acute ischaemic stroke (Vila *et al.*, 2000a; Zaremba *et al.*, 2001), including those with pronounced white matter lesions (Tarkowski *et al.*, 1997). Serum concentrations of TNF-α are elevated in most studies of acute ischaemic stroke patients (Intiso *et al.*, 1997; Carlstedt *et al.*, 1997; Vila *et al.*, 2000a; Zaremba *et al.*, 2001), and raised plasma TNF-α concentrations in patients with lacunar infarction are associated with early neurological deterioration and poor functional outcome (Castellanos *et al.*, 2002). In one study, however, no such rise in serum TNF-α concentrations was seen (Fassbender *et al.*, 1994a). A significant increase in TNF-α release from stimulated blood cells persists for at least 3 months after stroke (Ferrarese *et al.*,

1999). Elevated serum sTNF-RI and sTNF-RII concentrations are associated with carotid atherosclerosis (Elkind *et al.*, 2002), and sTNF-RI concentrations are increased in patients with acute ischaemic stroke and infection (Fassbender *et al.*, 1997).

Transforming growth factor

Experimental evidence exists for a role for members of the TGF-β family in acute neurodegeneration, including cerebral ischaemia (Allan and Rothwell, 2001). Administration of TGF-α has been shown to reduce infarct volume after focal transient cerebral ischaemia in the rat (Justicia *et al.*, 2001). Increased expression of TGF-β_1 mRNA and protein occurs in brain tissue after ischaemic stroke in humans, particularly in infarct border zones (Krupinski *et al.*, 1996). This observation may be in keeping with a neuroprotective action of TGF-β_1 within the ischaemic penumbra. Serum concentrations of TGF-β have been reported to decrease in patients with acute stroke, regardless of stroke subtype (Kim *et al.*, 1996) although a subsequent study demonstrated no difference in serum TGF-β_1 concentrations between acute ischaemic stroke patients and control subjects (Slevin *et al.*, 2000). Because TGF-β expression is prominent in the recovery phase of some models of CNS disease, it has been proposed that this cytokine contributes to disease recovery (Benveniste, 1998).

Interleukin-10

The cytokine IL-10 is primarily anti-inflammatory and is neuroprotective in experimental focal cerebral ischaemia (Spera *et al.*, 1998). Elevated numbers of peripheral blood mononuclear cells secreting IL-10 are seen in patients with acute ischaemic and haemorrhagic stroke (Pelidou *et al.*, 1999). CSF concentrations of IL-10 are also elevated in acute ischaemic stroke (Tarkowski *et al.*, 1997). A protective role for this molecule is supported by clinical studies suggesting that subjects with low IL-10 production levels have an increased risk of stroke (van Exel *et al.*, 2002), and suffer neurological worsening in acute ischaemic stroke (Vila *et al.*, 2003).

Other cytokines

Various other cytokines may have important roles in the regulation of inflammation following cerebral ischaemia. Limited experimental and/or clinical evidence exists for several such molecules, including interferon (IFN)-γ (Tomimoto *et al.*, 1996; Li *et al.*, 2001), IL-2 (Kim *et al.*, 2000), IL-4 (Kim *et al.*, 2000), IL-16 (Schwab *et al.*, 2001), IL-17 (Li *et al.*, 2001), IL-18 (Zaremba and Losy, 2003) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Tarkowski *et al.*, 1999).

Chemokines

Chemokines are a family of regulatory polypeptides, generally smaller than other cytokines, with roles in cellular communication and inflammatory cell recruitment in host defence. IL-8 and monocyte chemoattractant protein-1 (MCP-1), of the CXC and CC chemokine sub-families respectively (CXC and CC relating to the position of cysteine residues), have been implicated in cerebral ischaemia. Overall the clinical data currently available suggest chemokines have a deleterious role after cerebral ischaemia by increasing leucocyte infiltration (Mennicken *et al.*, 1999).

Interleukin-8

A systemic increase of mononuclear cells expressing IL-8 mRNA in blood, and increased concentrations of plasma IL-8, occurs in patients with acute ischaemic stroke. It has been suggested that IL-8 may have a role in recruiting peripheral neutrophils to sites of cerebral ischaemia (Kostulas *et al.*, 1998). Furthermore, IL-8 concentrations are higher in CSF compared with plasma in patients with ischaemic stroke, indicating that the CNS may be the predominant site of production (Kostulas *et al.*, 1999). CSF IL-8 concentrations differ between patients with large infarcts or grey matter infarcts, and patients with small lesions mainly located in the white matter. In the former group, IL-8 concentrations are initially elevated and then fall rapidly, suggesting a role for IL-8 in acute inflammation. In the latter group, IL-8 concentrations remain elevated up to 3 months, which may be suggestive of a neuroprotective role (Tarkowski *et al.*, 1997).

Monocyte chemoattractant protein-1 (MCP-1)

This potent chemokine is specific for monocytes, and is thought to have a significant role in monocyte/macrophage infiltration in experimental cerebral ischaemia. A significant increase of CSF MCP-1 concentration occurs in acute ischaemic stroke patients (Losy and Zaremba, 2001).

Nitric oxide synthase (NOS) and cyclooxygenase (COX)

The roles of NOS and COX in post-ischaemic inflammation after experimental cerebral ischaemia have been reviewed (del Zoppo *et al.*, 2000). Three NOS isoforms exist, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible or immunological NOS (iNOS). It has been proposed that nitric oxide (NO) produced early by eNOS may be beneficial via vasodilatation, whilst NO produced later by nNOS and iNOS may contribute to ischaemic injury. Infarct volumes after MCAo are smaller following selective iNOS inhibition, or in mice lacking the iNOS gene. In rodent models, iNOS mRNA, protein and enzymatic activity are present in the inflammatory cell infiltrate in ischaemic regions and in cerebral blood vessels. iNOS expression has also been demonstrated in neutrophils and blood vessels in cerebral infarcts of patients who died within 24 h of ischaemic stroke (Forster *et al.*, 1999).

COX-2 mRNA and protein are present at the border of the ischaemic territory in experimental cerebral ischaemia, and COX-2 reaction products, including prostaglandins and reactive oxygen species, are thought to exacerbate ischaemic damage (del Zoppo *et al.*, 2000). In post-mortem specimens from acute ischaemic stroke patients, COX-2 is up-regulated both locally, in regions of ischaemic damage (Iadecola *et al.*, 1999) and more globally, including regions remote from the infarct (Sairanen *et al.*, 1998). Microglia are the major source of COX-2 expression in post-mortem brain tissue of patients with chronic cerebral ischaemia (Tomimoto *et al.*, 2000). iNOS and COX-2 up-regulation in the human brain suggests an involvement of these two enzymes in the mechanisms of cerebral ischaemia in clinical stroke.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in extracellular matrix modelling, but may contribute to the neuroinflammatory response. In experimental cerebral ischaemia, MMPs contribute to the disruption of the blood-brain barrier (BBB) that leads to vasogenic cerebral oedema and haemorrhage (Mun-Bryce and Rosenberg, 1998). In patients with acute ischaemic stroke, elevated MMP-9 occurs in both brain tissue (Clark *et al.*, 1997) and in circulation, together with decreased serum level of tissue inhibitor of MMP-2 (TIMP-2) (Horstmann *et al.*, 2003). Human brain tissue also shows MMP-2 several months after an infarct (Clark *et al.*, 1997). Both in cerebral ischaemia and haemorrhage, MMPs contribute to the acute tissue damage and to the opening of the BBB. High plasma MMP-9 concentration after acute cerebral infarction is associated with neurological impairment (Montaner *et al.*, 2001), thrombolysis failure where recanalisation does not occur (Heo *et al.*, 2003), and predicts haemorrhagic transformation (Castellanos *et al.*, 2003). Whilst MMP-9 appears to be associated with injury in the early phases of stroke, the fact that MMP-2 is found in the later reparative phases, suggests that MMPs have both deleterious and beneficial effects (Rosenberg, 2002).

Adhesion molecules

The interaction between leucocytes and the vascular endothelium is mediated by three main groups of cell adhesion molecules, selectins (comprising P-selectin, E-selection and L-selectin), integrins (eg lymphocyte function-associated antigen-1 [LFA-1] and Mac-1) and the immunoglobulin (Ig) superfamily (which includes intercellular adhesion molecule-1 [ICAM-1] and vascular adhesion molecule-1 [VCAM-1]) (DeGraba, 1998). Data currently available suggest that adhesion molecules may have roles not only in leucocyte infiltration into the brain during ischaemic stroke, but also in the pathogenesis of atherosclerosis and in the conversion of plaque to a symptomatic state.

Localised ICAM-1 expression occurs in histologically normal human carotid bifurcation (Endres *et al.*, 1997), a high-risk region for the development of atherosclerotic plaque, whilst endothelial ICAM-1 expression is increased in

symptomatic versus asymptomatic carotid plaque (DeGraba *et al.*, 1998). Patients with carotid atherosclerosis who may be described as 'pre-stroke' have raised concentrations of soluble (s)ICAM-1 (Hwang *et al.*, 1997; Blann *et al.*, 1999) and E-selectin (Hwang *et al.*, 1997). Chronic alteration of adhesion molecule expression also occurs in the presence of risk factors for atherosclerosis, with an increase in sICAM-1 serum concentration and a decrease in sL-selectin (Fassbender *et al.*, 1995). Adhesion molecules are also expressed by human brain microvascular endothelium. The expression of ICAM-1, VCAM-1 and E-selectin by cerebral microvascular endothelial cells is increased by IL-1β, TNF-α, lipopolysaccharide (LPS) (Hess *et al.*, 1994; Stanimirovic *et al.*, 1997), and *in vitro* ischaemia-like insults (Stanimirovic *et al.*, 1997). ICAM-1 expression by brain microvessels is significantly increased in the cerebral infarcts of patients who died after ischaemic stroke (Lindsberg *et al.*, 1996).

Several studies have also demonstrated a rise in serum concentrations of soluble adhesion molecules after cerebral ischaemia. sICAM-1 concentrations are elevated (Shyu *et al.*, 1997) and may peak (Bitsch *et al.*, 1998) in patients within 24 h of acute ischaemic stroke. sVCAM-1 concentrations are elevated between days 1 and 5 (Fassbender *et al.*, 1995; Blann *et al.*, 1999) with a peak at 5 days (Bitsch *et al.*, 1998). A transient rise in circulating concentration of soluble endothelial leucocyte adhesion molecule-1 (sELAM-1) also occurs (Fassbender *et al.*, 1995). Furthermore, elevated serum levels of sICAM-1 and sE-selectin occur in patients with both large- and small-vessel cerebrovascular disease (Fassbender *et al.*, 1999). One study reported decreased concentration of sICAM-1 in acute stroke in parallel with increased *in vitro* neutrophil adhesion (Clark *et al.*, 1993). At present it is uncertain whether measurement of soluble adhesion molecules adds useful information about stroke risk beyond that provided by CRP measurement (Tan and Blann, 2003).

1.3.2 Cellular components of inflammation

The major inflammatory cells to be activated and accumulate within the brain following cerebral ischaemia are blood derived leucocytes and resident microglia. Leucocytes clearly perform vital roles in normal host defence, and

there is increasing evidence that neutrophils in particular may be mediators of secondary brain damage in cerebral ischaemia and reperfusion. The experimental work addressing neutrophils and monocytes/macrophages in this context has been reviewed in detail (Kochanek and Hallenbeck, 1992). Microglia are the first non-neuronal cells to respond to CNS injury, are the major CNS source of cytokines and other immunomolecules and become phagocytic when fully activated by neuronal death.

Leucocytes

In subjects exposed to the atomic bombs in Japan under routine follow-up, elevated total leucocyte counts have been associated with a higher risk of cerebral infarction, and neutrophil counts showed a significant positive relationship with incidence of stroke (Prentice *et al.*, 1982). Epidemiological studies have shown similar correlations between leucocyte count and the risk of myocardial infarction (Ernst *et al.*, 1987). Although the contribution of smoking to this association has been debated, a component of the correlation between leucocyte count and vascular disease has been demonstrated to be independent of smoking (Ernst *et al.*, 1987).

In patients with acute ischaemic stroke, peripheral leucocyte counts are increased (Pozzilli *et al.*, 1985a; D'Erasmo *et al.*, 1991). Labelled leucocyte uptake by infarcted areas (Pozzilli *et al.*, 1985b) and infiltration in areas of perfusion defect (Wang *et al.*, 1993) have also been described. Selectively labelled neutrophil accumulation in the brain occurs 6-12 h after stroke onset, progresses for up to 24 h and then declines (Akopov *et al.*, 1996). Histopathological studies in human post-mortem specimens have shown that neutrophil accumulation around the infarct core is maximal at 48-72 h, and is replaced by mononuclear cells by 4-6 days (Adams and Sidman, 1968). Furthermore, in serial cerebrospinal fluid samples of patients with acute stroke, neutrophil outflow is maximal on day four and modest rises in macrophages and monocytoids reach maxima towards the end of the first week (Sörnäs *et al.*, 1972).

Microglia

Most of the data pertaining to microglia in cerebral ischaemia derives from experimental rather than clinical work. 'Resting' or ramified microglia constitute 5-20% of the total CNS glial population and following focal cerebral ischaemia they undergo substantial transformation of morphology and metabolic activity. They become enlarged with stout processes and undergo a rapid and profound gene upregulation, with the degree of activation being dependent on time and the extent of injury (Zhang *et al.*, 1997; Kato and Walz, 2000). Observations in rodents have shown that microglia and macrophages are the principal CNS source of cytokines such as IL-1β, TNF-α, and TGF-β (Rothwell, 1999; Gregersen *et al.*, 2000; Lehrmann *et al.*, 1998). The relevance of microglia to stroke in the clinical setting *in vivo* is being elucidated by novel imaging approaches outlined below.

1.3.3 Classic acute phase reactants and body temperature

The acute phase response (APR) comprises a variety of systemic changes in response to tissue injury, infection and inflammation, and is cytokine mediated, mainly by IL-6 and IL-1 (Ramadori and Christ, 1999). Classic 'positive' acute phase reactants which are elevated in patients with acute cerebral infarction include the plasma proteins CRP, serum amyloid A protein and fibrinogen (Syrjänen *et al.*, 1989a).

C-reactive protein

CRP is a trace protein in healthy subjects, having a median concentration around 1 mg/l. It is the major acute phase protein in humans, and its concentration can rise 100 fold or more in response to injury, inflammation or infection (Pepys, 1981). Recently, high-sensitivity (hs) assays have suggested that subtle elevations in CRP concentration may be an indicator of underlying systemic inflammation and atherosclerotic disease. Elevated CRP concentration predicts the risk of first ischaemic stroke among apparently healthy men and women (Ridker *et al.*, 1997; Ridker *et al.*, 1998a), including thromboembolic stroke in men (Curb *et al.*, 2003), future ischaemic stroke and TIA in the elderly (Rost *et al.*, 2001), and fatal stroke in the elderly (Gussekloo *et al.*, 2000). Plasma CRP

concentration is elevated in acute stroke patients with or without infection (Grau *et al.*, 1995b), and is an independent predictor of survival or non-fatal vascular events after ischaemic stroke (Muir *et al.*, 1999; Di Napoli *et al.*, 2001). It has been suggested that a very early increase (within 3 h of stroke) occurs (Di Napoli, 2001), and that a CRP increase 12 – 24 h after symptom onset may predict an unfavourable outcome (Winbeck *et al.*, 2002) although only limited data on its early time course exist for stroke. Inflammation is also likely to be involved in the progression and complication of intracranial large-artery occlusive disease, as hsCRP serum level in these patients also predicts further ischaemic events (Arenillas *et al.*, 2003). hsCRP seems to be a stronger predictor of cardiovascular events than low density lipoprotein (LDL) cholesterol: hsCRP levels of <1, 1 to 3, and >3 mg/l correspond to low-, moderate-, and high-risk groups for future cardiovascular events (Ridker, 2003).

Erythrocyte sedimentation rate and fibrinogen

The erythrocyte sedimentation rate (ESR) is the rate of fall of red blood cells in a column of blood and reflects an increase in plasma concentration of large proteins such as fibrinogen and immunoglobulins. These promote erythrocyte aggregation, thereby causing them to sediment more rapidly. Elevated fibrinogen level is a risk factor for stroke (Wilhelmsen *et al.*, 1984), perhaps reflecting advanced atherosclerosis (Kofoed *et al.*, 2003). Significant hyperfibrinogenaemia occurs in patients with acute cerebral infarction in association with leucocytosis and an increase in leucocyte aggregation (D'Erasmo *et al.*, 1991; Belch *et al.*, 1998). Recent data show that carriers of the A allele of the fibrinogen –455G/A polymorphism, associated with increased plasma fibrinogen, may be predisposed to lacunar stroke (Martiskainen *et al.*, 2003). Elevated ESR in patients with acute ischaemic stroke is an independent predictor of poor short-term outcome within the first month (Chamorro *et al.*, 1995; Balestrino *et al.*, 1998) and early stroke recurrence (Chamorro *et al.*, 1997).

Body temperature

In animal models of stroke, body temperature and brain temperature profoundly influence outcome. Hypothermia reduces infarct size (Corbett and Thornhill, 2000), whilst hyperthermia exacerbates ischaemic neuronal injury (Ginsberg and

Busto, 1998). Data from clinical studies of stroke relating to body temperature tend to conflict, and further work is required. In a recent meta-analysis of 9 studies with 3790 patients, it was concluded that elevated body temperature is common in acute stroke patients and is associated with increased morbidity and mortality (Hajat *et al.*, 2000). Infections may account in part for this rise in body temperature, but fever generated by infection alone is an insufficient explanation as body temperature has been independently and significantly related to initial stroke severity, lesion size, mortality and outcome in survivors (Reith *et al.*, 1996). A significant rise in body temperature in major stroke was confirmed in a more recent study (Boysen and Christensen, 2001). However, this study failed to confirm a negative prognostic influence of elevated body temperature in the first hours after stroke on outcome at 3 months. Furthermore, in patients with major stroke, low body temperature on admission was related to adverse outcome.

1.3.4 Imaging CNS inflammation in stroke

Imaging the inflammatory components in clinical stroke *in vivo* is important as it permits the identification of associations between neurological outcome and brain tissue damage. Such imaging facilitates the study of inflammation as a dynamic process, and is likely to assist in the development of therapeutic interventions. Various techniques have been employed to image leucocyte infiltration in ischaemic stroke, including gamma camera imaging of reinjected ^{111}indium labelled leucocytes (Pozzilli *et al.*, 1985b) and single-photon emission computed tomography (SPECT) imaging of technetium-99m hexamethylpropyleneamine oxime (^{99m}Tc HMPAO)-labelled leucocytes (Wang *et al.*, 1993). Neutrophil invasion can be demonstrated in acute cerebral infarcts within 24 h of symptom onset using ^{111}indium labelled autologous neutrophils (figure 1.4) (Price *et al.*, 2002). ^{99m}Tc HMPAO-labelled leucocyte brain SPECT has also shown neutrophils to intensively accumulate in cerebral infarctions. This accumulation correlates with the severity of brain tissue damage and poor neurological outcome (Akopov *et al.*, 1996).

PK11195 is a specific ligand for the peripheral benzodiazepine binding site (PBBS). After cerebral ischaemia in rodents, increased binding of PK11195 is

colocalized with invading cells of mononuclear-phagocytic lineage, in and around infarcted tissue. However, activated microglia are responsible for increased PK11195 binding in lesions where the BBB is intact (Banati *et al.*, 1997). In patients with ischaemic stroke, [^{11}C]PK11195 positron emission tomography (PET) has demonstrated activated microglia in both middle cerebral artery (MCA) or posterior cerebral artery (PCA) territory infarctions (Gerhard *et al.*, 2000) and in secondary ipsilateral thalamic lesions in patients with MCA territory infarction (figure 1.5) (Pappata *et al.*, 2000). Such microglial activation is present for up to several weeks after the onset of cerebral infarction.

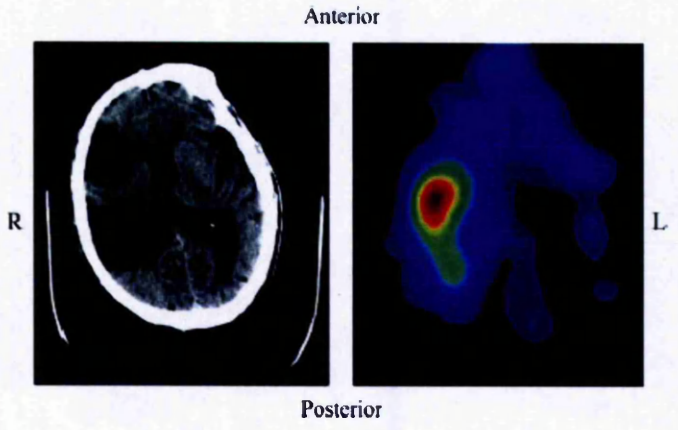


Figure 1.4 *Corresponding trans-axial non-enhanced computed tomogram (left) and ^{111}indium autologous neutrophil scan (right) images at 16 and 44 h, respectively, after clinical onset of a right middle cerebral artery ischaemic stroke (courtesy of CJ Price, University of Cambridge, UK).*

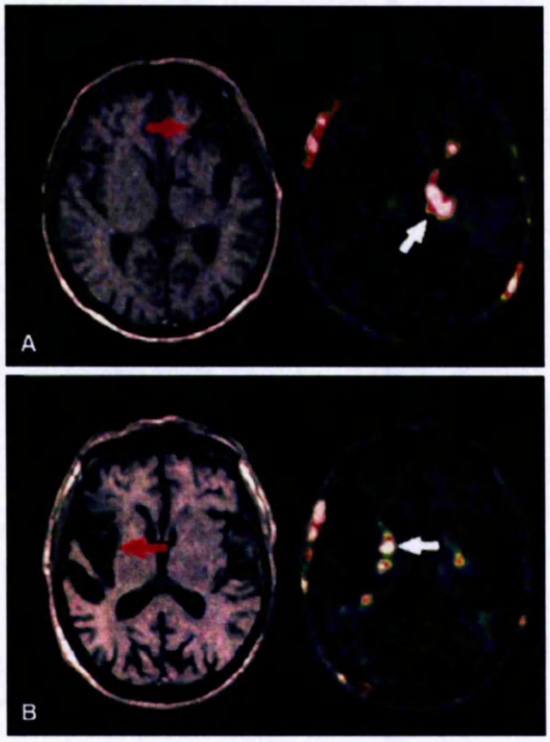


Figure 1.5 *Transverse sections of the magnetic resonance images (left) and coregistered parametric images of [^{11}C]PK11195 binding superimposed on the corresponding magnetic resonance imaging (MRI) planes (right) of two patients 2 months (A) and 7 months (B) after stroke. Increased binding can be seen in the thalamus ipsilateral to the infarct in both patients (white arrow). The red arrows indicate the infarct as seen on the corresponding MRI planes. (Reprinted with permission from Pappata et al., 2000)*

Novel MRI techniques such as perfusion-weighted imaging (PWI) and DWI, when used in combination, have been proposed as a method by which tissue in the ischaemic core can be differentiated from penumbral tissue (Heiss *et al.*, 2001a). This might be useful in the identification and monitoring of patients suitable for thrombolytic and/or neuroprotective therapies. MRI has been used in experimental cerebral ischaemia to monitor evolving cellular responses associated with inflammation (Schroeter *et al.*, 2001), and it is possible that this technique may have clinical applications.

Future developments in the imaging of inflammatory components in stroke, as in many other conditions, may include the use of cytokines to assist with the molecular characterisation of immune processes and the development of new therapeutic strategies (Signore *et al.*, 2000).

1.4 Inflammatory conditions associated with stroke

Stroke has been reported to occur in a wide range of systemic inflammatory conditions (table 1.1), but it must be borne in mind that the majority of these conditions are rare, and stroke is an unusual complication in most cases. Stroke has been largely ascribed to an associated inflammatory vasculitis in these conditions. However, it is now recognised that mechanisms other than vasculitis are also important in the pathogenesis of stroke in this setting, some of which are common to both inflammation and infection-associated stroke, and will be discussed later. Of the primary inflammatory vascular disorders, giant cell arteritis (temporal arteritis) is probably the most common cause of ischaemic stroke. It is a multisystem vasculitis of the elderly most commonly affecting the branches of the external carotid artery, associated with fever, anaemia, headache and elevated ESR. TIA, cerebral infarction and cerebral haemorrhage may all occur. The frequency of TIA/stroke in temporal artery biopsy-proven disease varies from 3% to 7% (Caselli *et al.*, 1988; Hu *et al.*, 2000).

Systemic lupus erythematosus (SLE), a chronic autoimmune multisystem connective tissue disease, may be complicated by stroke, or occasionally present with stroke as an early manifestation (Haas, 1982). Both cerebral infarcts and

haemorrhages may occur. Although vasculitis had previously been considered to be a frequent cause of stroke in SLE, other mechanisms such as hypertension mediated by immunologic abnormalities, anticardiolipin antibodies (aCL), coagulopathy and cardiac emboli from Libman-Sacks endocarditis appear to be more important (Devinsky *et al.*, 1988; Khamashta *et al.*, 1990; Kitagawa *et al.*, 1990; Mitsias and Levine, 1994; Roldan *et al.*, 1996).

Table 1.1. Inflammatory conditions associated with cerebrovascular disease

Condition	References
Primary vasculitis	
Giant cell arteritis	Caselli *et al.*, 1988; Hu *et al.*, 2000
Primary angiitis of the CNS	Hankey, 1991
Takayasu arteritis	Lupi-Herrera *et al.*, 1977; Hall *et al.*, 1985
Polyarteritis nodosa	Reichhart *et al.*, 2000
Kawasaki disease	Templeton and Dunne, 1987
Churg-Strauss syndrome	Sehgal *et al.*, 1995
Wegener's granulomatosis	Nishino *et al.*, 1993
Henoch-Schoenlein purpura	Belman *et al.*, 1985
Behçet's disease	Sigal, 1987; Krespi *et al.*, 2001
Unspecified systemic vasculitis	Moore and Fauci, 1981
Secondary vasculitis	
Systemic lupus erythematosus	Haas, 1982; Devinsky *et al.*, 1988; Kitagawa *et al.*, 1990; Mitsias and Levine, 1994
Progressive systemic sclerosis	Lee and Haynes, 1967
Rheumatoid disease	Sigal, 1987
Sjögren's syndrome	Sigal, 1987
Other systemic inflammatory and immune conditions	
Sneddon's syndrome	Kalashnikova *et al.*, 1994; Lossos *et al.*, 1995a
Inflammatory bowel disease	Lossos *et al.*, 1995b
Sarcoidosis	Brown *et al.*, 1989

Elevated anticardiolipin antibody titres in patients with ischaemic or haemorrhagic stroke vary in frequency between 1% and 38% (Montalbán *et al.*, 1994; Muir *et al.*, 1994, Tuhrim *et al.*, 1999), and may represent an independent stroke risk factor (Tuhrim *et al.*, 1999). Very few patients have the features comprising the antiphospholipid syndrome (APS) which include recurrent venous and arterial thrombosis and recurrent miscarriage in the presence of aCL. Antiphospholipid antibodies of other specificities such as those to β_2-glycoprotein 1, phosphatidyl serine, or phosphotidyl inositol may be equally or more important (Tanne *et al.*, 1998).

In addition to these systemic vasculitides and inflammatory conditions, various rare primary CNS vasculitides, including a granulomatous primary angiitis of the CNS, have been associated with cerebrovascular syndromes (Hankey, 1991). Isolated angiitis of the CNS with cerebral infarction has also been described in children (Lanthier *et al.*, 2001).

1.5 Infection and stroke

1.5.1 Acute infection and stroke

Infections observed to precede stroke have most often been bacterial in origin, although a wide range of organisms and infections are associated with stroke. Stroke is well known to occur in infections such as neurosyphilis, bacterial meningitis and infective endocarditis. The latter condition is complicated by cerebral infarction in approximately 20% of cases (Valtonen *et al.*, 1993). Perhaps less well appreciated, but important from a clinical perspective, many strokes are associated with common infections. The prevalence of infection in the month preceding ischaemic stroke has been estimated to be at least 20% (Grau *et al.*, 1995a). Acute infection, mostly respiratory and of bacterial origin, during the preceding month is a risk factor for cerebral infarction (Syrjänen *et al.*, 1988). This association remains significant after controlling for the effects of other risk factors. Several other studies support this observation, particularly in the week preceding stroke (Ameriso *et al.*, 1991; Bova *et al.*, 1996; Macko *et al.*, 1996a; Grau *et al.*, 1998a), and suggest antecedent infection as a risk factor for stroke in all age groups (Grau *et al.*, 1995a). Respiratory tract infection has been linked to large-vessel and/or cardioembolic ischaemic stroke, particularly in those without vascular risk factors (Paganini-Hill *et al.*, 2003). Patients with recent infection tend to present with a more severe neurological deficit than patients without infection (Grau *et al.*, 1995b) although not all studies accord with this observation (Bova *et al.*, 1996). Numerous problems face clinical investigators examining the role of preceding infection. Consistent identification and classification of infections can be particularly difficult when done retrospectively.

1.5.2 Chronic or recurrent infection and stroke

Chlamydia pneumoniae

C. pneumoniae, a ubiquitous intracellular bacterium, is able to cause a persistent infection of macrophages/monocytes, endothelial cells and vascular smooth muscle cells, and may play a role in many systemic diseases, including the pathogenesis of atheroma (Campbell *et al.*, 2000). By the sixth decade of life more than 50% of individuals show seropositivity (Grayston *et al.*, 1990). Direct clinical evidence that *C. pneumoniae* causes atherosclerotic lesions is lacking, but chronic infection, combined with other factors, may contribute to atherogenesis in some patients (Ross, 1999). However, despite the demonstration of the organism in atherosclerotic plaques in the carotid artery (Grayston *et al.*, 1995; Chiu *et al.*, 1997) and middle cerebral artery (Virok *et al.*, 2001), the presence of the organism appears not to be associated with the development of carotid atherosclerosis (Yamashita *et al.*, 1998), symptomatic disease (LaBiche *et al.*, 2001), or have any detectable impact on plaque instability (Gibbs *et al.*, 2000).

A positive association between elevated *C. pneumoniae* antibodies, indicative of chronic infection and cerebrovascular disease has been reported in several studies (Wimmer *et al.*, 1996; Cook *et al.*, 1998; Fagerberg *et al.*, 1999; Gil Madre *et al.*, 2002; Bucurescu and Stieritz, 2003). These observations have been extended to an elderly, multiethnic population (Elkind *et al.*, 2000). In addition, acute recrudescence of infection (diagnosed by specific immunoglobulin M titres) is apparently associated with acute stroke and TIAs (Cook *et al.*, 1998). Increased serum levels of immune complexes containing chlamydial lipopolysaccharide (and anti-cytomegalovirus antibodies) have also been associated with increased stroke incidence and worse clinical outcome (Tarnacka *et al.*, 2002). At least four other studies have failed to provide convincing support for an association between *C. pneumoniae* infection and stroke (Glader *et al.*, 1999; Heuschmann *et al.*, 2001; Ngeh *et al.*, 2003; Tanne *et al.*, 2003). The role of chronic *C. pneumoniae* infection as a risk factor for stroke remains unclear.

Other infections

Chronic infection with *Helicobacter pylori*, another Gram-negative bacterium, has also been implicated as a risk factor for stroke. Although any association between *H. pylori* and risk of stroke was abolished in two studies after adjustment for adult socio-economic status and/or vascular risk factors (Whincup *et al.*, 1996; Moayyedi *et al.*, 2003), other studies have suggested that seropositivity for *H. pylori* may be an independent risk factor for ischaemic stroke (Markus and Mendall, 1998; Grau *et al.*, 2001a). Another report demonstrated an association only with stroke caused by small-artery occlusion (Heuschmann *et al.*, 2001).

Recurrent or chronic respiratory infection, independent from recent acute respiratory infection, may also be associated with cerebral infarction or TIA (Grau *et al.*, 1997). Although other microorganisms such as herpes viruses have been identified in carotid atherosclerotic plaque (Chiu *et al.*, 1997), immunoglobulin G antibodies to herpes simplex virus or cytomegalovirus in apparently healthy men do not predict future stroke risk (Ridker *et al.*, 1998b).

Periodontal disease (gingivitis or periodontitis) caused by Gram-negative bacteria is common in adults. An association between dental infection and risk of cerebral infarction has been suggested, independent from other risk factors (Syrjänen *et al.*, 1989b; Grau *et al.*, 2004). Other studies have produced conflicting results. Whilst one study supported periodontal disease as an independent risk factor for cerebrovascular disease (Wu *et al.*, 2000), particularly ischaemic stroke, another failed to confirm the association (Howell *et al.*, 2001). In the latter study, self-reported periodontal disease did not independently predict subsequent cardiovascular disease, including non-fatal stroke.

A wide range of neurologic complications can occur in chronic meningitis. In one recent clinical series, cerebral infarction occurred in 47% of cases of tuberculous meningitis, and 32% of cases of cryptococcal meningitis, commonly in the internal capsule and basal ganglia (Lan *et al.*, 2001).

Although neurological complications of human immunodeficiency virus (HIV) infection are common, it has been unclear whether any association exists

between the acquired immune deficiency syndrome (AIDS) and stroke (Pinto, 1996). The prevalence of cerebral infarction in patients with AIDS ranges from 4% to 29% in autopsy series, but cerebral infarcts in HIV-infected patients without non-HIV CNS infection are uncommon (Connor *et al.*, 2000). A more recent population-based study suggested an association between AIDS and both ischaemic stroke and intracerebral haemorrhage (Cole *et al.*, 2004).

The potential association between chronic infection and coronary artery disease has been examined in greater detail than for stroke, but the link also remains uncertain (Danesh *et al.*, 1997). The parallels with stroke are evident, and problems such as confounding by causal risk factors and uncertainty over the temporal sequence of infection and stroke may only be resolved, particularly in the case of *C. pneumoniae*, by randomised intervention studies.

1.6 Mechanisms of inflammation and infection-associated stroke

A wide range of mechanisms has been suggested to link inflammation and infection to the pathophysiology of ischaemic stroke. These various pathophysiological pathways are summarised in figure 1.6.

1.6.1 Atherosclerosis, inflammation, infection and vascular risk factors

Atherosclerosis is an inflammatory disease (Ross, 1999), and inflammation contributes to the risk of plaque rupture (Blake and Ridker, 2001), one of the key events in the occurrence of acute atherothrombotic or atherothromboembolic clinical events such as stroke. It is likely that traditional risk factors interact with inflammatory mechanisms in a more complex manner than has been envisaged previously. For example, increased blood pressure is significantly associated with elevated levels of sICAM-1 and IL-6 in apparently healthy men, and may support a role for hypertension as a pro-inflammatory stimulus (Chae *et al.*, 2001). Plasma hsCRP concentrations correlate with various cardiovascular risk factors including male gender, smoking, body mass index, lipid levels and blood pressure (Saito *et al.*, 2003). Furthermore, higher CRP levels are seen in

individuals with periodontal disease or evidence of *C. pneumoniae* or *H. pylori* infection (Mendall *et al.*, 1996; de Maat and Kluft, 2001). Hypertension is an important factor in the development of cerebrovascular disease in SLE, where there is renal involvement mediated by immunologic abnormalities with high anti-DNA antibody titres (Kitagawa *et al.*, 1990), as well as in unusual primary vasculitides such as Takayasu's arteritis, also owing to renal involvement (Lupi-Herrera *et al.*, 1977).

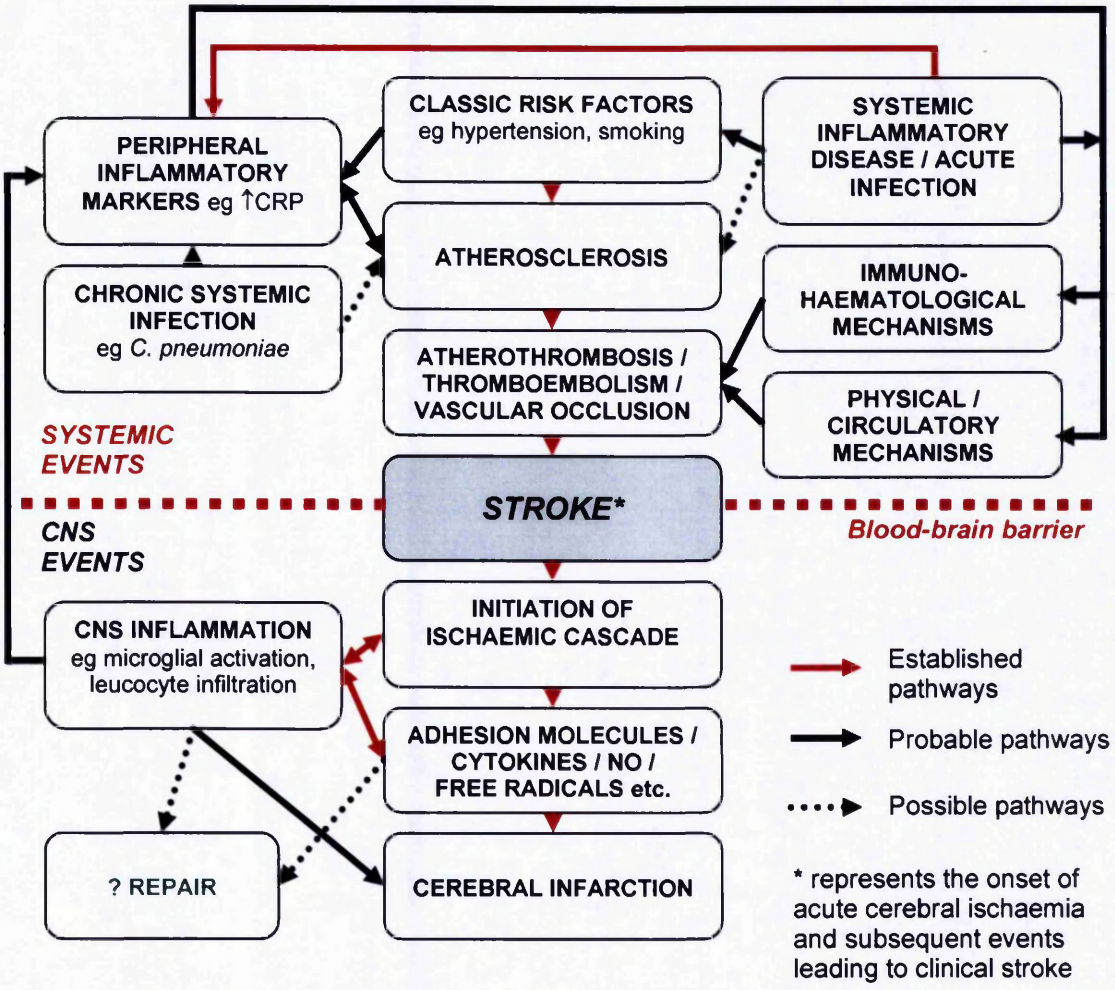


Figure 1.6 *Pathways of interaction between inflammation, infection and ischaemic stroke*

Multiple mechanisms have now been proposed to link inflammatory and infective stimuli to the development and progression of atherosclerotic plaque. These include a direct pro-inflammatory effect of CRP on human arterial endothelium (Pasceri *et al.*, 2000), causing the induction of adhesion molecule

expression with the subsequent recruitment of monocytes and other immune cells to the arterial wall. Monocyte-derived macrophages and activated T-cells release various growth factors and cytokines including TNF-α and IL-1 (Barath *et al.*, 1990; Galea *et al.*, 1996). It is under the influence of these molecules that endothelial and smooth muscle cells release further growth factors resulting in smooth muscle cell proliferation and fibrous plaque formation characteristic of advanced atherosclerotic lesions. As mentioned above, *C. pneumoniae* and other microorganisms are possible inflammatory triggers of atherogenesis. In addition, the progression of atherosclerosis may be accelerated by the treatment of certain systemic inflammatory conditions with corticosteroids, as in SLE (Bulkley and Roberts, 1975). Significant correlations have also been reported between peripheral differential leucocyte counts and the severity of carotid atherosclerosis in male non-smokers (Huang *et al.*, 2001).

1.6.2 Immuno-haematological mechanisms

Disturbances in immune function and alterations in the coagulation system have been described in inflammation and infection-associated stroke. A pro-thrombotic state has been suggested to predispose to large-vessel atherothrombosis and ischaemic stroke. In addition, microvascular obstruction may also contribute to a reduction in tissue perfusion and worsen cerebral ischaemia.

Reduced circulating antithrombotic activated protein C (APC), elevated C4b-binding protein (C4BP) and a low ratio of tissue plasminogen activator (t-PA) to plasminogen activator inhibitor (PAI) are examples of components of the coagulation system which are modified in inflammation or infection-associated stroke (Macko *et al.*, 1996b). Increased fibrin D-dimer levels, cardiolipin immunoreactivity and fibrinogen levels also occur in acute ischaemic stroke patients with antecedent infection (Ameriso *et al.*, 1991). Seasonal variations in clotting factors may also be important. Elevated winter levels of plasma fibrinogen and factor VII clotting activity occur, possibly related to winter infections (Woodhouse *et al.*, 1994). Increased von Willebrand factor (vWF) levels also occur in acute stroke patients for up to 3 months and subjects with

carotid atherosclerosis, perhaps reflecting continued endothelial damage or repair (Blann *et al.*, 1999). Not all studies concur however, as a range of markers of coagulation and fibrinolysis, including anti-thrombin III, plasminogen, PAI-1, thrombin-antithrombin complexes, prothrombin fragment F1+2, fibrin monomer or D-dimer, factor VII and factor VIII, vWF, C4BP activity, protein S, anticardiolipin antibodies and thrombomodulin have been reported not to differ between stroke patients with and without infection (Grau *et al.*, 1995b; Grau *et al.*, 1998). The CD40-CD40 ligand (CD40L-CD154) has emerged as an important pathway in local inflammation of the vascular wall and haemostasis; the CD40 system is upregulated in patients with acute cerebral ischemia (Garlichs *et al.*, 2003).

Increased levels of CRP and pro-inflammatory cytokines can contribute to a procoagulant state. CRP may promote localised coagulation, and therefore thrombosis, by stimulating monocytes to produce tissue factor, a membrane-bound glycoprotein that initiates the extrinsic pathway of coagulation (Cermak *et al.*, 1993). Greater circulating IL-6 levels in acute ischaemic stroke patients have been associated with decreased levels of free protein S, leading to the suggestion that IL-6 may partly modulate this anticoagulant pathway (Vila *et al.*, 2000b).

In SLE, antiphospholipid antibodies such as aCL and lupus anticoagulant may well be important in the development of occlusive cerebrovascular disease via inhibition of prostacyclin formation, causing platelet aggregation. Several other effects of these antibodies have been suggested, including inhibition of prekallikrein, alterations of antithrombin III, decreased fibrinolysis, decreased release of plasminogen activator, inhibition of protein C activation and the induction of endothelial injury (Kitagawa *et al.*, 1990). Thrombotic thrombocytopaenic purpura has also been implicated in SLE patients with cerebral infarction (Devinksy *et al.*, 1988). In inflammatory bowel disease, the cerebral arterial and venous circulation may be affected by hypercoagulability-related thrombosis, vasculitis, and consumption coagulopathy leading to haemorrhagic events (Lossos *et al.*, 1995b).

In patients with bacteraemic infections, thromboembolic complications are relatively common, occurring in around 20% of patients with infective endocarditis and 10% of septicaemia cases (Valtonen *et al.*, 1993). Numerous mechanisms may be responsible, many of which involve activation of the coagulation system. These include reduced levels of antithrombin III, protein C and S, increased platelet aggregation and adhesion, impaired fibrinolysis and antiphospholipid antibody formation. In addition, endotoxins, other bacterial toxins and pro-inflammatory cytokines such as IL-1 and TNF-α may well contribute to thrombosis via effects on endothelial function.

1.6.3 Physical and circulatory mechanisms

As already discussed, inflammation and infection are likely to play a part in the event which causes at least 50% of ischaemic strokes and transient ischaemic attacks, acute arterial obstruction. This is usually caused either by atherothrombosis or atherothromboembolism. Less frequently low-flow distal to a severely narrowed or occluded artery will be responsible for clinical events.

Valvular heart disease is common in SLE and associated with the presence of antiphospholipid antibodies (Khamashta *et al.*, 1990). Cardioembolism is an important cause of stroke in such patients (Roldan *et al.*, 1996). Cerebral vasculitis as a cause of stroke is reported in a range of vasculitides and inflammatory processes, including giant cell arteritis (Caselli *et al.*, 1988), Takayasu's arteritis (Lupi-Herrera *et al.*, 1977) and Wegener's granulomatosis (Nishino *et al.*, 1993).

Virally-induced inflammatory vasculopathies have been associated with thrombotic occlusion of the internal carotid and cerebral arteries (Eidelberg *et al.*, 1986; Leber *et al.*, 1995; Grau *et al.*, 1998b). Focal MCA vasculitis has also been implicated in stroke associated with *Mycoplasma pneumoniae* infection (Fu *et al.*, 1998). Intraneuronal migration of the varicella zoster (VZ) virus from the trigeminal ganglion along the trigeminal nerve to the cerebral arteries has been proposed as a reasonable mechanism for varicella-associated ischaemic stroke (Askalan *et al.*, 2001). VZ virus is present within the media of affected large

cerebral arteries in adults with herpes zoster ophthalmicus and delayed cerebral infarction (Eidelberg *et al.*, 1986; Melanson *et al.*, 1996). Varicella lesions have also been suggested to cause stroke via a local irritant effect in the region of the superior cervical ganglion causing sympathetic stimulation (Ganesan and Kirkham, 1997). An inflammatory carotid arteritis with intimal damage, peripheral embolisation and cerebral lesions ranging from transient ischaemia to infarction has been described in children with preceding throat infection (Bickerstaff, 1964).

In cerebrovascular neurosyphilis there is meningovascular inflammation resulting in progressive vascular insufficiency predominantly affecting the MCA territory (Dalal and Dalal, 1989). A basal exudate in neurotuberculosis entraps the circle of Willis with involvement of lenticulostriate branches from the MCA mainstem (Dalal and Dalal, 1989).

Cervical artery dissection is another important cause of stroke and TIA, particularly in young and middle-aged patients, and a significant association has been reported between recent infection and cervical artery dissection in this age group (Grau *et al.*, 1999; Guillon *et al.*, 2003). Pre-existing abnormalities of extracellular matrix proteins may increase susceptibility to infection-associated injury of the arterial wall.

1.7 Implications for treatment

Current approaches to the primary and secondary prevention of ischaemic stroke focus on modifiable vascular risk factors as outlined above. Commonly used drugs include antiplatelet agents, antihypertensives, lipid-lowering agents and anticoagulants. The only approved treatments for acute ischaemic stroke are aspirin (Gubitz *et al.*, 2003) and intravenous recombinant tissue plasminogen activator (rt-PA) (Wardlaw *et al.*, 2003), but only a minority of patients are eligible for thrombolytic therapy, and as mentioned above, the efficacy of thrombolysis itself may be restricted by reperfusion-associated inflammation. A clearer understanding of the mechanisms underlying inflammation and infection-

associated stroke will pave the way for novel therapeutic approaches, in addition to providing new insights into current strategies.

1.7.1 Anti-inflammatory effects of current treatments

It is conceivable that a range of the current stroke treatments possess anti-inflammatory effects in addition to their traditionally accepted actions. The discussion here will focus mainly on the possible anti-inflammatory mechanisms of the antiplatelet agent acetylsalicylic acid (aspirin), lipid-lowering agents β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), and modulators of the renin-angiotensin-aldosterone system (RAS).

Antiplatelet agents

Aspirin has a firmly established position in the prevention of death, myocardial infarction and stroke in high-risk patients (Antithrombotic Trialists' Collaboration, 2002). The risk reduction of a first myocardial infarction associated with the use of aspirin appears to be directly related to CRP level (Ridker *et al.*, 1997), which raises the possibility of an anti-inflammatory action of aspirin as well as its antiplatelet effect mediated via COX inhibition. Experimentally, aspirin and sodium salicylate prevent glutamate-induced neurotoxicity via blockade of NF-κB induction (Grilli *et al.*, 1996), which lends support to aspirin having more actions than COX inhibition. Blockade of NF-κB activation has been proposed as another treatment approach in cerebral ischaemia, since this transcription factor acts early after the onset of ischaemia on multiple pro-inflammatory genes (Carroll *et al.*, 2000). However, modulation of NF-κB may be problematic as this transcription factor also appears to have a role in the resolution of inflammation (Lawrence *et al.*, 2001). Clinical observations show that ischaemic stroke patients with prior aspirin treatment have lower plasma levels of TNF-α, IL-6 and ICAM-1 (Castellanos *et al.*, 2002). Combined aspirin and clopidogrel has been noted to reduce platelet P-selectin expression and plasma CRP concentration after acute ischaemic stroke (Cha *et al.*, 2002).

Statins

Several clinical trials and meta-analyses of the statins have shown a significant reduction in ischaemic stroke in patients with coronary artery disease, both with and without elevated serum cholesterol concentrations (Crouse *et al.*, 1998). However, the relationship between serum cholesterol and stroke from the epidemiological data remains unclear. Anti-inflammatory and/or neuroprotective properties of statins, in addition to their lipid-lowering effects, have therefore been proposed. Pravastatin lowers CRP levels in a manner largely independent of effects on LDL cholesterol in subjects with and without a history of cardiovascular disease (Albert *et al.*, 2001). Statin use after ischaemic stroke is associated with CRP reduction and improved prognosis independent of lipid lowering, with the greatest risk reduction seen in patients with higher CRP levels (Di Napoli and Papa, 2001). Several carotid ultrasound studies have demonstrated beneficial effects of statins on intimal-medial thickness, an indicator of atherosclerotic disease. Possible mechanisms by which statins achieve atherosclerotic plaque stabilisation or even regression may include reduced macrophage activation within the vessel wall, inhibition of MMP-induced plaque rupture, and prevention of smooth muscle cell proliferation (Kwak *et al.*, 2003), whilst reduced serum cholesterol encourages an anti-thrombotic state, for example by reducing platelet aggregation or by lowering PAI-1 levels (Mohler *et al.*, 1999). Potential neuroprotective mechanisms may include endothelial upregulation of eNOS and inhibition of iNOS, an effect associated with augmented cerebral blood flow and reduced infarct size, reduction of leucocyte-endothelium interaction, modulation of CNS cytokine production and antioxidant effects (Vaughan and Delanty, 1999). Further data are emerging on the clinical effects of statins in stroke prevention (Shepherd *et al.*, 1999; Heart Protection Study Collaborative Group, 2002; Welch, 2002; Sever *et al.*, 2003).

Angiotensin converting enzyme inhibitors and angiotensin II receptor blockers

These blood pressure lowering agents modify the RAS. Recent clinical trial evidence has established the clinical benefits of angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers in stroke (The HOPE Investigators, 2000; PROGRESS Collaborative Group, 2001; Dahlöf *et al.*,

2002). However, such therapy may possess benefits beyond blood pressure lowering. Angiotensin II has pro-inflammatory effects via augmentation of expression of VCAM-1, MCP-1 and IL-6, and increased production of reactive oxygen species (Libby, 2001); other data supporting the inflammatory role of the RAS have been reviewed recently (Suzuki *et al.*, 2003). Inhibition of angiotensin II via ACEIs or angiotensin II receptor blockers may therefore have anti-inflammatory effects.

1.7.2 Novel therapeutic strategies

Neuroprotection

The term 'neuroprotection' encompasses a diverse range of potential therapies, including modulators of the excitatory amino acid system, modulators of calcium influx, metabolic activators, anti-oedema agents, inhibitors of leucocyte adhesion, free radical scavengers and other agents. Many of these interventions do not possess direct anti-inflammatory effects, and are therefore beyond the scope of this introduction, whilst some, such as antileucocyte strategies, are discussed below. More than 100 clinical trials have addressed the safety and efficacy of over 50 neuroprotective agents (Liebeskind and Kasner, 2001). To date, there has been a disappointing failure to translate results seen in animal models to humans (Muir, 2002). This highlights the need to take a standardised approach to pre-clinical and clinical methodology in future clinical trials of neuroprotective agents (STAIR-1, 1999; STAIR-II, 2001; Fisher, 2003). One of the many barriers to the successful use of neuroprotective drugs is the small amount of viable penumbral tissue remaining in stroke patients at the time of presentation. It is possible that only where reperfusion is sufficient, ie in the setting of combined thrombolysis and neuroprotection, will neuroprotective stategies prove efficacious (Heiss *et al.*, 2001b; Kaste *et al.*, 2001).

Antimicrobial agents and vaccines

Serological evidence for an association between *C. pneumoniae* and coronary heart disease exists just as for stroke. Several studies have examined the potential role of antichlamydial antibiotics in the secondary prevention of coronary artery disease events, but a reduction in clinical events has not been observed

consistently (Grayston, 1999). Possible effects of these agents include suppression of reactivation of chronic infection within atherosclerotic plaque, and an anti-inflammatory effect leading to attenuation of intra-plaque inflammation. Antichlamydial agents may merit further evaluation as an alternative therapeutic approach in ischaemic stroke. Antibiotic use and cerebrovascular disease in the elderly has been studied, and penicillin appeared to show a protective association that may merit further investigation (Brassard *et al.*, 2003).

Subjects vaccinated against influenza are less prone to viral (influenza) infections and subsequent secondary bacterial infections. A recent study found a negative association between influenza vaccination and brain infarction, particularly in patients under 75 (Lavallée *et al.*, 2002). Whether this protection is attributable to reduced infections or the identification of a subgroup with low stroke risk because of a better lifestyle, is open to debate. The use of vaccination is a potentially promising approach to stroke prevention.

Antileucocyte strategies

Considerable interest has been devoted to the use of monoclonal antibodies to competitively block leucocyte adhesion to ICAM-1. The aim has been to reduce the negative effects associated with leucocyte infiltration into the site of cerebral infarction. It has also been suggested that the prevention of leucocyte adhesion using anti-adhesion molecule agents may extend the therapeutic window during which thrombolytic therapy may be administered, and that the simultaneous use of such treatments may further reduce the extent of brain injury (Bowes *et al.*, 1995; Bednar *et al.*, 1998). Despite showing promise in pre-clinical studies, the first human trial using an anti-ICAM-1 murine monoclonal antibody was associated with adverse clinical outcome (Enlimomab acute stroke trial investigators, 2001). The experimental rationale and possible mechanisms for the negative outcome of this study have been discussed elsewhere (Furuya *et al.*, 2001).

Other, experimental antileucocyte interventions in cerebral ischaemia such as the use of other monoclonal antibodies to block the neutrophil / endothelial cell

interaction, or antineutrophil serum and antineoplastic agents to achieve neutrophil depletion, have also been suggested (Härtl *et al.*, 1996). Recently a study of UK-279,276 (neutrophil inhibitory factor) in acute ischaemic stroke was stopped early because of futility (Krams *et al.*, 2003). The suppression of microglial function has also been put forward as a potential alternative or adjunctive therapeutic approach (Wood, 1995).

Cytokine targets

Evidence for any benefit in stroke by manipulation of cytokine pathways is derived entirely from experimental work at this stage. As mentioned above, inhibition of TNF-α activity may be a useful approach, although the situation is complicated by the apparent dual role of TNF-α (Shohami *et al.*, 1999). Although anti-TNF strategies have proved beneficial in other clinical settings such as inflammatory bowel disease (Blam *et al.*, 2001), to date there have been no clinical trials of anti-TNF agents in stroke. IL-6 also apparently mediates anti-inflammatory and neuroprotective effects in addition to its pro-inflammatory role, and therefore its manipulation may have either detrimental or beneficial effects. In contrast, IL-1 is well placed as a therapeutic target. As discussed below, a number of studies have demonstrated that inhibiting the release or actions of IL-1 markedly reduces ischaemic cerebral damage. IL-1 has little role in normal brain function, and therefore its blockade should not be associated with significant adverse effects within the CNS. IL-1ra is the most advanced approach to modify IL-1 action, and is safe and of benefit in the treatment of patients with rheumatoid arthritis (Bresnihan *et al.*, 1998).

Other approaches

Mechanisms of cell death in cerebral ischaemia are controversial, but both necrosis and apoptosis occur. Whilst necrosis is associated with inflammation, apoptosis *per se* evokes little or no associated inflammation. However, it is worthy of mention that cysteine proteases called caspases, as the executioners of apoptosis, may be potential therapeutic targets in stroke (Schulz *et al.*, 1999). iNOS inhibitors may have some value in patients ineligible for early thrombolysis since these agents have demonstrated an extended therapeutic

window of up to 24 h experimentally (del Zoppo *et al.*, 2000). Further insights into the therapeutic potential of nitric oxide donors in patients with acute stroke should be provided by ongoing studies (Bath, 2004). The situation with COX-2 inhibition is less clear since COX-2, although probably deleterious in ischaemia, may also possess regenerative properties (del Zoppo *et al.*, 2000). MMPs may offer another potential therapeutic target once their beneficial and deleterious effects in stroke have been better defined

Induced hypothermia is another potential therapy currently under investigation. The rationale for cooling in clinical stroke is based on experimental findings showing that hypothermia decreases the final cerebral infarct volume and extends the duration the brain is able to withstand ischaemia prior to the occurrence of permanent damage. A pilot study of induced hypothermia has shown that this approach appears to be feasible and safe (Krieger *et al.*, 2001), but optimal treatment conditions and clinical efficacy remain to be established in larger studies. Paracetamol has been shown to decrease body temperature in acute ischaemic stroke patients (Dippel *et al.*, 2001), but further studies are required to establish whether antipyretic agents lead to improved outcome.

1.8 Interleukin-1 biology and role in cerebral ischaemia

1.8.1 Interleukin-1

Interleukin-1 was the first cytokine identified to act on the brain, and was originally described as "the endogenous pyrogen". It is now clear that endogenous and exogenous IL-1 have numerous actions in the brain, including induction of fever, suppression of appetite and weight loss, sleep modulation, alterations in endocrine, immune, and nervous system functions, behavioural changes, in addition to influences on synaptic plasticity, neuronal transmission, epilepsy, and neuronal cell death (Rothwell, 2003).

The term interleukin-1 classically describes two closely related proteins, IL-1α and IL-1β, which have considerable structural homology. A third, related protein, IL-1ra, is a highly selective, competitive receptor antagonist which

blocks all known actions of IL-1, but to-date, has no other known actions (Dinarello, 1998). IL-1ra was the first naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule to be described. Its IL-1 inhibitory actions were first described in the early 1980s. The balance between IL-1 and IL-1ra appears to be important in determining the response to infection and inflammation. This subject has recently been reviewed in detail (Arend, 2002). All three proteins are formed as precursors, but whereas pro-IL-1α and pro-IL-1ra are biologically active, the inactive pro-IL-1β must undergo cleavage by the enzyme interleukin-1 converting enzyme (ICE or caspase-1) to release the active form. Cleavage and cellular release of IL-1 is not yet well characterised, but does not occur via classical mechanisms because the leader sequence required for cellular release through the usual pathways is lacking from IL-1β. Activation of purinergic $P2X_7$ receptors by extracellular adenosine triphosphate (ATP) can induce cleavage release of both IL-1α and IL-1β (Rothwell, 2003).

Both IL-1α and IL-1β are believed to exert their actions through binding to the IL-1 receptor type I (IL-1-RI), which requires the accessory protein (IL-1AcP) for signal transduction. While IL-1α and IL-1β induce intracellular signals, IL-1ra does not elicit signal transduction, thus inhibiting IL-1(α and β) activity. A second receptor, IL-1-RII, to which all three proteins also bind, does not transduce intracellular signalling, but acts as a decoy receptor. The two receptors exist in soluble forms, permitting regulation of IL-1 biological activity. In addition, in recent years, several new "members" of the IL-1 ligand and receptor families have been identified on the basis of sequence homology. New nomenclatures have been suggested for the ligands (IL-1F1-10) and receptors (IL-1R1-9) (Sims *et al.*, 2001). Although several of these genes are expressed in the brain, their functions remain unknown.

1.8.2 IL-1 expression in cerebral ischaemia

Constitutive expression of IL-1(α and β) is minimal in the normal human and rodent brain, but is induced rapidly in response to cerebral ischaemia in several models of experimental stroke. IL-1β mRNA and protein are elevated in ischaemic areas within 15 minutes and 1 h of the insult respectively (Touzani *et*

al., 1999). IL-1β over-expression is also seen in the contralateral hemisphere in focal ischaemic models such as MCAo. The cellular morphology suggests the IL-1β mRNA is derived mainly from macrophages or activated microglia. This observation is supported by the massive increase in microglial IL-1β immunoreactivity after MCAo in rats, accompanied by a dramatic influx of neutrophils and macrophages into the core of the infarct (Davies *et al.*, 1999). IL-1β is expressed in other sites, including the endothelium of cerebral vessels, following MCAo (Zhang *et al.*, 1998). Astrocytes, invading immune cells and possibly neurones are also capable of expressing IL-1. Other members of the IL-1 family and related molecules, including IL-1ra and the IL-1 receptors, have been studied in cerebral ischaemia, albeit in less detail, and the temporal evolution of each appears to differ.

1.8.3 Involvement of IL-1 in ischaemic brain injury

Numerous lines of evidence strongly implicate IL-1 in ischaemic brain injury. The degree of oedema, infarct volume and number of infiltrating neutrophils elicited by MCAo in rats is markedly enhanced by central injection of recombinant human IL-1β (rhIL-1β) after reperfusion, whilst anti-IL-1β antibody and the IL-1 blocker zinc protoporphyrin (ZnPP) attenuate these effects (Yamasaki *et al.*, 1995). Icv, striatal or cortical injection of IL-1β at the onset of MCAo in rats, suggests that IL-1β acts in the striatum to mediate exacerbation of ischaemic damage, including remote effects on the cortex (Stroemer and Rothwell, 1998). In contrast, administration of IL-1ra significantly reduces (usually by at least 50%) ischaemic brain damage in various models when injected icv (Relton and Rothwell, 1992; Loddick and Rothwell, 1996; Stroemer and Rothwell, 1997; Stroemer and Rothwell, 1998) or systemically (Martin *et al.*, 1994; Garcia *et al.*, 1995; Lin *et al.*, 1995; Relton *et al.*, 1996). Neuroprotection is maintained even when injected at a delay of up to 3 h after MCAo (Mulcahy *et al.*, 2003; figure 1.7) and 8-12 h after global ischaemia or brain trauma (Touzani *et al.*, 1999). Because IL-1ra is a large protein (17kDa), it is unlikely to diffuse readily into normal brain, but an active transport system for IL-1 and IL-1ra may facilitate transfer into the brain (Gutierrez *et al.*, 1994).

Overexpression of IL-1ra in the brain, using an adenoviral vector (Betz *et al.*, 1995; Yang *et al.*, 1997) or IL-1β blockade by injection of anti-IL-1β antibodies (Yamasaki *et al.*, 1995) also causes a significant reduction of ischaemic injury. Pharmacological ICE inhibition, caspase-1 gene deletion or mutation (overexpression of a dominant negative caspase-1 mutant) also reduce ischaemic brain damage in rodent models (Loddick *et al.*, 1996; Hara *et al.*, 1997a; Hara *et al.*, 1997b; Friedlander *et al.*, 1997). The reduction in injury seen with non-selective caspase inhibitors may arise via inhibition of IL-1β activity and/or via inhibition of apoptosis. Lipocortin-1, originally identified as a mediator of the anti-inflammatory actions of glucocorticoids, also inhibits ischaemic and excitotoxic brain damage, perhaps via modification of the actions of IL-1 (Rothwell and Relton, 1993).

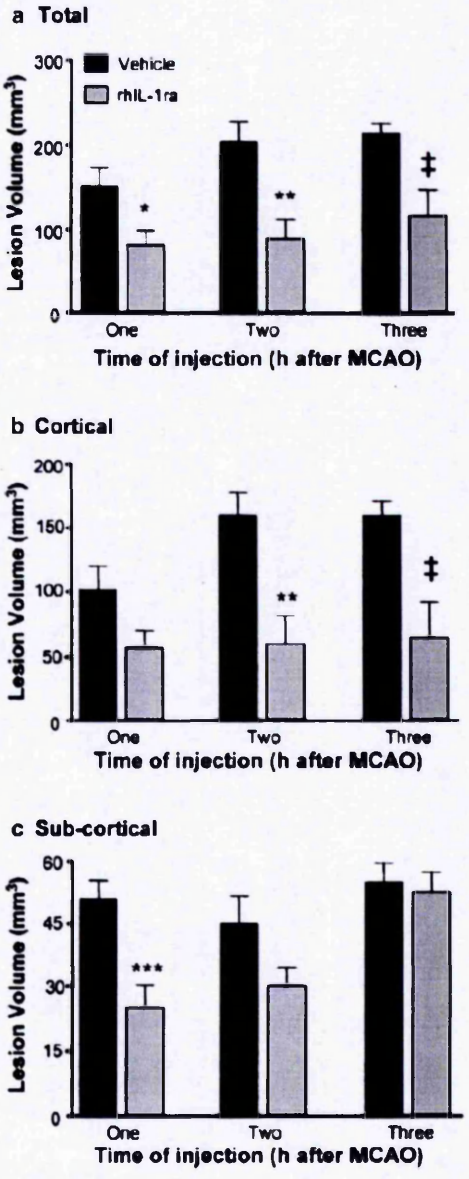


Figure 1.7
*Effect of delayed intracerebroventricular injection of vehicle or rhIL-1ra on (a) total, (b) cortical and (c) subcortical lesion volume assessed 24 h after transient (60 min) MCAO. *p < 0.05 **p < 0.01 compared to vehicle (Student's t-test) ‡ p < 0.05 compared to vehicle (Student's t-test with Welch correction). Reproduced with permission from Mulcahy et al., 2003.*

1.8.4 Potential mechanisms of action of IL-1 in cerebral ischaemia

Although there is compelling evidence to support a role for IL-1 in ischaemic brain injury, the mechanisms of action remain unknown. Direct effects of IL-1 on neurotransmission, glia and endothelial cells have been described. IL-1 stimulates the synthesis of growth factors, neuropeptides and other cytokines including anti-inflammatory and anticoagulant cytokines such as TGF-β, IL-10 and IL-4 as well as pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, IL-2 and IFN-γ (Touzani *et al.*, 1999). The study of IL-1 mechanisms of action in the brain is hampered by its lack of direct neurotoxicity *in vivo* in the absence of other insults, as well as the difficulty of reproducing *in vitro* those effects observed on neuronal death *in vivo*.

IL-1 might induce hyperthermia, which is known to exacerbate ischaemic damage, although IL-1 has been shown to worsen ischaemic damage without changes in body temperature (Stroemer and Rothwell, 1998). IL-1 might alter brain temperature independently of core temperature, but it seems unlikely that this accounts for its injurious effects. IL-1β expression in the cerebral microvasculature (Zhang *et al.*, 1998) might imply an effect on blood flow. IL-1β can also increase production of vasoactive substances such as prostanoids and endothelin, but little indicates that IL-1β has a significant impact on cerebral perfusion.

IL-1 induces adhesion molecule expression and facilitates leucocyte recruitment. IL-1 is also known to prompt the release of other mediators including prostaglandins, collagenases and phospholipase A_2 as well as other cytokines including IL-6, TNF-α, IFN-γ, IL-8, and IL-1 itself. Modulation of other elements in the ischaemic and inflammatory cascades by IL-1β has been described, including the increase of intracellular calcium levels in neuronal culture, stimulation of NOS, arachidonic acid release, corticotrophin releasing factor release, complement and β-amyloid activation and microglial activation. Interestingly, IL-1 is also able to stimulate synthesis of anti-inflammatory cytokines such as IL-1ra, IL-4, IL-10 and TGF-β which implies, in contrast to its damaging effects, a tendency to negative feedback on the inflammatory response.

Potentially neuroprotective actions such as inhibition of calcium currents, enhancement of γ-aminobutyric acid (GABA) activity, and neurotrophic factor stimulation have also been described. The potential mechanisms of action of IL-1 in cerebral ischaemia have been reviewed (Touzani *et al.*, 1999).

1.8.5 IL-1 in clinical stroke

To date, there have been only a few clinical reports on IL-1 and IL-1ra in stroke patients. Elevated serum concentrations of IL-1β protein have not been detected (Fassbender *et al.*, 1994a; Tarkowski *et al.*, 1995), although intrathecal production of IL-1β may occur (Tarkowski *et al.*, 1995). Increased IL-1β messenger ribonucleic acid (mRNA) expression in peripheral mononuclear cells 1-3 days after onset symptoms, returning to normal by 20-31 days, showed moderate correlation with degree of neurological impairment (Kostulas *et al.*, 1999). Plasma concentrations of IL-1ra are elevated in patients within 4 ± 2 days (mean ± SD) of acute ischaemic stroke, with and without infection, in comparison with healthy controls (Beamer *et al.*, 1995). The IL-1α-889 TT genotype is associated with increased IL-1α production by monocytes (Dominici *et al.*, 2002), and an increased frequency of the T allele is found in patients with cerebral infarction compared with controls (Um *et al.*, 2003). The IL-1ra gene exhibits a variable number of tandem repeats polymorphism in intron 2, and allele 2 is associated with increased IL-1 and decreased IL-1ra production in monocytes. The IL-1ra 1/1 genotype has been suggested as a susceptibility factor for ischaemic stroke (Seripa *et al.*, 2003).

1.9 Conclusion

Inflammation and infection are increasingly recognised as key elements in the pathogenesis and outcome of ischaemic stroke. It is now possible, using laboratory and imaging techniques in carefully selected clinical populations, to extend experimental approaches to human studies. Greater understanding of the role and potential for modulation of the inflammatory response may have profound implications for patient management. IL-1 is strongly implicated in ischaemic brain damage and is therefore an attractive therapeutic target in stroke.

1.10 Summary of aims and objectives

The aim of this thesis was to test two primary hypotheses, namely:

(1) Peripheral inflammatory responses occur in patients with acute stroke, and cytokines, in particular IL-1, have a role in the regulation of these responses, and

(2) Recombinant human IL-1ra (rhIL-1ra) treatment is safe, well tolerated, and biologically active in patients with acute stroke.

To achieve this, two clinical studies were undertaken.

The first was a prospective study of ischaemic stroke patients recruited within twelve hours of onset of symptoms, with control subjects individually matched for age, sex and degree of atherosclerosis. Patients with acute ischaemic stroke were studied early after onset of symptoms in order to determine the extent to which a peripheral inflammatory response is activated following acute ischaemic stroke. In the same patients and controls, IL-1β, TNF-α and IL-6 production by peripheral blood was investigated in addition to circulating plasma cytokine and soluble cytokine receptor concentrations.

The second study was a randomised, double-blind, placebo-controlled study of rhIL-1ra in patients with acute stroke. The primary aim of this study was to assess the safety of rhIL-1ra in acute stroke patients. Secondary aims of this study were to obtain information on biological activity and efficacy, based on 3-month survival, well validated stroke scales, a range of biological markers and computed tomography cerebral infarct volume.

CHAPTER 2

General clinical methodology

2.1 Introduction

This chapter describes aspects of methodology for the work reported in chapters 3, 5 and 7 in order to minimize later repetition, including the location of the research, the clinical services in which patients were managed, patient recruitment, ethical approval, research assessments, sample collection and laboratory methods, imaging techniques, assessment of atherosclerosis, data storage and statistical analysis. More specific methods are reported in subsequent chapters.

2.2 Location

Salford Royal Hospitals NHS Trust is an acute teaching hospital trust with 875 beds and 3000 staff. The Trust provides approximately 80% of its services to the former Salford and Trafford Health Authority (now part of the Greater Manchester Strategic Health Authority, figure 2.1) predominantly from a single site, Hope Hospital.

Figure 2.1
Location of Salford and Trafford districts within Greater Manchester Strategic Health Authority

The population of Salford is slightly older than the average for England and more of the population describes themselves as white (97.8%) compared to 93.8% for England and 94.1% for Greater Manchester. The Salford population has high

levels of deprivation, with 71% in paid employment compared to 79% for England, and 15% receiving income support compared to 9% for England. The former Salford and Trafford Health Authority was in the most deprived quartile of health authorities, with higher rates of illness than the national average, and higher rates of mortality, particularly in heart disease which accounts for twice as many deaths as it does nationally (Commission for Health Improvement, 2001).

2.3 *Stroke services at Hope Hospital*

2.3.1 *Clinical service*

Neurosciences services for Greater Manchester, which includes the Stroke Service at Hope Hospital, have been amalgamated onto the Hope Hospital site. The Stroke Service is run by Dr Pippa Tyrrell (Senior Lecturer in Stroke Medicine and Honorary Consultant Stroke Physician), supported by Dr Charles Sherrington (Consultant Cerebrovascular Neurologist), two Stroke Specialist nurses and support staff. The Stroke Service has 600 acute admissions per year. Resources include an 18-bedded Stroke Rehabilitation Unit. The Specialist nurses coordinate the integrated stroke care pathway, carry out follow-up clinics and supervise patients in clinical trials. There are strong links with other disciplines, including Neurology, Neuroradiology, Neurorehabilitation, Elderly Care, Rheumatology, General Medicine and Cardiology. Full investigational facilities are available including three MRI scanners, two CT scanners, angiogram suite, duplex and cardiology. There are close links with primary care through Stroke Association Family Support Organisers and the multidisciplinary Stroke Strategy Group, which includes representatives from the Trust, Health Authority, Stroke Association and Primary Care Trust.

2.3.2 *Clinical research*

The current research forms part of a larger collaborative project examining the role of inflammation in acute stroke and ischaemic brain injury with Professor Nancy Rothwell (School of Biological Sciences, University of Manchester) and Dr Stephen Hopkins (Injury Research, Hope Hospital). Two full-time and one part-time clinical research fellows and one full-time

research nurse, as well as additional laboratory staff, were employed during the conduct of the work constituting this thesis.

2.4 *Patient and control subject recruitment*

In order to optimise round-the-clock recruitment of consecutive eligible patients to both clinical studies and perform the necessary subsequent clinical research assessments and other study procedures (including sample collection, plasma preparation, whole-blood stimulation protocol, and test treatment related procedures in the case of the second study) the clinical research fellows (myself [HCAE], Dr Carole Gavin [née Libetta, CML], and Dr Craig Smith [CJS]) undertook an on-call rota throughout the duration of this research. CML undertook the rota between April 2000 and June 2001, CJS between April 2000 and January 2003, and HCAE between April 2000 and February 2003. Dr Elisa Barberan, Rachel Georgiou (née Drennan), and Paula Beech also assisted with patient recruitment and the clinical research assessments. Two further clinical research fellows employed on a subsequent programme grant, Dr Johann Selvarajah and Dr Adrian Parry-Jones took up their posts in February 2003 and undertook the rota between March and July 2003.

Patients presenting to Hope Hospital (via Accident and Emergency or by GP referral), or in-patients with suspected stroke were referred immediately to the clinical research team by staff working in the relevant departments. Potential control subjects were identified following their response to project publicity and given an information sheet. Those wishing to participate gave their contact details to the research team, and where appropriate were invited to attend for an initial assessment. Specific patient and control subject eligibility criteria are discussed in the relevant chapters.

2.5 *Ethical approval*

Both of the clinical studies from which work is reported in this thesis received ethical approval by the Salford and Trafford Local Research Ethics Committee

(LREC), and by the University of Manchester Senate Ethics Committee. When necessary, protocol amendments also received approval from the Salford and Trafford LREC.

2.6 Clinical assessments

2.6.1 Study procedures

Assessment of patients included history, clinical examination and aural temperature (Braun Thermoscan LF20 digital thermometer), National Institutes of Health Stroke Scale (NIHSS) score (Lyden *et al.*, 1994), Barthel Index (BI) (Mahoney and Barthel, 1965), modified Rankin Scale (mRS) (van Swieten *et al.*, 1988), and Oxfordshire Community Stroke Project (OCSP) classification (Bamford *et al.*, 1991). Infections or other events likely to provoke a marked inflammatory response in the 6 weeks prior to each assessment were recorded using available clinical, radiological and laboratory data. Assessment of control subjects included history, clinical examination and aural temperature. Hypertension, diabetes mellitus, hypercholesterolaemia, atrial fibrillation, and ischaemic heart disease were defined according to local clinical practice (discussed below).

2.6.2 Clinical definitions

Conventional stroke risk factors

A patient was defined as hypertensive if he/she was prescribed one or more antihypertensive agents for this indication at baseline, or was commenced on treatment for hypertension during the study period. Implicit in this definition is a sustained elevation of blood pressure over several separate readings, typically with a systolic BP of 140mmHg or greater and/or a diastolic BP of 90mmHg or greater, although specific cut-points for systolic and diastolic values were not set as guidelines for such values are contentious and subject to change. Diabetes mellitus was defined on the basis of a past history of the condition or diagnosis during the study period, in accordance with local practice (based on WHO criteria). Smoking history was recorded as lifelong non-smoker, ex-smoker, or current smoker. A patient was defined as having hypercholesterolaemia if he/she

was prescribed a lipid-lowering agent at baseline, or was commenced on treatment for hypercholesterolaemia during the study period. During the period when this work was conducted a total serum cholesterol concentration of 5 mmol/l or above would be regarded as hypercholesterolaemic; however no cut-point was applied to our definition owing to the lack of access to study pre-baseline data. The definition of atrial fibrillation was based upon the presence of this arrhythmia on the baseline electrocardiogram, or if the patient had a previous history of paroxysmal atrial fibrillation for which treatment was prescribed at baseline. The definition of ischaemic heart disease was based on a past history of the condition.

Infections

The definition of acute infection was based on the presence of typical symptoms with elevated aural temperature or corresponding serological, cultural, haematological or radiological finding that indicated acute infection. For the work reported in chapter 3, infections or other events that might provoke a marked inflammatory response in the 6 weeks prior to each assessment were recorded using such available clinical, radiological and laboratory data.

2.6.3 Stroke subtype classification

Oxfordshire Community Stroke Project classification

The OCSP classification (see CRF, appendix) was developed primarily as an epidemiological tool from a data set based on the clinical examination of patients at a mean of 4 days post-stroke (Bamford *et al.*, 1991). This clinically-based classification system distinguishes anterior (carotid) circulation and posterior (vertebrobasilar) circulation strokes. It further subdivides strokes into those restricted to deep, subcortical areas (supplied by small perforating arteries), those restricted to superficial cortical areas (supplied by small pial branch arteries), and those involving both deep and cortical structures (involving the whole area of supply of the cerebral artery). This subdivision into lacunar (LACS), partial anterior circulatory (PACS), total anterior circulatory (TACS) and posterior circulatory (POCS) syndromes is important because of differences in pathophysiology and prognosis between these stroke subtypes.

2.6.4 *Measurement of impairment and disability*

National Institutes of Health Stroke Scale

This is a measure of neurological impairment used to describe stroke severity. It is based on the neurological examination, and scores are given depending on the presence, absence, or severity of various impairments. The NIHSS (see CRF, appendix) is used to stratify patients at baseline in acute stroke trials and to monitor change in the acute phase. Validity and inter-observer reliability have been established (Brott *et al.*, 1989).

Barthel Index

The BI (see CRF, appendix) is a 21-point scale, either 0 – 20 in 1-point increments or 0 – 100 in 5-point increments, where zero represents the worst score (Mahoney and Barthel, 1965). It contains the 10 activities normally considered the core of any assessment of activities of daily living (ADL), namely dressing, bathing, transfers, grooming, managing stairs, walking, feeding, going to the toilet, wheelchair skills and continence. The BI is quick and easy to use, valid and reliable, is widely used in clinical practice and research, and has been proposed as the standard index of disability.

Modified Rankin scale

The original version of this scale was developed in 1957 (Rankin, 1957), and it was modified later to take into account language disorders and cognitive deficits. The mRS (see CRF, appendix), using six grades to measure overall independence with reference to prior activities and abilities, was found to have acceptable interobserver reliability (van Swieten *et al.*, 1988). It is widely used.

2.7 ***Blood samples and laboratory methods***

2.7.1 *Subject state, preparation and timing of sample collection*

Blood samples were taken with the subject seated or lying. Control subjects were rested for 30 minutes prior to blood sampling. With the exception of samples taken on admission and 24 hours after admission, all blood was collected at 09:00 in order to minimize the effects of diurnal variation.

2.7.2 Sample collection, plasma preparation, whole-blood stimulation and enzyme-linked immunosorbent assays

Blood samples were drawn by venepuncture and collected into tubes containing EDTA (Sarstedt, UK) for determination of ESR using an automated Westergren method on a Starrsed III analyser, and full blood count (FBC) and differential WBC count using a Coulter Gen-S analyser. Samples were also collected into serum gel tubes for serum biochemical profile and plasma glucose (Roche Integra 700 analyser). A detailed description of the methodology for sample collection and plasma preparation for plasma cytokines and other inflammatory markers is given in chapters 3 and 5. The methodology employed for whole-blood stimulation and assessment of cytokine induction is described in chapter 5. Enzyme-linked immunosorbent assays used during this work are described in detail in chapters 3, 4 and 5.

2.7.3 Sample storage

Blood, plasma, and urine samples were labelled, catalogued and stored systematically in freezer boxes and racks in a Sanyo MDF -86°C freezer (Sanyo Scientific, UK). The freezer was fitted with a temperature alarm with a remote alarm contact system.

2.8 Imaging techniques

2.8.1 Computed tomography

CT brain scans were performed on an IGE CT Pace Plus 3rd generation CT scanner (IGE Medical Systems Ltd, Milwaukee, WI) within 24 h of admission to exclude patients with PICH or stroke mimic from further analysis. During the course of the phase II study of rhIL-1ra, the scanner was replaced with a LightSpeed CT scanner (GE Medical Systems, Europe).

2.8.2 Measurement of infarct volume

Patients with ischaemic stroke underwent a second CT brain scan at 5 to 7 days for measurement of infarct volume. Paired admission and 5 to 7 day CT brain scans were compared to determine recent visible infarction. Analysis of recent

visible infarct volume was performed using a semi-automated technique, with scans viewed as digital images. On each slice with visible recent infarction, the area was traced manually with a cursor. The volume of infarction was automatically calculated by the IGE CT Pace Plus scanner volumetry program based on the area traced on each slice, slice thickness and number of slices. For each 5 to 7 day scan with recent visible infarction, five observers (CJS, HCAE, CML and two consultant neuroradiologists) independently performed infarct volume analyses, blinded to each others' values. The mean of the five determinations was then calculated in each case. This method was shown to have acceptable interobserver variation (Gavin *et al.*, 2004), although measurements by a single observer had greater reliability, and therefore in the second study reported in chapter 7, a single observer (HCAE) performed the infarct volume analysis.

2.9 *Assessment of atherosclerosis*

Ankle-brachial pressure index (ABPI) was determined using a hand-held Doppler device with an 8MHz transducer (Huntleigh Mini Dopplex, Huntleigh Healthcare, UK, figure 2.2) and a sphygmomanometer with an appropriately sized blood pressure cuff. Systolic pressure was measured at the ankle using Doppler ultrasound to detect the presence of arterial flow distal to the cuff. Pressures were compared to the contralateral leg and to the higher brachial systolic pressure, and the ABPI calculated by dividing each ankle pressure by the highest brachial systolic pressure.

Most patients and control subjects (for the work reported in chapters 3 and 5) also had a carotid duplex scan (Toshiba Acuson 128 Ultrasound Scanner, UK). ABPI was used to classify patients and control subjects where carotid duplex data were unavailable. Patients and control subjects were divided into two groups, one without significant atherosclerosis (< 50% carotid artery stenosis bilaterally, or lowest ABPI ≥ 0.92), and one with significant atherosclerosis (≥ 50% carotid artery stenosis on at least one side, or lowest ABPI < 0.92) (Howell *et al.*, 1989; Benavente *et al.*, 1998).

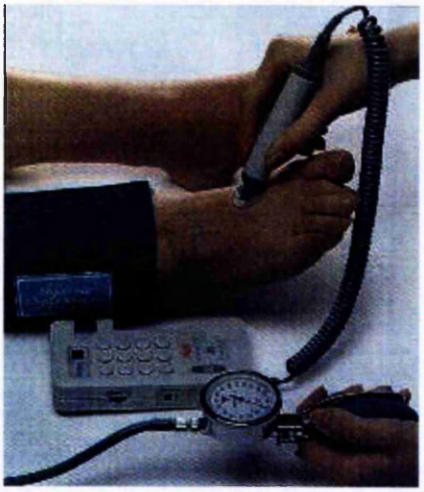


Figure 2.2 *Measuring ABPI using a hand-held Doppler device. (Reproduced with permission from Huntleigh Healthcare, UK)*

2.10 Computerisation of data and data storage

When initially collected, clinical data were recorded using a dedicated case report form (CRF, see appendix for the CRF relevant to work reported in chapters 6 and 7). CRFs and associated documentation were stored in a secure location. Wherever possible, such data were then computerised using Microsoft® Excel. Data were stored in accordance with the Data Protection Act 1998 and local guidelines taking account of research governance recommendations.

2.11 Discussion

The study population was relatively ethnically uniform, having a greater proportion of white subjects than might be expected from the population of England as a whole. Ensuring adequate representation of ethnic minorities in clinical research is clearly important (Rathore and Krumholz, 2003), although any degree of ethnic bias in the work reported here reflects the demographics of the population in the hospital catchment area. Bias attributable to non-consent/non-assent is likely to have been minimal in this work as the consent/assent rate was very high in both studies.

Ideally, a limit of six hours from onset of symptoms would have been preferred for the work reported in chapters 3 and 5. Specific arguments for this preferred

time window are provided later. However, owing to time constraints of the overall project for patient recruitment, and because recruitment of patients within this interval is difficult usually owing to their late presentation to hospital, a time window of 12 h was used.

Defining such entities as hypertension and hypercholesterolaemia is fraught with difficulties, as their clinical definitions have a somewhat dynamic nature. The work reported here has been conducted at a time when long-standing dogmas in clinical stroke practice are being overturned by the wealth of information emerging from clinical studies. For example, it is becoming increasingly recognized that blood pressure targets should be as low as possible (PROGRESS Collaborative Group, 2001). It is likely that national guidelines on treatment targets for blood pressure will change. Although a total serum cholesterol concentration of 5 mmol/l has been used as a threshold for treatment in relevant individuals, it is likely that this cut-point will be driven down by recent data demonstrating reduced risk of vascular events by lowering the concentration in patients with baseline cholesterol > 3.5 mmol/l (Heart Protection Study Collaborative Group, 2002).

Potential weaknesses in methodology exist in respect of subject preparation and timing of sample collection. In the original study protocol it was intended that patients and control subjects would be fasted prior to sample collection, because it has been suggested that cytokine profiles may vary between the fed and fasted states (Orban *et al.*, 1999). However, as it was found to be impracticable to consistently meet this criterion, it was not adhered to. Similarly, it had been intended that patient and control subject samples would be seasonally matched because seasonal variations in cytokine profiles have been described (Tang *et al.*, 1996; Mann *et al.*, 2000). However, owing to the difficulties of identifying suitable age-, sex-, and atherosclerosis-matched control subjects, it became too difficult logistically to adhere to seasonal matching. Bias in this respect was therefore minimized by collecting from both groups across the same time period.

Potential sources of bias exist in relation to clinical measurements. Owing to failure of the first infrared digital ear thermometer used in the early part of the

work it was not possible for all aural temperature measurements to be made using a single device. Concern has recently been expressed with regard to the accuracy of infrared ear thermometry and its low level of agreement with other methods of temperature measurement (Craig *et al.*, 2002). Similarly, scope exists for variability in blood pressure measurements because different automated devices (eg Dinamap, GE Medical Systems) exist in different clinical departments, and some measurements were made manually using a traditional sphygmomanometer. In order to reduce sources of variability in the handling of study samples, any research personnel involved in the collection of samples and preparation of plasma received specific prior training.

The clinically based OCSP classification has clear advantages over other systems of classification of stroke subtypes such as the Trial of Org 10172 in Acute Stroke Treatment (TOAST) system (Adams *et al.*, 1993), because the information it requires is quick, easy and inexpensive to collect on virtually all patients without recourse to additional investigations.

There are a large number of stroke-specific impairment measures, but the NIHSS is probably one of the most widely used. Furthermore, the demonstration of its increased inter-observer reliability with the use of video training makes it especially attractive to use (Lyden *et al.*, 1994).

Although not originally stroke-specific, the BI and mRS are both very widely used disability scales for stroke. Cut-points on the BI and mRS in clinical trials remain controversial, and depend on various factors, such as the expected outcome of the intervention under test, trial power calculations based on entry criteria, and marketing intentions of the sponsors (in the case of commercial studies). In various recent clinical stroke trials, both scales have been trichotomised, for example BI categories 0-50, 55-90, 95-100 and mRS categories 0-1, 2-3, 4-5 (NINDS, 1995). A category for death is usually added, although death may not relate to the cause of disability. These issues were discussed at an early stage in the research (KR Lees, personal communication, July 1, 2001).

CT was used during this work as it is quick, readily available and easy to use in almost all patients. MRI, by contrast, is neither always readily available, nor appropriate in certain patient groups such as those who are very ill. CT is arguably also the first-line neuroimaging investigation of choice for acute stroke, as discussed in chapter 1. A second CT scan was performed at 5-7 days for determination of infarct volume because the majority of infarcts visible on CT can be seen by this time, but later than this fogging may render infarcts invisible for a period.

Although the definition of 'significant atherosclerosis' in the present work is arbitrary, 50% carotid artery stenosis was also the threshold for inclusion in clinical trials of endarterectomy for asymptomatic carotid stenosis (Benavente *et al.*, 1998). Where it was not possible to undertake carotid ultrasound, owing either to lack of availability of the investigation or where the patient was unable to tolerate the test, ABPI data were used as the measure of atherosclerosis. An ABPI cut-point of 0.92 was selected because values less than this are generally accepted as abnormal (Howell *et al.*, 1989). Potential sources of bias do exist in these methods of classifying degree of atherosclerosis. Carotid ultrasound, for example, is a highly operator-dependent technique (Mead *et al.*, 2000), but we ensured that dedicated technicians from a vascular laboratory undertook all of these scans. ABPI measurements are also subject to certain limitations, but their reproducibility can be optimised with an experienced observer (Kaiser *et al.*, 1999).

CHAPTER 3

An early and sustained peripheral inflammatory response in acute ischaemic stroke: relationships with infection and atherosclerosis

3.1 Introduction

Central nervous system (CNS) and peripheral inflammation is an important feature of the pathophysiological response to ischaemic stroke (Emsley and Tyrrell, 2002), and may also predispose to the initial development of cerebral ischaemia (Rost *et al.*, 2001). Ischaemic stroke is associated with increased peripheral markers of the inflammatory response, including CRP (Syrjänen *et al.*, 1989a; Vila *et al.*, 1999; Muir *et al.*, 1999), ESR (Vila *et al.*, 1999; Chamorro *et al.*, 1995), total peripheral WBC count (Pozzilli *et al.*, 1985a), peripheral neutrophil count (Vila *et al.*, 1999) and body temperature (Boysen and Christensen, 2001). Increases in these peripheral markers of inflammation and the acute phase response appear to predict poor outcome after stroke (Vila *et al.*, 1999; Muir *et al.*, 1999; Chamorro *et al.*, 1995; Azzimondi *et al.*, 1995; Di Napoli *et al.*, 2002).

In addition, cytokines such as IL-6 have been associated with both CNS and peripheral responses to stroke (Loddick *et al.*, 1998; Kim *et al.*, 1996). Several clinical studies have demonstrated elevated concentrations of circulating (Beamer *et al.*, 1995; Tarkowski *et al.*, 1995; Kim *et al.*, 1996; Fassbender *et al.*, 1997; Ferrarese *et al.*, 1999; Vila *et al.*, 2000a) or cerebrospinal fluid (Tarkowski *et al.*, 1995) IL-6 in acute stroke patients. However, distinguishing between the cytokine response to brain injury, peripheral inflammatory stimuli, and early expression that might contribute to stroke severity is problematical.

Our understanding of the role of inflammation in stroke is deficient in several respects. Relatively few data have been collected within the first few hours of onset of symptoms of ischaemic stroke. Secondly, the extent to which observed peripheral inflammatory or acute phase responses are driven by cerebral infarction per se has not been distinguished clearly from the effects of pre-existing inflammatory or infective processes, or inflammatory/infective processes complicating the stroke.

Changes in inflammatory and acute phase markers have been attributed to preceding infectious or inflammatory conditions, which occur commonly prior to

a stroke (Grau *et al.*, 1995a), or infectious complications after ischaemic stroke rather than to cerebral infarction itself (Syrjänen *et al.*, 1989a). Elevated circulating IL-6 and CRP concentrations have been reported in patients with atherosclerosis (Erren *et al.*, 1999), which is an inflammatory process (Ross, 1999) and is an important risk factor for both coronary artery disease and ischaemic stroke. Thus it is important to take account of the extent of atherosclerosis when commenting on peripheral inflammatory markers.

I have therefore undertaken a prospective study of ischaemic stroke patients recruited within twelve hours of onset of symptoms, with control subjects individually matched for age, sex and degree of atherosclerosis. By studying patients early after onset I aimed to determine (1) the extent to which a peripheral inflammatory response is activated following acute ischaemic stroke, and (2) whether there was evidence for pre-existing inflammation, in stroke patients relative to their matched controls. Data concerning the relationship between the inflammatory response and radiological and clinical outcome have been reported separately (Smith *et al.*, 2004).

3.2 Subjects and Methods

3.2.1 Patients and control subjects

The study was approved by the LREC. Patients over 18 years of age, within 12 hours of onset of symptoms of acute stroke, where either written informed consent or assent from a relative was available, were eligible. Patients were excluded if there was any improvement in symptoms since onset, the time of onset of symptoms could not be reliably determined, or there was evidence of active malignancy.

Control subjects matched for age (± 5 years), sex and degree of atherosclerosis, who volunteered in response to project publicity or following identification from vascular disease registers, were also recruited from the local population. Control subjects were eligible if they were individuals living in the community, over 18 years of age, with no history of stroke or transient ischaemic attack and able to

give written, informed consent, but were excluded if there was clinically evident infection necessitating medical treatment, or a history of cognitive impairment sufficient to interfere with daily life.

3.2.2 Clinical assessments

Assessment of patients included history, clinical examination and aural temperature (Braun Thermoscan LF20 digital thermometer), NIHSS score (Lyden *et al.*, 1994), BI (Mahoney and Barthel, 1965), mRS (van Swieten *et al.*, 1988), and OCSP classification (Bamford *et al.*, 1991). Infections or other events likely to provoke a marked inflammatory response in the 6 weeks prior to each assessment were recorded using available clinical, radiological and laboratory data. Assessment of control subjects included history, clinical examination and aural temperature. Hypertension, atrial fibrillation, diabetes mellitus and ischaemic heart disease were defined according to local clinical practice, based on standard criteria.

3.2.3 Blood samples

Venous blood samples were taken from patients at baseline, the next 09:00 time point where admission was before 07:00 or after 11:00, 24 h after admission, plus 5 to 7 days, 3 months and 12 months at 09:00. Blood was drawn from resting control subjects at 09:00, and again at a time matched to the patient's time of admission if this was before 07:00 or after 11:00. Samples drawn by venepuncture were collected into tubes containing EDTA (Sarstedt, UK) for determination of ESR using an automated Westergren method on a Starrsed III analyser, and FBC and differential WBC count using a Coulter Gen-S analyser.

Blood for CRP, IL-6 and cortisol concentrations was collected into tubes containing pyrogen-free heparin, and wrapped in cool-gel packs. One hour after collection, blood was centrifuged at 2000g for 30 minutes at 4°C. Plasma was separated, frozen and stored at -70°C until analysis. Plasma IL-6 was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). IL-6 was captured on 96-well microplates (Costar, Cambridge, MA) with a monoclonal antibody (clone IL-6-16; CLB, Amsterdam, The Netherlands) and secondarily

bound with a rabbit polyclonal antibody against recombinant human IL-6 (a gift from L. Aarden, CLB). Detection employed a donkey anti-rabbit IgG (Jackson Immunoresearch, Burlingame, PA), conjugated with peroxidase and developed with orthophenylene-diamine (Sigma, Poole, UK). Standard IL-6 was calibrated against the 1st international IL-6 standard (89/548, National Institute for Biological Standards and Control, South Mimms, UK). CRP was measured using a competitive ELISA, essentially as previously described (Holt *et al.*, 1991). Cortisol concentration was determined using a solid phase time-resolved fluoroimmunoassay (DELFIA®, Perkin Elmer™ Life Sciences, manufactured by Wallac Oy, Finland). Minimum sensitivities were 9 pg/ml (IL-6), 100 μg/l (CRP), and 30 nmol/l (cortisol). Inter-assay coefficients of variation, determined in the appropriate working range, were 12% at 130 pg/ml and 32% at 44 pg/ml (IL-6), 15% at 480 mg/l and 7% at 23 mg/l (CRP), and <10% between 150 and 940 nmol/l (cortisol).

3.2.4 Radiology

Computed tomography (CT) brain scans were performed on an IGE CT Pace Plus 3rd generation CT scanner within 24 h of admission to exclude patients with primary intracerebral haemorrhage or stroke mimic from further analysis.

3.2.5 Assessment of degree of atherosclerosis

ABPI was determined using a hand-held Doppler device (Huntleigh Diagnostics, UK). Most patients ($n = 27$) and control subjects ($n = 32$) also had a carotid duplex scan (Toshiba Acuson 128 Ultrasound Scanner, UK). ABPI was used to classify patients and control subjects where carotid duplex data were unavailable. Patients and control subjects were divided into two groups, one without significant atherosclerosis (< 50% carotid artery stenosis bilaterally, or lowest ABPI ≥ 0.92), and one with significant atherosclerosis (≥ 50% carotid artery stenosis on at least one side, or lowest ABPI < 0.92).

3.2.6 Statistical Analysis

Primary outcome measures were CRP and ESR at study entry in stroke patients, with and without significant atherosclerosis, compared with control subjects.

Secondary outcome measures were CRP, ESR, WBC count, IL-6, cortisol, and aural temperature (a) at all time points compared with controls and (b) versus presence of infection. Log transformed CRP, ESR, WBC count, IL-6, cortisol and aural temperature data were compared using paired t-tests. Possible differences in baseline characteristics were examined using analysis of variance (ANOVA). The achieved sample size of 36 patient-control subject pairs had 80% power at the 5% significance level to detect a difference of 0.67 standard deviations (SD) in primary analyses between control subjects and patients.

3.3 Results

Forty-three patients were recruited between April 2000 and January 2001. Seven patients were excluded (see figure 3.1). Thirty-six patients, recruited at a median interval of 4.75 h (range 1.5 h to 11.75 h) from onset of symptoms of acute stroke, were included in the analysis. Fourteen patients died by 12 months: certified causes of death were index stroke (8), recurrent stroke (1), pulmonary embolism (1), left ventricular failure secondary to myocardial infarction (1), sepsis (3). Baseline characteristics are shown in table 3.1.

3.3.1 Infections and other events associated with an inflammatory response

Two patients reported infections in the six-week period prior to study entry (both chest infections). One further patient suffered a myocardial infarction 5 days prior to the index stroke. Eight further patients developed definite or suspected infections in the first week after admission (four chest infections, two urinary tract infections [UTIs], one infected intravenous cannula site, one pyrexia with no identified source of infection). Two of these patients, as well as another three patients developed further infections (recurrent peritonitis, gram negative sepsis, chest infection, 2 UTIs) in the six weeks prior to their 3-month assessments. Three other patients were symptomatic of infection (2 UTIs and one upper respiratory tract infection) during the six weeks before their 12-month assessments.

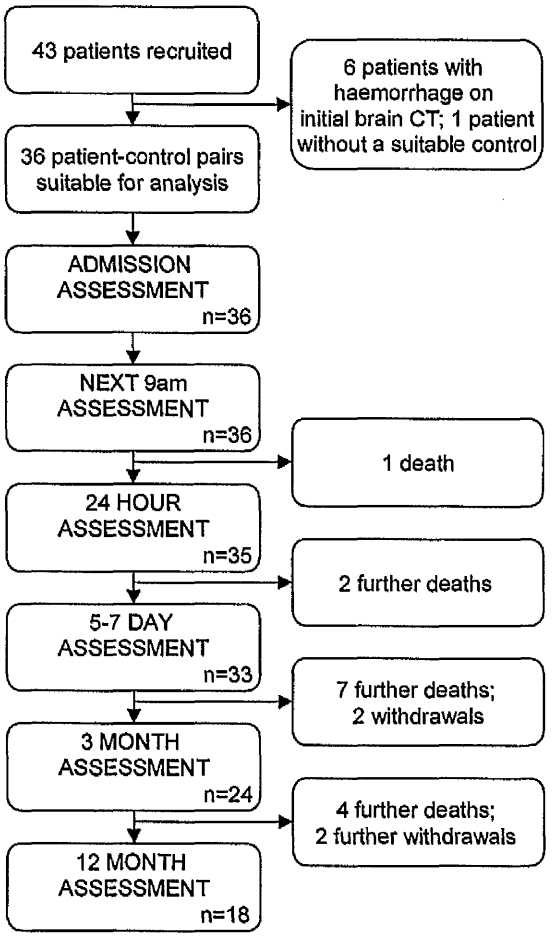


Figure 3.1.
Study profile

3.3.2 Atherosclerosis

Seventeen patients and 17 control subjects were classified as having significant atherosclerosis. No differences in any inflammatory markers were seen between those individuals (including patients and controls) with or without significant atherosclerosis at any time points.

Table 3.1. *Control subject and patient characteristics*

Baseline characteristics	Controls	Patients		
Mean age ± S.D. (years)	68.7 ± 12.6	69.6 ± 13.0		
Male	24 (67%)	24 (67%)	*Stroke characteristics*	
Caucasian	36 (100%)	36 (100%)	*OCSP classification*	
Systolic blood pressure*	142 (100, 200)	151 (60, 232)	TACS	16 (46%)
Diastolic blood pressure*	79 (60, 120)	82 (40, 146)	PACS	12 (34%)
Body mass index* (kg/m^2)	26 (17, 36)	26 (18,41)	LACS	5 (14%)
'Significant' atherosclerosis	17 (47%)	17 (47%)	POCS	2 (6%)
Current or former smoker	23 (64%)	25 (69%)	*NIHSS score**	
Smoking history unknown	0 (0%)	1 (3%)	Admission	14 (3, 24)
Hypertension	11 (31%)	23 (64%)	5-7 days	11 (1, 42)
Atrial fibrillation	2 (6%)	8 (22%)	*mRS**	
Diabetes mellitus	6 (17%)	3 (8%)	Pre-stroke	0 (0, 4)
Ischaemic heart disease	7 (19%)	8 (22%)	*BI**	
*Prevalence of selected drugs***			Pre-stroke	100 (20, 100)
Paracetamol	Not known	20 (56%)		
Statins	11 (31%)	20 (56%)		
Antiplatelet agents	14 (39%)	33 (92%)	*Infections / other events associated with an inflammatory response*‖	
Warfarin	2 (6%)	4 (11%)	Pre-stroke	3 (8%)¶
ACEIs† or A-IIR‡ antagonists	5 (14%)	10 (28%)	Pre-stroke or 1st week after stroke	11 (33%)¶
NSAIDs§	4 (11%)	7 (19%)		
Beta-blockers	7 (19%)	15 (42%)	Pre-3 months	5 (21%)¶
Inhaled corticosteroids	2 (6%)	6 (17%)	Pre-1 year	3 (17%)¶
Azathioprine	1 (3%)	0 (0%)		

* data shown as median (min, max)
** at recruitment for control subjects; during study period for patients
† angiotensin-converting enzyme inhibitors
‡ angiotensin-II receptor
§ non-steroidal anti-inflammatory drugs
‖ in 6 weeks prior to assessment
¶ number of patients (% of survivors) with infection at each time point

3.3.3 C-reactive protein

CRP concentration was significantly higher in patients at admission, and remained elevated until 3 months compared with control subjects, the greatest difference occurring at 5 to 7 days (figure 3.2a and table 3.2). By 12 months no differences were seen between patient and control subject CRP concentrations. A similar, but less marked difference was seen in patients without infection (figure 3.2a); nonetheless, elevations in CRP concentration in this group were seen at each time point except for 12 months. CRP concentration was significantly higher in patients sampled 4-12 hours after the onset of symptoms (median 8.9 mg/l, $n = 19$) compared with those sampled at 0-4 hours (median 2.3 mg/l, $n = 17$) ($p \leq 0.01$) and control subjects ($p \leq 0.001$) (figure 3.3). Variability between individual patients is illustrated in figure 3.4a.

3.3.4 Erythrocyte sedimentation rate

Although admission ESR in patients was not raised, ESR at 5 to 7 days was elevated compared with control subjects (figure 3.2b and table 3.2). This increase remained at 3 months but not at 12 months. A similar response was observed in patients without evidence of infection (figure 3.2b). Variability between individual patients is illustrated in figure 3.4b.

3.3.5 White blood cell count

Total WBC and neutrophil counts were elevated at all time points from admission until 3 months (figure 3.2c and table 3.2). By 12 months no differences from control subjects were apparent in either total WBC or neutrophil count. Similar differences in total WBC and neutrophil counts were seen when patients with evidence of infection were excluded, although they were somewhat less marked. The monocyte count showed elevation compared to control subjects at 5 to 7 days, whilst the lymphocyte count showed no differences at any time point from control subjects (data not shown).

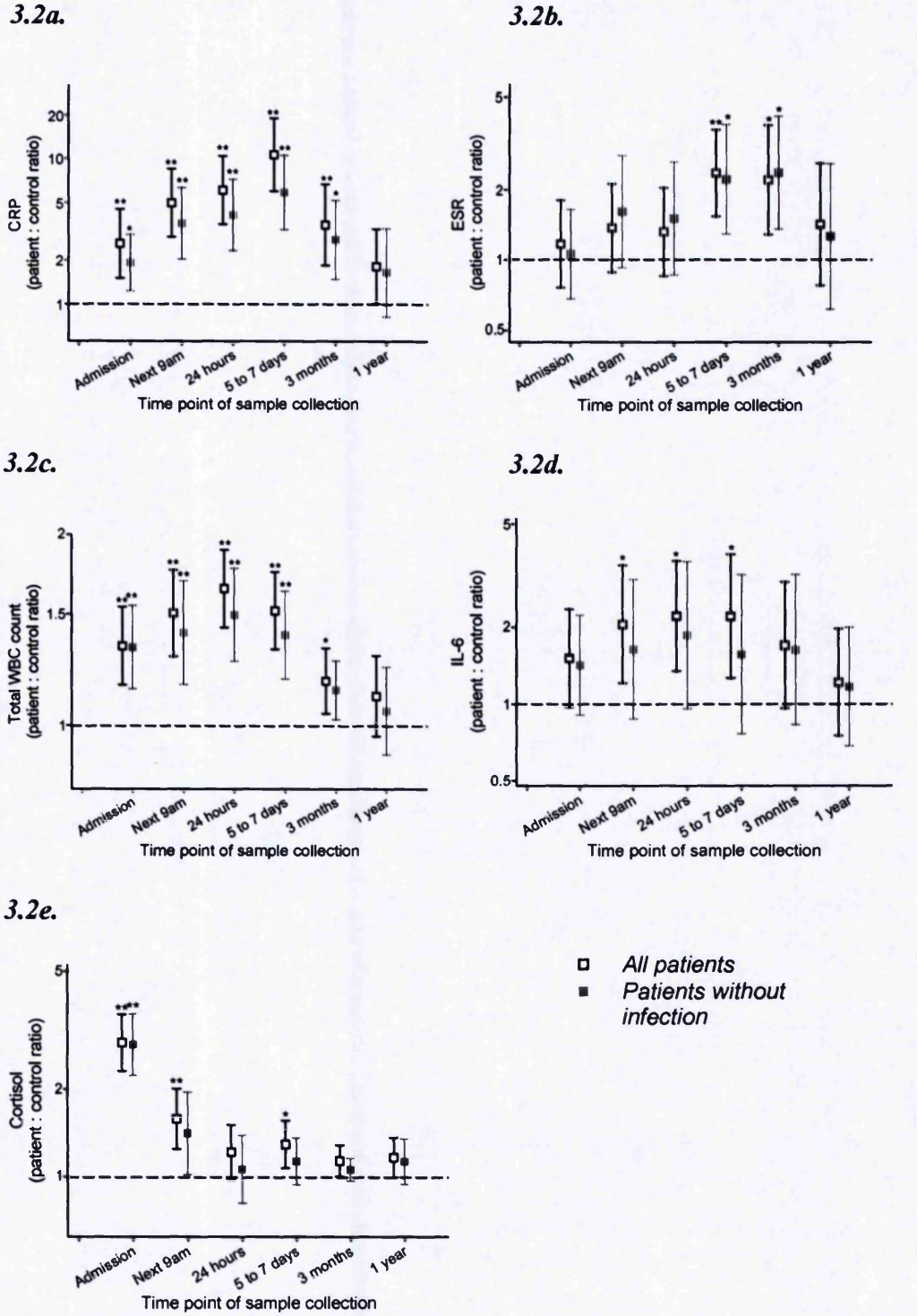


Figure 3.2(a-e). *Peripheral inflammatory marker kinetics in all patients and patients without infection (in the six weeks preceding assessment). Patient values are expressed as multiples (ratios) of their corresponding controls (with 95% confidence intervals) to account for skewed distributions. The 24 hour time point includes "next 09:00" data where admission was between 07:00 and 11:00.*
**p ≤0.01; **p ≤0.001 (relative to control)*

Table 3.2 *Peripheral inflammatory markers in control subjects and patients*

	Controls		*Patients*				
	09:00	Admission matched[a]	Admission	24 hours	5-7 days	3 months	12 months
	$n = 36$	$n = 36$	$n = 36$	$n = 35$	$n = 33$	$n = 24$	$n = 18$
Plasma CRP (mg/l)	2.1 (0.3, 53.7)	1.9 (0.3, 53.7)	4.3** (0.8, 197.7)	13.4** (1.1, 159.0)	17.7** (1.9, 199.7)	6.7** (0.3, 87.9)[b]	2.9 (0.7, 24.9)
ESR (mm/h)	12 (2, 63)	10 (2, 70)	13 (2, 83)[c]	14 (2, 71)[b]	30** (4, 80)[b]	24* (2, 68)[d]	14 (2, 62)
Total WBC count ($x10^9$/l)	6.2 (3.4, 9.9)	7.6 (4.3, 11.6)	9.8** (4.2, 19.9)	10.1** (5.3, 24.4)[c]	8.8** (4.3, 21.6)	6.9* (4.3, 13.5)[b]	7.2 (4.1, 8.9)
Neutrophil count ($x10^9$/l)	3.7 (1.5, 7.9)	4.5 (2.1, 8.0)	7.6** (2.5, 15.9)	7.9** (3.6, 23.0)[c]	5.9** (2.6, 18.5)	4.4* (2.5, 10.9)[b]	4.4 (2.4, 7.0)
Monocyte count ($x10^9$/l)	0.5 (0.3, 1.0)	0.6 (0.3, 1.1)	0.5 (0.0, 1.3)	0.6* (0.3, 1.8)[c]	0.6* (0.3, 1.7)	0.5 (0.2, 0.9)[b]	0.5 (0.2, 0.7)
Plasma IL-6 (pg/ml)	9.0 (9.0, 265.6)	9.0 (9.0, 265.6)	13.5 (9.0, 235.7)	22.5* (9.0, 311.3)	23.5* (9.0, 276.4)	9.0 (9.0, 303.0)[b]	9.8 (9.0, 237.7)
Plasma cortisol (nmol/l)	323 (201, 637)	260 (47, 437)	663** (106, 1377)	436 (83, 1322)	420* (204, 1135)	370 (236, 657)[b]	425 (218, 552)
Temperature (°C)	36.3 (35.0, 37.6)	36.5 (35.2, 37.2)	36.4 (34.8, 38.0)	37.2* (34.5, 38.5)	36.6 (34.0, 38.8)	36.6 (35.5, 37.7)[b]	36.2 (35.4, 37.4)

[a] admission matched data includes 09:00 values if admission was between 07:00 and 11:00
[b] 1 missing case; [c] 2 missing cases; [d] 3 missing cases
*$p \leq 0.01$ **$p \leq 0.001$ (relative to controls) n = number of observations
data shown as median (min, max)

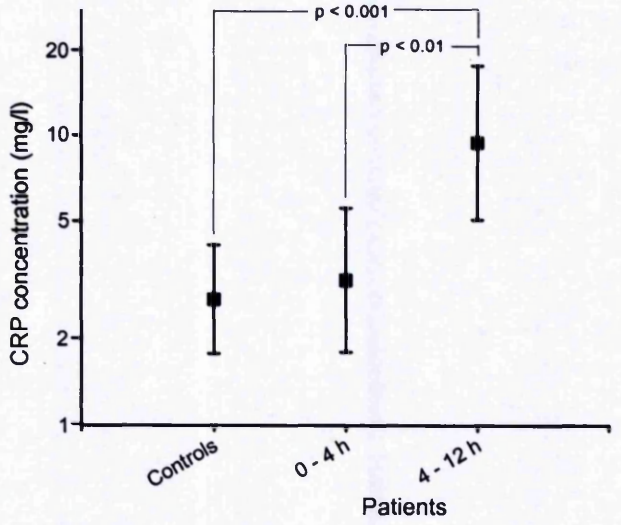


Figure 3.3.
CRP concentration (mean with 95% confidence intervals) in patients sampled 0-4 h and 4-12 h after onset of symptoms compared with controls.

3.3.6 Interleukin-6

An increase in plasma IL-6 concentration was seen at the next 09:00 time point after admission, 24 hours and 5 to 7 days (figure 3.2d, table 3.2). This elevation did not persist beyond 5 to 7 days. Differences from control subjects were not seen when patients with evidence of infection were excluded. Variability between individual patients is illustrated in figure 3.4c.

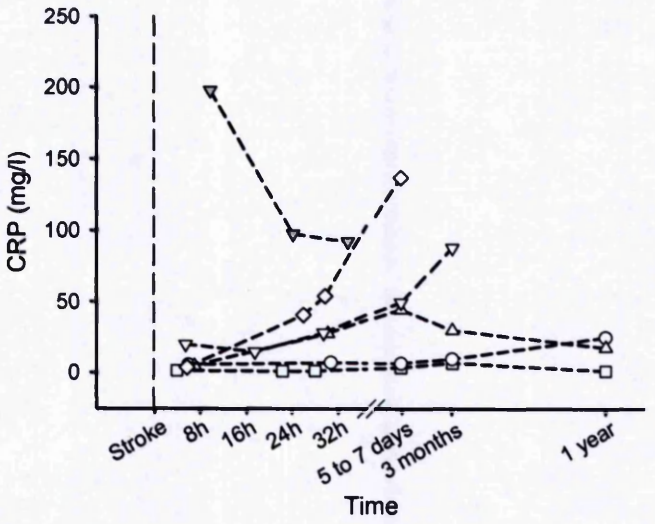
3.3.7 Cortisol

An early, extensive elevation was seen in plasma cortisol concentration on admission, which was less marked by the next 09:00 time point (figure 3.2e, table 3.2). By 24 hours no differences from control subjects remained, nor were any further increases seen beyond this time. A similar response was observed in patients with or without evidence of infection.

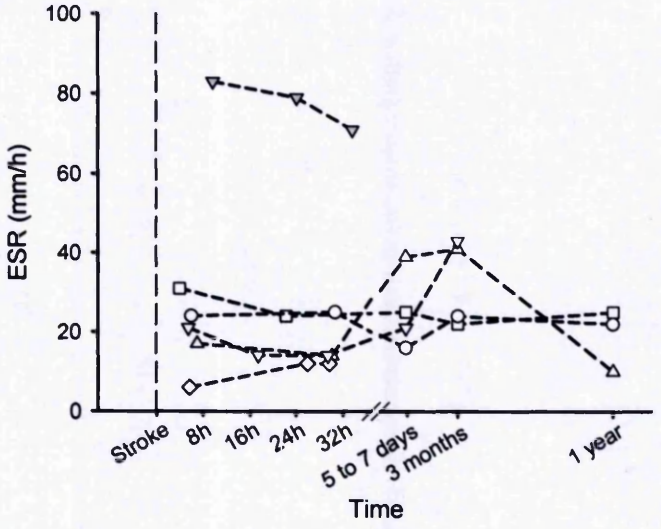
3.3.8 Aural temperature

Aural temperature exhibited a modest elevation 24 hours after stroke, but otherwise no differences were seen compared to control subjects (table 3.2). This elevation was not seen in patients without evidence of infection.

3.4a



3.4b



3.4c

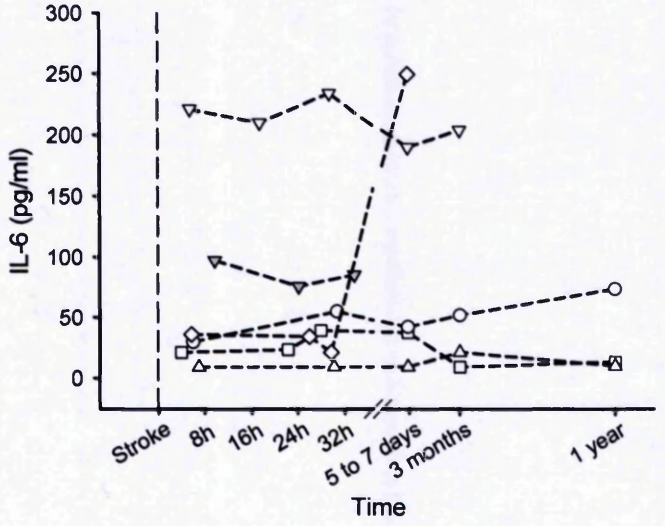


Figure 3.4a-c.
Variability between individual patients markers. Every sixth patient included in the analysis is shown, for illustrative purposes only.

Admission NIHSS score (OCSP classification) for each patient are shown below:

1	□	*12*	*(LACS)*
2	○	*16*	*(PACS)*
3	△	*12*	*(PACS)*
4	▽	*22*	*(TACS)*
5	◇	*15*	*(TACS)*
6	▽	*9*	*(PACS)*

No infections or other events associated with an inflammatory response occurred in patients 1, 2 or 6 in the 6 weeks prior to each assessment.
Patient 3 – urinary tract infection day 90;
patient 4 – myocardial infarction 5 days pre index stroke;
patient 5 – aspiration pneumonia day 3

3.4 Discussion

These data provide evidence of an early and sustained peripheral inflammatory response to acute ischaemic stroke in patients, with or without evidence of infection, characterised by elevated plasma CRP, IL-6 and cortisol concentrations, ESR, and total WBC count. By contrast with previous studies, these data have been collected at multiple pre-specified time points, beginning very early after the onset of symptoms, up to 12 months. To my knowledge, this is the first report demonstrating the sequential modification of these peripheral inflammatory markers, in patients with acute ischaemic stroke. The very early increase in these markers after stroke, sustained for at least 3 months, may have two possible explanations. First, that stroke induces a very early inflammatory response that is sustained for a long time and might thus predispose to further cardiovascular events. Second, the data may indicate a pre-existing inflammatory condition in stroke patients which could contribute to the development of stroke.

Various cardiovascular risk factors, including increasing age, smoking, raised body mass index, elevated lipid levels and high blood pressure, are known to influence concentrations of CRP and inflammation-sensitive proteins (de Maat and Kluft, 2001; Engström *et al.*, 2002). Previously recognised hypertension was more prevalent in the present patient population, but this did not significantly influence the peripheral inflammatory response reported here (data not shown). Statin, antiplatelet agent and β-blocker use was much more common amongst the stroke patients during the study period than in the control subjects, all of which may have inhibitory effects on the inflammatory response. This would tend, if anything, to have lessened the differences seen between the patients and control subjects in the present study for the markers measured.

The degree of atherosclerosis was assessed using objective, reproducible and non-invasive techniques. A 50% carotid artery stenosis threshold for 'significant atherosclerosis' is supported by the degree of carotid stenosis chosen for inclusion in clinical trials of endarterectomy for asymptomatic carotid stenosis, which ranged from 50% to 99% (Benavente *et al.*, 1998). ABPI was used as a readily available alternative measure of atherosclerosis in the absence of carotid

ultrasound data. In the present study a threshold of 0.92 for 'significant atherosclerosis' was selected because values less than this are accepted to be abnormal (Howell *et al.*, 1989). An ABPI of less than 0.8 has now been shown to be independently associated with carotid stenosis > 50% (Cina *et al.*, 2002).

A particular feature of the present study is that patients were recruited early after onset of symptoms of acute stroke (median < 5 h) and further assessments were performed within the first 24 hours of admission. Most other studies of peripheral inflammatory markers in acute stroke have studied patients between 24 hours and 3 days after onset (Syrjänen *et al.*, 1989a; Vila *et al.*, 1999; Muir *et al.*, 1999; Chamorro *et al.*, 1995; Di Napoli *et al.*, 2002), and therefore did not address the acute response at such an early stage as the present study. At later time points, it may be difficult to distinguish the influence of the stroke itself from complicating infections or other events. To limit the known effects of diurnal variation on inflammatory markers (Perdiz *et al.*, 1996), blood samples were collected at 09:00 wherever possible. A potential limitation of this study is that patients with the most severe strokes were amongst the non-survivors at 12 months. This may have caused an under-estimation of the magnitude of the inflammatory response observed at the later time points.

Published data in respect of two patients have provided evidence for an early increase in CRP after stroke (Di Napoli, 2001). In the present, larger study CRP concentration was significantly higher in patients sampled 4-12 hours after stroke onset, compared with those sampled at less than 4 hours, suggesting a rapid rise in CRP concentration in direct response to the stroke. In patients with or without infection, CRP was maximal at 5 to 7 days and remained elevated for at least 3 months. No elevation in ESR was seen until 5 to 7 days, consistent with ESR being a more slowly evolving marker of the acute phase response, and primarily determined by elevation of fibrinogen concentration. The persistent, significant elevations in both CRP and ESR at 3 months are in agreement with previous work suggesting that there is a protracted inflammatory phase in stroke survivors up to 1 year (Beamer *et al.*, 1998).

It is possible that a pre-existing inflammatory condition was present in the present patient population, and responsible for at least an element of the early measurements. The concept of a pre-existing inflammatory condition is supported by evidence that low-grade inflammation, identified by an elevated CRP concentration, may be a risk factor for stroke (Rost *et al.*, 2001). Median admission CRP for all patients was raised relative to controls, although this increase was not significant for patients sampled less than 4 hours from onset. However, an important and novel feature of the present study design is the individual matching of patients and control subjects for age, sex and degree of atherosclerosis, all of which are related to differences in peripheral inflammatory markers (Erren *et al.*, 1999; de Maat and Kluft, 2001). Since atherosclerosis is a known inflammatory stimulus (Ross, 1999) the present control population is likely to have elevated inflammatory markers compared with an entirely normal population lacking atherosclerosis, which may be expected to reduce differences between control subjects and patients. Indeed, the plasma CRP concentration for both controls and patients sampled at 0 – 4 hours lies within the range associated with increased risk of cardiovascular disease (Ridker, 2003).

Elevated total WBC counts are also associated with higher risk of cerebral infarction (Prentice *et al.*, 1982) and, certainly in the present study, total WBC count was elevated on admission in patients with or without infection, compared with controls. One may also take the view that increased levels of inflammatory markers 12 months after stroke are indicative of elevated baseline values. Persistent elevations in fibrinogen, shown by others (Beamer *et al.*, 1998), and modest increases in peripheral inflammatory markers in the present study, at 12 months tend to support this.

Increased peripheral WBC counts have been reported previously in patients with ischaemic stroke recruited up to 3 days after onset (Pozzilli *et al.*, 1985a; D'Erasmo *et al.*, 1991). The peaks in peripheral total WBC and neutrophil counts observed at 24 hours in the present study, are consistent with the observation of labelled neutrophil accumulation in the brain, which progresses for up to 24 hours in patients with ischaemic stroke, and then diminishes (Akopov *et al.*, 1996). These data, and the present observation of elevated monocyte counts at 5

to 7 days, also accord with histopathological evidence of initial neutrophil accumulation around the infarct core, which is replaced by mononuclear cells by 4 to 6 days (Adams and Sidman, 1968). This pattern of WBC involvement has also been shown in serial cerebrospinal fluid samples of patients with acute stroke, where neutrophil outflow is maximal on day 4 and modest rises in macrophages and monocytoids reach maxima towards the end of the first week (Sörnäs *et al.*, 1972).

Circulating IL-6 concentrations have been reported previously to be increased significantly in patients with acute cerebral ischaemia or stroke for up to 14 days (Beamer *et al.*, 1995; Kim *et al.*, 1996; Perini *et al*, 2001). In these studies patients with evidence of infection were excluded. In the present study, IL-6 concentrations demonstrated similar kinetics, a plateau being achieved by 24 h and persisting until at least 5-7 days. Infections appear to have been influential in the present study, because patients lacking infection showed no increase in IL-6 concentration. Variability between individual patients' inflammatory markers, including CRP, ESR and IL-6, was seen in the present study, extending earlier observations on CRP, where variable concentrations were noted following cerebral infarction (Syrjänen *et al.*, 1989a). Genetic influences may also be important in this regard, for example, in a recent study of acute stroke patients it was suggested that inter-individual variation in IL-6 levels may depend on the IL-6 promoter haplotype (Acalovschi *et al.*, 2003).

Endocrine abnormalities, including increased activity of the hypothalamic-pituitary-adrenal axis (HPAA), occur commonly in acute stroke. Increased cortisol production rates occur after stroke, and a higher mortality rate has been described in stroke patients with elevated plasma cortisol concentrations and catecholamine excretion (Franceschini *et al.*, 2001). In the present study, elevated cortisol concentrations were observed on admission, which returned to control concentration by 24 hours. This early activation of the HPAA is in agreement with earlier work (Fassbender *et al.*, 1994b); although the duration of elevation in cortisol concentrations seen here is much shorter (< 24 hours) than previously suggested (up to day 5).

IL-6 can stimulate the HPAA in man (Späth-Schwalbe *et al.*, 1994) and has been proposed to partly mediate hypercortisolism after stroke (Johansson *et al.*, 1997). However, although plasma IL-6 did not rise until cortisol concentrations were declining, suggesting that the observed acute increase in cortisol is certainly not driven by IL-6, it is conceivable that the persistent modest elevation in cortisol concentration up to 12 months, relative to controls, may be related to the similarly maintained elevation of IL-6 concentration. The data suggest that stress associated with the stroke itself was primarily responsible for the observed cortisol response, and that infection did not appear to contribute to the early elevation in cortisol concentrations. Although pharmacological concentrations of corticosteroids reduce inflammation and cytokine production, IL-6 appears to be resistant to relatively modest variations in cortisol concentrations such as those seen in the present study (DeRijk *et al.*, 1997).

Elevated body temperature is common after stroke and is associated with worse outcome (Azzimondi *et al.*, 1995; Reith *et al.*, 1996); however, infections may have been responsible for this relationship since data on the underlying causes of pyrexia were not available in one study (Azzimondi *et al.*, 1995). In the present study, an elevation in aural temperature was seen at 24 hours, but this elevation was not present in patients lacking evidence of infection (data not shown). Although the observed temperature response was small, it is interesting that it peaked at the same time as IL-6 was reaching its maximum, in view of the demonstrated pyrogenic activity of IL-6 (Rothwell *et al.*, 1991). It is possible that potentially greater temperature responses may have been blunted by the prevalence of paracetamol and aspirin use in the stroke population studied.

In conclusion, I present new insights into the kinetics of the peripheral inflammatory response to acute ischaemic stroke by describing the sequential changes of various peripheral inflammatory markers. Furthermore, these data raise the possibility of a pre-existing inflammatory condition contributing to the development of stroke which warrants further prospective studies. Establishing that inflammatory processes are influential in predisposing to stroke, and determining clinical outcome, may lead to potential targets for therapeutic intervention.

CHAPTER 4

Development of enzyme-linked immunosorbent assays for interleukin-1β and interleukin-1 receptor antagonist in human plasma

4.1 Introduction

Since the principle of immunoassay was first described for the quantification of plasma insulin in human subjects (Yalow and Berson, 1959), this method has enabled the measurement of minute concentrations of complex molecules. Diverse immunoassay designs have been used in a wide range of applications, including not only medical diagnosis, but also in pharmaceutical, veterinary, forensic, military and food sciences. The characteristics of all immunoassays are dependent upon three particular properties of antibodies. First, antibodies are proteins with binding sites derived from a huge number of possible combinations of amino acid sequences, and can thus bind a wide range of chemicals, biomolecules, cells and viruses. Second, owing to their very high degree of specificity, minute concentrations of analyte may be assayed in the presence of many other closely related substances, as in the case of human plasma. Third, antibodies bind their target with a very strong, noncovalent bond that survives the later processing steps that are necessary in a typical assay. Immunoassays are accurate and precise even at the very low analyte concentrations found in biological fluids.

The fundamental immunological reaction of all immunoassays occurs between antibody and antigen, taking between a few seconds to many hours to achieve equilibrium. Several factors influence the equilibration time, such as the average distance an analyte molecule has to move before coming into contact with an antibody molecule, pH, ionic strength and temperature. This reaction can be described by the Law of Mass Action:

$$[Ag] + [Ab] \overset{K_{eq}}{\leftrightarrow} [Ag\text{–}Ab]$$

where [Ag] = antigen concentration
[Ab] = antibody concentration
[Ag–Ab] = antigen–antibody complex
K_{eq} = equilibrium constant

K_{eq} is important because it represents the final ratio of bound to unbound analyte and antibody, and is a key measure of an antibody's ability to function well in an immunoassay (Davies, 2001a).

Immunoassays exists in several formats. In a radioimmunoassay (RIA), a radioactive label is used for detection of the formation of an antigen-antibody complex. Non-isotopic immunoassays are now preferred, not least because they avoid the use of radioactive substances. In a latex particle agglutination immunoassay, antigen-antibody complexes are visualized by the attachment of latex particles and quantified using techniques such as turbidimetry or nephelometry (Ullman, 2001). In the sandwich ELISA format, the antibodies form a sandwich around the analyte. Antibody or antigen is coated onto a solid-phase, and signal is generated by using an antibody-enzyme conjugate. In the presence of an appropriate substrate, such an enzyme label can be used to generate coloured, fluorescent or luminescent end-products that can be read using optical and electronic equipment (Davies, 2001a).

Sandwich ELISAs support standard curves by the inclusion of serial dilutions of a standard solution of known concentration. Standard curves (also known elsewhere as calibration curves) are plotted as concentration against the corresponding mean signal (eg colour) value of replicates. Analyte concentrations can then be interpolated from the standard curve, a process that is facilitated by the use of curve fitting computer program.

Originally described for a quantitative assay of IgG (Engvall and Perlmann, 1971), sandwich ELISA is an example of a two-site immunometric assay in which the analyte is required to have at least two epitopes sufficiently spatially separated that two antibodies may bind at the same time. This increases the specificity of the assay, and confers on ELISA the ability to discriminate between molecules with overlapping biological functions that are not resolvable in a bioassay, where biological activity is measured by comparing the effects of a substance on living tissue with a reference standard. Monoclonal antibodies, which possess single-epitope specificity, are preferred in ELISAs. ELISAs have now become a standard method for the detection and quantification of concentration of soluble cytokines and other proteins in large numbers of plasma samples, in some cases detecting cytokines in the low picogram per millilitre range. ELISAs are used frequently because they offer many advantages over other types of assay, including relative ease and rapidity of performance,

availability of components and the use of non-radioactive reagents. The use of monoclonal antibodies confers additional advantages, including their virtually limitless supply by virtue of their method of production from hybridomas derived from the fusion of B lymphocytes and immortalized myeloma cells (usually of murine origin), and their constant properties.

Although commercial ELISA kits are available for human IL-1β and IL-1ra, in-house ELISAs were developed for two main reasons. First, marked variation in sensitivity and specificity can exist between commercial ELISA kits (Wang *et al.*, 1997). Although relatively time consuming in the short-term, in-house development allowed close scrutiny of assay performance, including sensitivity and specificity, and rigorous attention to quality control issues. Once developed, these ELISAs would be useable in future projects at this centre. Second, commercial assay kits carry significant cost implications compared to the relatively modest costs of buying individual antibodies. Given the finite nature of the budget for the work, economic considerations did need to be taken into account.

4.2 *Aims*

The aims of the work described in this chapter were to develop and evaluate sensitive and accurate ELISAs for (1) human interleukin-1β and (2) human interleukin-1 receptor antagonist in plasma.

4.3 *Materials and Methods*

4.3.1 *Outline ELISA procedure*

A suitable ELISA plate is coated with a primary antibody (coating antibody) specific to the analyte of interest (IL-1β or IL-1ra) diluted in an appropriate buffer solution (coating buffer). The plate is sealed and incubated in a humidified chamber in appropriate conditions (optimized for duration and temperature). At the end of this incubation, unbound antibody is removed by washing the plate using a standard method. An appropriate blocking reagent (block buffer) is added

in order to block additional binding sites in each well, followed by a further incubation. After a further wash, samples and standards are added, and incubated with the primary antibody, which captures the analyte. After a further wash to remove unbound analyte, a conjugated detection antibody (secondary antibody, eg biotin conjugated) is added and incubated for a further period. This detection antibody binds to a different epitope of the analyte, to complete the sandwich. After a further wash to remove unbound detection antibody, a detection reagent (eg streptavidin-horse radish peroxidase [HRP]) is added and incubated for a short time. The plate is washed prior to the addition of a substrate solution (eg orthophenylene diamine [OPD] / hydrogen peroxide) that produces a coloured end-product. Developed colour is proportional to the concentration of analyte present in the sample. Colour development is stopped using an appropriate reagent (stop solution, eg H_2SO_4), and the intensity of the colour is measured.

4.3.2 Materials

IL-1β assay antibodies and cytokine protein standards

Coating antibodies were mouse monoclonal anti-human IL-1β, catalogue number MAB601 (R&D Systems Europe Ltd., Abingdon, UK) and mouse monoclonal anti-human IL-1β (Diaclone Research, Besançon, France, catalogue number 855.010.005). Secondary antibodies were goat polyclonal anti-human IL-1β, catalogue number AF201NA (R&D Systems); peroxidase-labelled donkey anti-goat immunoglobulin, code number 705-035-147 (Jackson ImmunoResearch Laboratories Inc., PA, USA), and biotinylated goat anti-human IL-1β, catalogue number BAF201 (R&D Systems). The working standard was recombinant human IL-1β, catalogue number 201-LB (R&D Systems), standardised against international standard human IL-1β obtained from the National Institute for Biological Standards and Control (NIBSC), South Mimms, UK, code number 86/680, as described below.

IL-1ra assay antibodies and cytokine protein standards

Coating antibodies were mouse monoclonal anti-human IL-1ra, obtained from Biosource, Nivelles, Belgium, catalogue number AHC0112 (clone 998A 2A2); mouse monoclonal anti-human IL-1ra, catalogue number MAB280 (clone

10309.211) (R&D Systems); and mouse monoclonal anti-human IL-1ra, catalogue number MCA1467 (clone 1384.92.17.8) (Serotec Ltd, Oxford, UK). Secondary antibodies were biotinylated mouse monoclonal anti-human IL-1ra, obtained from Biosource, catalogue number AHC0219 (clone A71B 6D11); biotinylated goat anti-human IL-1ra, catalogue number BAF280 (R&D Systems); biotinylated mouse monoclonal anti-human IL-1ra, catalogue number MCA1466B (clone 1390) (Serotec); and polyclonal sheep anti-human IL-1ra immunoglobulin (a gift from S. Poole, NIBSC, UK) used in conjunction with stock HRP-conjugated anti-sheep immunoglobulin. Working standards were recombinant human IL-1ra, catalogue number 280-RA (R&D Systems) and Anakinra (referred to as r-metHuIL-1ra in this chapter to distinguish it from other forms of hIL-1ra) (100mg/l) obtained from Amgen, CA, USA. These were standardised against international standard human IL-1ra from NIBSC, code number 92/644, as described below.

Other materials and equipment

Streptavidin-HRP (1.25 mg/ml) was obtained from Zymed Laboratories Inc., CA, USA, catalogue number 43-4323. 1,2-phenyldiamine dihydrochloride tablets (orthophenylene-diamine, OPD) were obtained from the Sigma-Aldrich, MO, USA. 96-well ELISA microplates were from the Costar Corporation, MA, USA. PBS Coating buffer (pH 7.2-7.4) contained NaCl 0.14M, KCl 2.70mM, KH_2PO_4 1.50mM, Na_2HPO_4 8.1mM. Wash buffer (pH 7.2-7.4) contained NaCl 0.5M, NaH_2PO_4 2.5mM, Na_2HPO_4 7.5mM, Tween®-20 0.1% v/v. Block buffer contained 0.5% bovine serum albumin (BSA) in wash buffer plus 0.2 mg/l sodium azide. Substrate buffer (pH 5.0) contained citric acid 34.5mM, Na_2HPO_4 66.7mM. Assay dilution buffer contained 0.1% BSA, 0.05% Tween®-20 in tris-buffered saline (pH 7.3) (20mM Trizma base, 150mM NaCl). PBS/0.1% BSA contained 0.1% BSA in phosphate-buffered saline (PBS). Animal sera included horse sera obtained from Gibco Life Technologies, Paisley, UK, catalogue number 16050 and Sigma-Aldrich, Gillingham, UK, catalogue number H1270; mouse serum from Sigma-Aldrich, MO, USA, catalogue number M-5905; sheep serum from Sigma-Aldrich, catalogue number S-2263; and stock foetal calf serum. High performance ELISA (HPE) buffer was from CLM (Amsterdam, The

Netherlands). H_2O_2 (30%), H_2SO_4, NaCl, KCl, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, Tween®-20, sodium azide, citric acid and Trizma base were obtained either from BDH Chemicals Ltd (Poole, UK) or Sigma-Aldrich. Two different ELISA plate readers were used during assay development. The first system was a BioTek Instruments biokinetics microplate reader EL340 (Bio-Tek Instruments, VT, USA) in conjunction with the Kineticalc 4 (KC4) v2.7 computer program (Bio-Tek Instruments). Later assays used a Wallac Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland) in conjunction with the Wallac 1420 software version 2 release 9 computer program (Wallac Oy), and plate data was subsequently analysed using KC4 v2.7. Other equipment used included a Titramax 100 orbital plate shaker (Heidolph Instruments, Schwabach, Germany) and a Jenway 3310 pH meter (Jenway Ltd, Essex, UK).

4.3.3 Data analysis

Raw optical density data from the ELISA plate readers were transferred electronically or manually into the KC4 v2.7 computer program for analysis. Estimates of concentration were computed by interpolation from a standard curve, generated using the four-parameter logistic model within the KC4 program, given by the following equation:

$$y = d + \frac{a-d}{1+(x/c)^b} \qquad \text{(after O'Connell } et\ al.\text{, 1993)}$$

where y is the response (optical density) and x is the analyte concentration. If x approaches 0, then y will be closer and closer to a; hence, a is the lower asymptote. If x approaches infinity, then y approaches d; hence, d is the upper asymptote. If $x = c$, $y = (a + d) / 2$; hence c is the predicted concentration at midlevel response (corresponding to the inflection point of curve). The parameter b relates to the slope of the linear section of the curve. Figure 4.1 shows the typical shape of the curve.

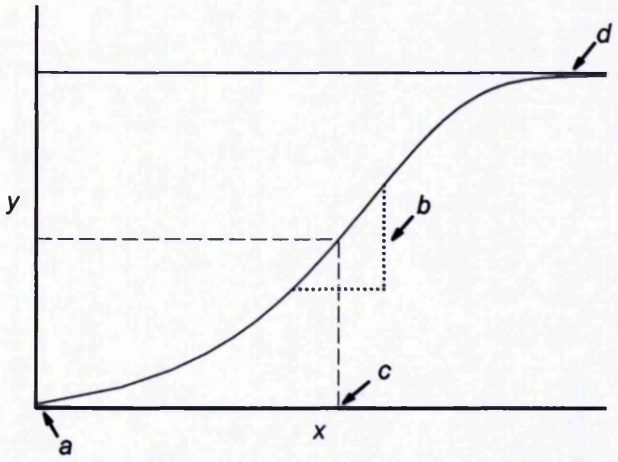


Figure 4.1
The four-parameter logistic model.
a – (theoretical) response at concentration = 0
b – measure of slope at inflection point
c – value of x at inflection point
d – (theoretical) response at concentration = ∞
x – analyte concentration
y – response

Determination of sensitivity and precision

The sensitivity of both assays was established by calculation of the mean optical density at a wavelength of 490 nm (OD_{490nm}) of control (zero standard) replicates plus 2 SD, and interpolation of the corresponding IL-1β or IL-1ra concentration from the standard curve. Overall minimum sensitivity for data presented in chapter 5 was the worst sensitivity from all of the assays used to measure samples. Intra-assay precision was assessed through the determination of percentage coefficient of variation (%CV) of sample replicates. In order to be able to assess inter-assay precision between batches of samples, low, medium and high concentration quality control (QC) samples were produced for both the IL-1β and IL-1ra assays.

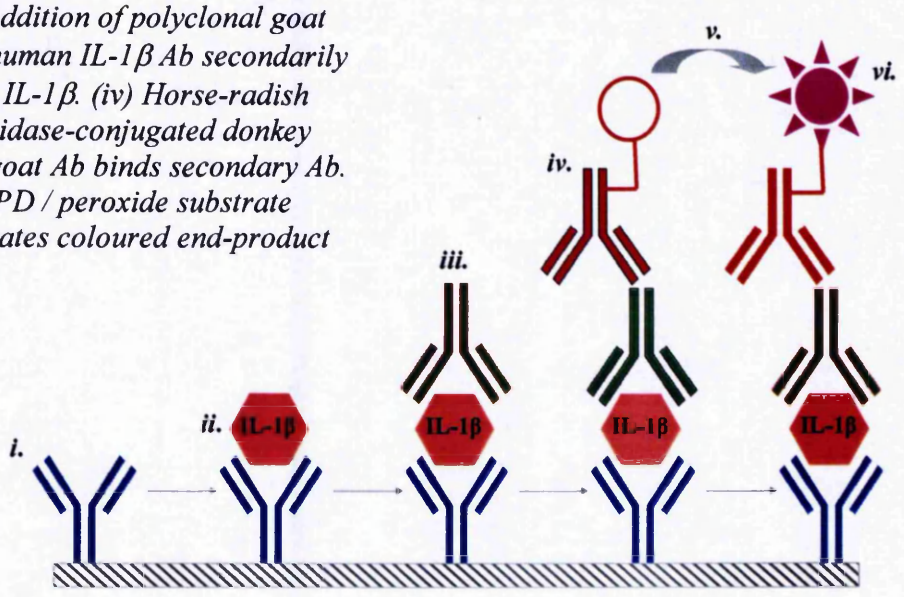
4.3.4 Final ELISA protocols

IL-1β assay procedure

The principle of the final IL-1β assay design is shown in figure 4.2. A 96-well microplate was coated with 100 µl per well of coating antibody (MAB601) diluted to 2.5 µg/ml in PBS coating buffer, covered with a sealer strip and incubated in a humidified chamber overnight at room temperature. Additional binding sites were blocked by incubation with 250 µl blocking buffer per well, at least overnight, in a humidified chamber, at 4°C. Samples were used neat. IL-1β working standard (201-LB, standardised against NIBSC material) was diluted in 50% horse serum in PBS / 0.1% BSA and tested at 80, 40, 20, 10, 5, 2.5 and 1.25 pg/ml to generate a standard curve. 100 µl of samples and standards were added

according to a predetermined plate plan. The plate was covered and incubated overnight at room temperature in a humidified chamber. Secondary antibody (AF201-NA) was diluted to 1 μg/ml and mixed with 1% mouse serum in assay dilution buffer. 100 μl was added per well. The plate was covered and incubated for 2 hours at room temperature in a humidified chamber. Peroxidase-labelled donkey anti-goat immunoglobulin was diluted to 0.2 μg/ml in assay dilution buffer, 100 μl added per well, and the plate was covered and incubated for a further 1 hour. An OPD tablet was dissolved in 12 ml substrate buffer. 5 μl 30% H_2O_2 was added to the substrate solution immediately prior to use, and 100 μl were added per well. The plate was covered, placed in the dark until sufficient colour developed, and the reaction was stopped with 25 μl 2M H_2SO_4 per well. The OD_{490nm} was read using an ELISA plate reader. The plate was washed 3 times in wash buffer between each step. All buffers were allowed to come to room temperature prior to use.

Figure 4.2 *Principle of the IL-1β assay. (i) Anti-human IL-1β monoclonal (coating) antibody (Ab) binds solid phase (ELISA plate). (ii) IL-1β in standard or test plasma binds to coating Ab. (iii) Addition of polyclonal goat anti-human IL-1β Ab secondarily binds IL-1β. (iv) Horse-radish peroxidase-conjugated donkey anti-goat Ab binds secondary Ab. (v) OPD / peroxide substrate generates coloured end-product (vi).*

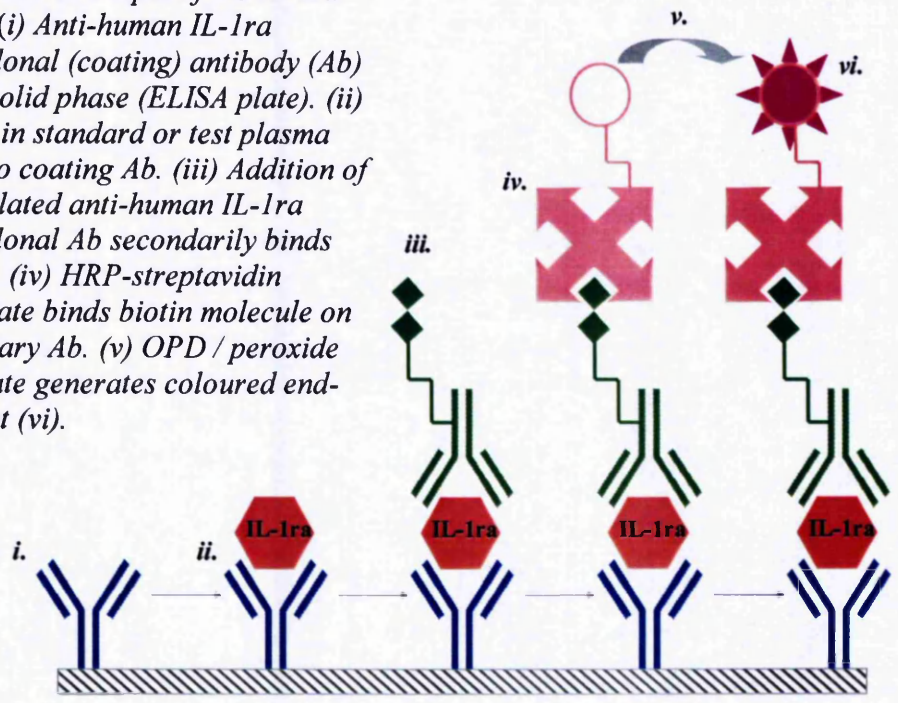


IL-1ra assay procedure

The principle of the final IL-1ra assay design is shown in figure 4.3. A 96-well microplate was coated and blocked using the same procedure as described for the

IL-1β assay. Samples were diluted 1:5 in assay dilution buffer. The working standard r-metHuIL-1ra (100 mg/ml) was diluted to a working concentration of 100 ng/ml by three serial 1:100 dilutions in assay dilution buffer, and then diluted in 20% horse serum in assay dilution buffer and tested at 800, 400, 200, 100, 50, 25 and 12.5 pg/ml to generate a standard curve. 100 µl of samples and standards were added per well according to a predetermined plate plan. The plate was covered and incubated overnight at room temperature in a humidified chamber. Secondary antibody was diluted to 0.4 µg/ml in assay dilution buffer and 100 µl was added per well. The plate was covered and incubated for 2 hours at room temperature in a humidified chamber. Streptavidin was diluted to 0.31 µg/ml in assay dilution buffer and 100 µl was added per well, the plate covered and incubated for a further 30 mins. An OPD tablet was dissolved in 12 ml substrate buffer. 5 µl 30% H_2O_2 was added to the substrate solution immediately prior to use, and 100 µl were added per well. The plate was covered, placed in the dark until sufficient colour developed, and the reaction was stopped with 25 µl 2M H_2SO_4 per well. The OD_{490nm} was read using an ELISA plate reader. The plate was washed 3 times in wash buffer between each step. All buffers were allowed to come to room temperature prior to use.

Figure 4.3 *Principle of the IL-1ra assay. (i) Anti-human IL-1ra monoclonal (coating) antibody (Ab) binds solid phase (ELISA plate). (ii) IL-1ra in standard or test plasma binds to coating Ab. (iii) Addition of biotinylated anti-human IL-1ra monoclonal Ab secondarily binds IL-1ra. (iv) HRP-streptavidin conjugate binds biotin molecule on secondary Ab. (v) OPD / peroxide substrate generates coloured end-product (vi).*



4.4 Results

During the development of assays for both IL-1β and IL-1ra, assay performance, including analyte recovery and calibration, sensitivity, specificity and precision, was assessed. Initial work was performed during the development of ELISA methods for IL-1β and IL-1ra respectively by Ms Karen Illingworth (KI) and Mrs Sylvia Scarth (SS), Injury Research, Hope Hospital, Salford. This consisted of seven IL-1β assays performed by KI testing different standard curve matrices, and eight IL-1ra assays performed by SS testing different coating antibody concentrations, streptavidin concentrations and block buffers. The following results, and the final assay procedures detailed in section 4.3.4, are based on work I carried out.

4.4.1 IL-1β assay optimization

Antibody pair selection

Three different antibody pairings were assessed during assay development (table 4.1). The R&D Systems MAB601-BAF201 and MAB601-AF201NA pairings behaved similarly other than the variation of signal in zero standards. The latter pairing was selected on the grounds that lower levels of background signal were seen in the zero standards at the time the final antibody pairing was selected.

Table 4.1 *Summary of anti-hIL-1β antibody pairings*

		Secondary antibody	
		R&D Systems polyclonal biotinylated goat anti-hIL-1β (BAF201)	*R&D Systems* polyclonal goat anti-hIL-1β (AF201-NA) with peroxidase-conjugated anti-goat immunoglobulin
Coating antibody	*R&D Systems* monoclonal mouse anti-hIL-1β (MAB601)	High signal in zero standards (typically ≥ 0.2) resulting in poor sensitivity	Low background signal (typically ≤ 0.2) and improved sensitivity
	Diaclone monoclonal mouse anti-hIL-1β	Pairing not assessed	Poor signal-to-noise ratio, poor sensitivity

Selection of standard curve matrix

Over a series of assays, various candidate standard curve matrices were compared with human plasma in order achieve a standard curve matrix with assay characteristics approximating those of the human plasma samples as closely as possible (figure 4.4). 50% horse serum diluted in PBS / 0.1% BSA buffer was found to be the optimal standard curve matrix.

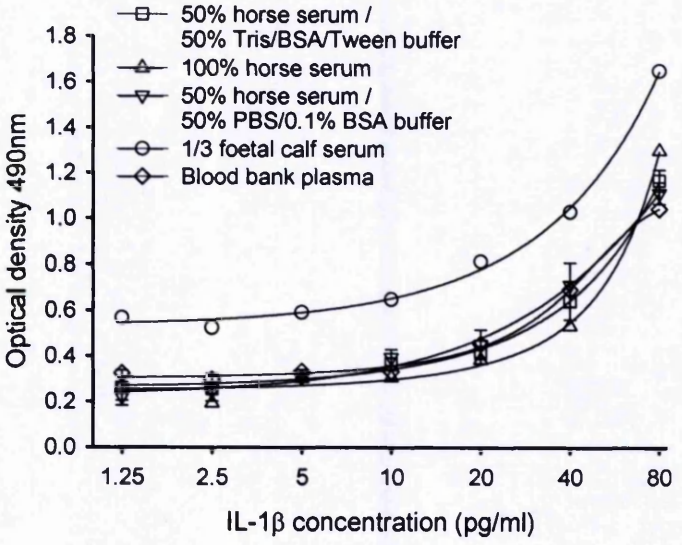


Figure 4.4
Comparison of IL-1β standard curves in a range of candidate matrices. Each point is the mean OD_{490nm} ± SD (bars) for duplicate determinations. Curves have been fitted using a four-parameter logistic curve fit method.

Influence of heterophilic antibodies

A recovery experiment was performed at an early stage during assay development using the R&D Systems MAB601 and BAF201 antibody pairing. In this experiment, plasma samples from seven control subjects had known amounts of exogenous IL-1β added, at three different concentrations. Plasma from one individual (PS) gave very high signal regardless of the concentration of exogenous IL-1β (mean ± SD OD_{490nm} without exogenous IL-1β of 1.23 ± 0.08), suggesting that this was a false positive result. It was hypothesized that this may have been due to the presence of heterophilic human anti-mouse antibodies in view of the assay employing a monoclonal mouse antibody. In order to test this hypothesis, the experiment was repeated, incorporating the pre-incubation of various concentrations of mouse serum with plasma samples from this individual. Following this modification, the measured concentrations of IL-1β approached expected concentrations (see figure 4.5).

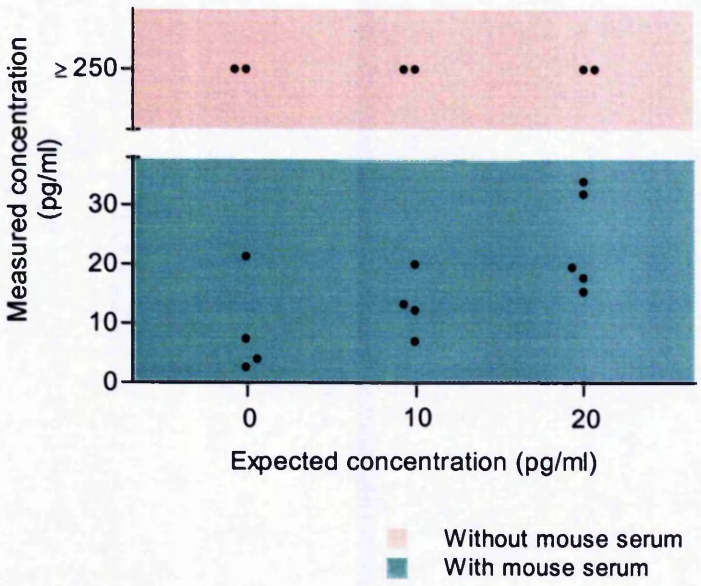


Figure 4.5
Vertical dot plot showing measured against expected concentration of IL-1β in subject PS with spikes of known IL-1β concentration in the presence and absence of mouse serum.

A range of concentrations of mouse serum (0.3%, 1%, 3% and 10%) were used during this exercise. The background signal (mean OD_{490nm} ± SD) seen with each of these concentrations was reduced to 0.11 ± 0.00, 0.10 ± 0.02, 0.14 ± 0.03 and 0.11 ± 0.1, therefore achieving a mean reduction in signal of 91%, 92%, 89% and 91% respectively. It was concluded that 1% mouse serum was a suitable concentration to use. Excessive signal was seen again in plasma from further individuals and it was therefore decided to overcome this problem by making the addition of 1% mouse serum an extra step in the assay. In view of the additional time that would have been required to add 1% mouse serum to every plasma sample in all subsequent assays, a further experiment was performed in which 1% mouse serum was mixed with the secondary antibody solution prior to its addition to the ELISA plate. This modification was similarly effective in reducing the false signal, and did not impair assay performance, with sensitivity being maintained in the low picomole per millilitre range.

Quality control samples

These are described in greater detail below for IL-1ra. A similar procedure was followed for IL-1β, with the preparation of low, medium and high concentration quality control samples (QCs) covering the range 15 – 50 pg/ml, although these QC samples also contained pre-determined concentrations of various other

cytokine and soluble cytokine receptors so that these QCs could be prepared in larger batches ready for use in a wider range of assays.

4.4.2 *IL-1ra assay optimization*

Antibody pair selection

A series of assays was carried out in order to identify the optimal coating and secondary antibody combination (table 4.2). The Biosource coating and secondary antibody combination was established as the most appropriate pairing. Next, various coating antibody concentrations were assessed. A coating antibody concentration of 1 μg/ml was found to produce uniformly low signal across the standard curve, whilst coating antibody concentration of 2.5 μg/ml and 5 μg/ml were found to achieve comparable, adequate standard curves. 2.5 μg/ml was selected as the optimal concentration for subsequent assays.

Table 4.2 *Summary of anti-hIL-1ra antibody pairings*

		Secondary antibody			
		R&D systems biotinylated goat anti-hIL-1ra	*Serotec* biotinylated mouse monoclonal anti-hIL-1ra	Polyclonal sheep anti-hIL-1ra Ig / peroxidase conjugated anti-sheep Ig	*Biosource* biotinylated mouse monoclonal anti-hIL-1ra
Coating antibody	*R&D systems* monoclonal mouse anti-hIL-1ra	Suppression of signal in human plasma ($OD_{490nm} \leq 0.4$)	Globally excess signal ($OD_{490nm} \geq 1.6$), including zero standards	Very high levels of background signal ($OD_{490nm} \geq 2.0$)	Pairing not assessed
	Serotec monoclonal mouse anti-hIL-1ra	Globally low signal ($OD_{490nm} \leq 0.4$) in buffer and human plasma	Globally low signal ($OD_{490nm} \leq 0.5$) in buffer and human plasma	Pairing not assessed	Pairing not assessed
	Biosource monoclonal mouse anti-hIL-1ra	Poor signal-to-noise ratio in human plasma	Pairing not assessed	Pairing not assessed	Optimal sensitivity; signal not suppressed in human plasma

Selection of standard curve matrix

As for the IL-1β assay, various candidate standard curve matrices were compared with human plasma. Over a series of assays, standard curves in human blood bank plasma were compared with those obtained in various concentrations of foetal calf serum, sheep serum, horse serum, HPE buffer, and assay dilution buffer. One of the main difficulties during this process was the apparent suppression of signal in human plasma, particularly when used neat, causing a reduction in the signal-to-noise ratio (OD of top standard / OD of zero standard) to values in the range 1.5 – 2.5, compared with values of 3.0 – 5.5 seen in other matrices. Dilution achieved a considerable improvement in signal-to-noise ratio and enabled a suitable standard curve to be achieved in 20% horse serum diluted in assay dilution buffer, exhibiting parallelism with that in 20% human blood bank plasma (figure 4.6).

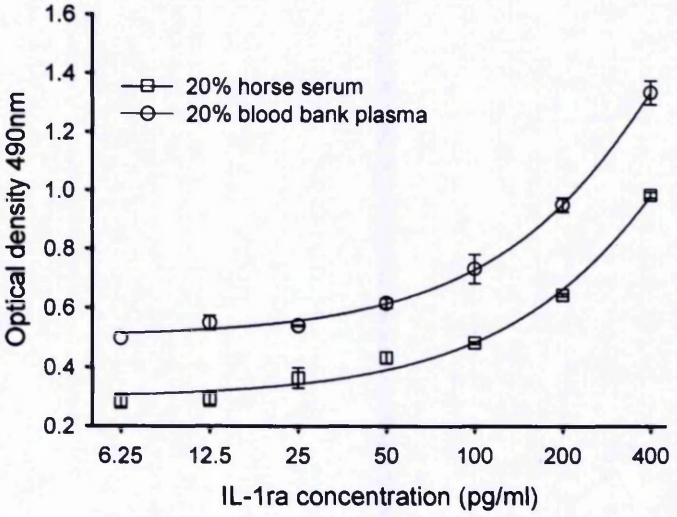


Figure 4.6
Comparison of IL-1ra standard curves in 20% horse serum and 20% blood bank plasma. Each point is the mean OD_{490nm} ± SD (bars) for duplicate determinations.

Quality control samples

In order to select an appropriate batch of blood bank human plasma (obtained from Hope Hospital, Salford) containing a low concentration of endogenous hIL-1ra, the concentration of endogenous hIL-1ra was determined in a sample of each of three different batches of blood bank human plasma (A, B and C). When diluted 1:5, plasma A was found to contain 85 pg/ml (425 pg/ml neat), B 36 pg/ml (180 pg/ml neat) and C 76 pg/ml (380 pg/ml neat). Batch B was therefore chosen as the plasma with which the QC samples would be produced. IL-1ra

concentrations were selected for low (blood bank plasma B only), medium and high QC samples so that when diluted 1:5 they would be appropriately distributed across the range of the assay. Three concentrations of r-metHuIL-1ra (0 pg/ml, 500 pg/ml and 2000 pg/ml) were added to each of three 3 ml aliquots of *neat* blood bank plasma B, and 75 µl aliquots of each QC sample were prepared. Prior to using these QC samples for the assessment of inter-assay precision, the concentration of IL-1ra in each was verified in a further assay (table 4.3). Although the measured concentrations of IL-1ra differed from the expected concentrations, the three QC samples were distributed appropriately across the range of the assay (figure 4.7).

Table 4.3 *Expected and measured IL-1ra concentrations in the QC samples*

	Expected [IL-1ra] (pg/ml)		Measured [IL-1ra] (pg/ml) ± SD	
QC sample	1:5 dilution	Neat	1:5 dilution	Neat (estimated)
Low	36	180	41 ± 14	205 ± 70
Medium	136	680	186 ± 20	930 ± 100
High	436	2180	671 ± 41	3355 ± 205

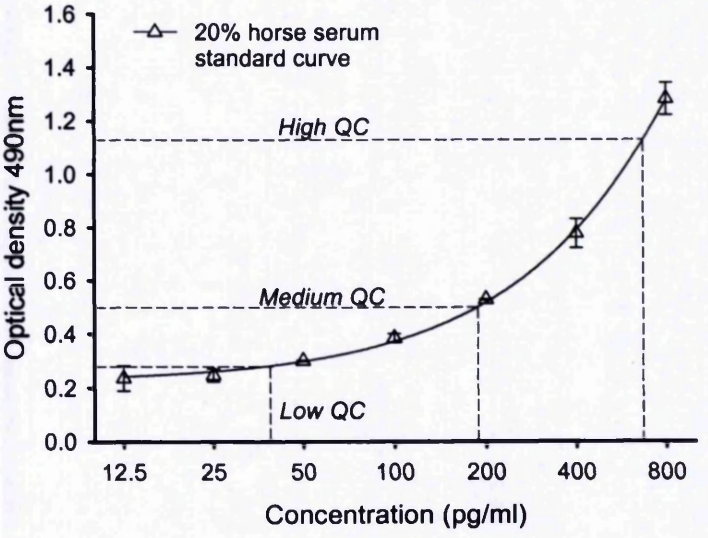


Figure 4.7 *[IL-1ra] in the low, medium and high QC samples (diluted 1:5) are interpolated against a standard curve in 20% horse serum. Each point is the mean OD_{490nm} ± SD (bars) for duplicate determinations. The curve has been fitted using a four-parameter logistic curve fit method.*

4.4.3 IL-1β and IL-1ra assay optimization: common points

Incubation times and temperatures were determined empirically over the course of multiple assays, with decisions being guided by problems such as high levels of background signal. It was found that an overnight incubation at room temperature was optimal for coating, and preferable to incubation overnight at

4°C. Although a block buffer incubation of 1 hour (at room temperature) was used initially, a longer (at least overnight) incubation at 4°C was later found to be preferable, since a lower mean ± SD OD_{490nm} of 0.09 ± 0.02 for the zero standards was achieved in one experiment using an overnight block buffer incubation step, compared with typical zero standard ODs in earlier experiments up to 0.4. Once blocked, ELISA plates were stored in a humidified chamber at 4°C for up to 2 months with no apparent loss of assay performance attributable to this change in protocol. Relatively short incubation times of 2 hours (at room temperature) were also used initially for samples and standards, but again, overnight incubation (at room temperature) was found to improve assay performance. Two different types of coating buffer were tried in the assay: PBS coating buffer and carbonate-bicarbonate buffer. No material difference in assay performance was seen between the two types of coating buffer, hence PBS coating buffer continued to be used.

4.4.4 IL-1β assay performance

Analyte recovery

In order to evaluate the accuracy of the assay, a recovery experiment was undertaken by the addition of known amounts of exogenous IL-1β at three different concentrations to plasma samples from five control subjects (0 pg/ml, 10 pg/ml and 20 pg/ml). The expected increase in concentration in each individual's spiked plasma sample was taken as the measured increase in concentration in spiked standard curve matrix (50% horse serum). Percentage recovery was calculated as follows:

$$\%\ recovery = \left(\frac{measured\ increase\ in\ concentration\ in\ plasma}{measured\ increase\ in\ conc.\ in\ 50\%\ horse\ serum} \right) x\ 100\%$$

The mean (± SD) percentage recovery for the 10 pg/ml spike was 67% (± 11%), and for the 20 pg/ml spike, 77% (± 12%).

Assay calibration

Assay calibration was performed between the working standard R&D hIL-1β and international standard NIBSC IL-1β material in three separate assays. Figure 4.8 shows an example.

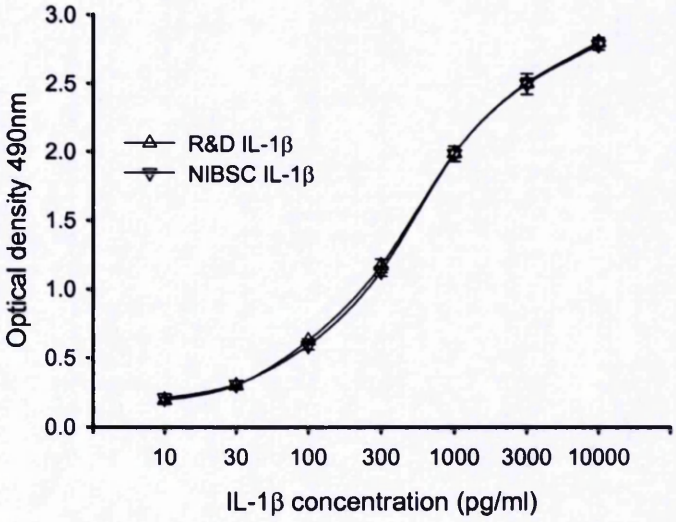


Figure 4.8
IL-1β calibration assay: R&D IL-1β versus international standard. Each point is the mean OD_{490nm} ± SD (bars) for duplicate determinations.

When using 50% Gibco horse serum as the assay matrix, a mean ratio of R&D IL-1β : NIBSC IL-1β values was calculated, based on standard curve points on the linear portion of the standard curve, and was found to be 121% (± 10%).

Specificity

IL-1β assay specificity was demonstrated by the lack of response to IL-1α or IL-1ra at any concentration ranging from 30 pg/ml to 3 ng/ml.

Sensitivity

During assay development, sensitivity varied considerably. The greatest variation in sensitivity (from <1 pg/ml to >20 pg/ml) occurred during the early assays before the final assay format was decided upon. Preliminary assays using the final format achieved sensitivities which were consistently lower, in the range 3 pg/ml to 9 pg/ml.

Stroke study sensitivity and precision data

The final IL-1β assay procedure was used in order to quantify plasma IL-1β concentration in serial samples from patients and control subjects in the study reported in chapter 5.

Twenty-nine batches of samples were run in total in order to achieve an overall minimum assay sensitivity of 7.9 pg/ml across the twelve batches from which data were accepted. Data from assays with a sensitivity that exceeded this value were discarded, and those samples re-assayed. In these accepted assays, mean ± SD %CV for the fourth standard curve dilution point at a mean ± SD concentration 9.9 ± 0.6 pg/ml was 36 ± 24%. Samples with replicate %CVs exceeding 50% were re-assayed, unless the mean interpolated concentration for the sample was less than the overall minimum assay sensitivity. Inter-assay precision between batches of samples was assessed through the determination of %CVs for QC samples. Inter-assay %CVs were 15% at 83.5 pg/ml, 24% at 63.9 pg/ml and 26% at 51.5 pg/ml.

4.4.5 IL-1ra assay performance

Analyte recovery

A recovery experiment was undertaken as described above for the IL-1β assay. Known amounts of exogenous IL-1ra were added at three different concentrations (0 pg/ml, 50 pg/ml and 100 pg/ml) to plasma samples from four control subjects. As for IL-1β, the expected increase in concentration in each individual's spiked plasma sample was taken as the measured increase in spiked standard curve matrix (20% horse serum). The mean (± SD) percentage recovery for the plasma sample with 50 pg/ml of exogenous IL-1ra was 97% (± 28%), and for the sample with 100 pg/ml, 107% (± 15%).

Assay calibration

Assay calibration was performed between the working standard Amgen r-metHuIL-1ra and international standard NIBSC IL-1ra material in three separate assays. Figure 4.9 shows an example. In 20% horse serum, a mean ratio of r-metHuIL-1ra : NIBSC IL-1ra values was calculated, based on standard curve

points on the linear portion of the standard curve, and this was found to be 132% (± 4.8%).

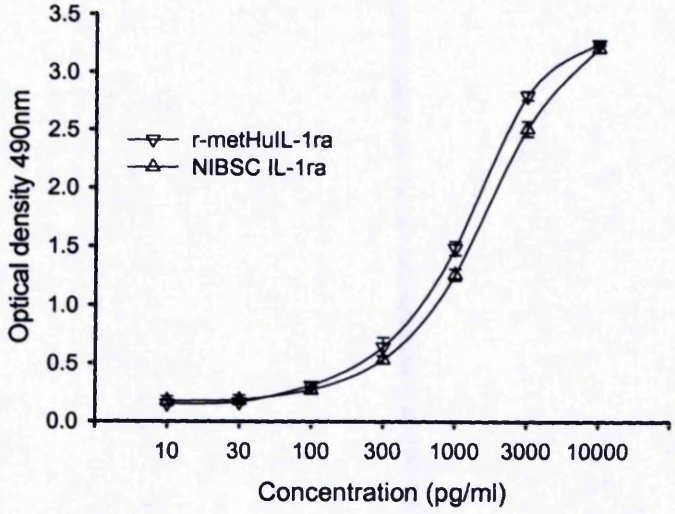


Figure 4.9
IL-1ra calibration assay: r-metHuIL-1ra versus international standard. Each point is the mean OD_{490nm} ± SD (bars) for duplicate determinations. Curves have been fitted using a four-parameter logistic curve fit method.

Specificity

IL-1ra assay specificity was demonstrated by the lack of response to IL-1α or IL-1β at any concentration ranging from 30 pg/ml to 3 ng/ml.

Sensitivity

During assay development, sensitivity varied considerably. The greatest variation in sensitivity (from <10 pg/ml to >300 pg/ml) occurred during the early assays before the final assay format was decided upon. Preliminary assays using the final format achieved sensitivities which were consistently lower, in the range 6 pg/ml to 100 pg/ml.

Stroke study sensitivity and precision data

The final IL-1ra assay procedure was used in order to quantify plasma IL-1ra concentration in serial samples from patients and control subjects in the study reported in chapter 5. Twenty-nine batches of samples were run in total in order to achieve an overall minimum assay sensitivity of 93 pg/ml across the sixteen batches from which data were accepted. Data from assays with a sensitivity that exceeded this value were discarded, and those samples re-assayed. In these accepted assays, mean ± SD %CV for the fourth standard curve dilution point at a mean ± SD concentration 108 ± 14 pg/ml was 13 ± 13%. Samples with

replicate %CV exceeding 50% were re-assayed, unless the mean interpolated concentration for the sample was less than the overall minimum assay sensitivity. Inter-assay precision between batches of samples was assessed through the determination of %CVs for QC samples. Inter-assay %CVs were 11% at 496 pg/ml and 19% at 136 pg/ml. Data for the low QC sample were disregarded as these values consistently fell below the assay sensitivity when running these batches.

4.5 *Discussion*

Immunoassay is the preferred method of cytokine assay, and the ELISA format is the most widely available, either as commercial kits or individual reagents and published protocols. One of the most important characteristics of a well-performing, stable assay is the selection of an appropriate antibody pair and a suitable antigen, and care was taken in this selection process in order to optimize the present assays. Previous experience of assay design in the laboratory dictated the choice of other reagents to some extent, although clearly there was an element of trial and error until appropriate combinations and concentrations were achieved.

Opinions vary over the determination of assay sensitivity. However, the most common practical approach is to assay multiple replicates of the control sample and define the limit of analytical sensitivity as being the concentration interpolated from the standard curve at an OD 2 or 3 standard deviations above the mean control OD (Davies, 2001b). In the present work, assay sensitivity was established by calculation of the mean OD_{490nm} of zero standard replicates plus 2 SD, and interpolation of the corresponding IL-1β or IL-1ra concentration from the standard curve. Values obtained at the limit of sensitivity are not always reliable because the precision at this concentration may be poor. A further potential problem with this method is that where the sensitivity lies below the concentration of the lowest standard, the interpolated value is based on a fitted standard curve assumed to closely reflect the true dose-response curve below the lowest standard, which may not always be the case. Functional sensitivity, a

concept introduced to overcome some of these problems, is defined as the lowest concentration in the assay for which the CV is less than an arbitrary percentage, eg 20%. Functional sensitivity is derived from knowledge of changes in precision over the assay range, or precision profile (Davies, 2001b).

Assay specificity refers to the ability of an antibody to produce a measurable response only for the analyte of interest. During the present work, the absence of cross-reactivity with other members of the IL-1 family was demonstrated, ie by showing a lack of antibody response to IL-1ra or IL-1α in the case of the IL-1β assay, and IL-1α or IL-1β in the case of the IL-1ra assay. However, this test does not excluded the possibility of cross-reactivity with other potential analytes. Reliance was therefore placed on the quality of the antibodies supplied.

Estimating concentration by interpolation requires the fitting of a model based on reference data points (x, y), then using the model to estimate an unknown x based on a new measured response, y. In the present context, the reference data points are provided by the standard curve. Various models may be used in this situation, but several have inherent limitations. For example, polynomial equations may be used for curve-fitting, but they can be unstable at the extreme boundaries of the fit. The four-parameter logistic function has been used here, and is widely used for ELISAs because it is a flexible model for data following a sigmoidal curve (O'Connell *et al.*, 1993).

Careful attention was paid to the selection of an appropriate standard matrix. As already discussed, a standard curve is required in order to assign values to the unknown samples. Ideally, such a standard curve should be prepared in a base matrix that is identical to that in the test samples, so that there should be parallelism between the sample and standard titration curves. However, in order to determine assay sensitivity, zero standards or control samples are required and by definition, such a zero standard should not contain the analyte in question. Normal human plasma is unsuited to this role as it may contain this particular analyte. Alternative matrices include buffer solutions containing protein or animal sera. The matrix chosen should behave in a similar way to the sample

matrix and be consistent from assay to assay, and should reflect any non-analyte constituents of patient samples that have a background effect in the assay. Various candidate standard curve matrices were assessed during the development of the present assays in order to achieve these goals. 50% horse serum and 20% horse serum were identified as the most suitable matrices for the IL-1β and IL-1ra assays respectively.

Recovery experiments allow an assessment to be made of the accuracy of an assay. In addition to being a test of whether an assay is correctly calibrated, recovery also assesses the influence of differences between sample and standard matrices. There are, however, several potential sources of error during a recovery experiment including inter-individual matrix variation, the base matrix (standard or sample) concentration, and the preparation, addition and concentration of exogenous analyte (Davies, 2001b). Care was taken with technique during the recovery experiment in order to minimize these potential errors as far as possible. Individual plasma samples were used rather than a relatively homogenous plasma pool. Sample-to-sample matrix differences, such as the presence or absence of heterophilic antibodies, can also be elicited in this way, as described earlier. Ideally, exogenous analyte concentrations should span the likely range of the assay in its given context, but because this was unknown during assay development, the choice of such concentrations was by necessity arbitrary. Recovery approximated 100% for the IL-1ra assay, although fell somewhat short of this in the case of the IL-1β assay. Recovery was lowest at the lowest IL-1β concentration, suggesting reduced accuracy at the lower end of the working range of the IL-1β assay.

There are several potential reasons for falsely high signal (or falsely low signal) in ELISAs, including heterophilic antibodies, non-specific binding, or interference from other materials in the plasma such as immunoglobulins (Boscato and Stuart, 1988), albumin (Place and Schroder, 1982) or other unidentified substances (Nemzek *et al.*, 1999). Endogenous heterophilic antibodies have been widely reported in many human plasma samples (Boscato and Stuart, 1988; Levinson, 1997). Such antibodies interfere with sandwich

ELISAs by bridging coating and secondary antibodies in the absence of analyte, thereby generating false positive signal. Examples of interfering antibodies are human anti-mouse antibodies (Kricka *et al.*, 1990) and several other types of heterophilic antibodies including multivalent human autoantibodies such as rheumatoid factors. During the present work, extremely high signal was obtained with plasma from one particular individual in the IL-1β recovery experiment. Excessive signal was seen irrespective of the concentration of exogenous IL-1β, and therefore this was presumed to be a false positive result. This problem was successfully overcome by the inclusion of mouse serum at the secondary antibody stage. This change in protocol was instituted permanently, including its application in the final IL-1β assay protocol.

Limitations of ELISAs also include the fact that the measured concentrations of cytokine protein (ie levels of immunoreactivity) may over-estimate or under-estimate the levels of bioactivity. ELISAs may utilize anti-cytokine antibodies that do not discriminate between mature forms of cytokine proteins and other forms lacking bioactivity, such as precursor forms, inactive fragments / degradation products, or cytokine protein bound in an inactive form to other proteins, such as soluble cytokine receptors (eg sIL-1-RII).

Precision is an estimate of the error in an analytical technique, expressed as the percentage coefficient of variation (%CV) at a particular analyte concentration. It is possible for an assay to be precise without necessarily being accurate. In the current assays, potential sources of variation which might contribute to error, or imprecision, include antibody characteristics, signal generation and detection, incubation temperatures, edge effects (due to temperature differentials across the assay plate), pipetting errors and timing of assay steps. Wherever possible, potential sources of systematic error were removed, for example through regular pipette calibration checks in the laboratory. Further attempts to minimize error included the preparation of standard curves from two independent dilution steps rather than as one series of serial dilutions, and the careful positioning of samples and standards on the assay plate such that no standard curve replicates occurred in the same row or column and to ensure the spatial separation of sample

replicates. The latter may result in apparently worse figures for precision, but is a more valid test within the assay. Intra-assay precision was determined from replicate %CVs. Inter-assay precision was assessed through the calculation of %CVs of quality control samples. These were prepared in-house from pools of samples rather than being commercially sourced for the sake of rigour, and were shown to contain concentrations of analyte distributed appropriately across the range of the assay.

Calibration experiments were conducted for both the IL-1β and IL-1ra assays in order to ensure comparability with internationally recognized and validated standards. For both IL-1β and IL-1ra, parallel dose-response curves were achieved when working standards were compared with international (NIBSC) standards, showing that the assays were detecting the same molecular species. The displacement of the two curves in each case represented the difference in antigen content, and a mean ratio of working : international standard was calculated from points on the linear portions of these curves so that final cytokine concentrations could be calibrated against the relevant international standard.

In conclusion, this chapter describes the development and evaluation of ELISAs for IL-1β and IL-1ra in human plasma. The various problems encountered during their development were overcome using a systematic approach, and particular attention has been paid to accuracy, precision, sensitivity, specificity and calibration, ensuring that the performance of these methods is adequate for their intended applications.

CHAPTER 5

Plasma cytokines, soluble receptors and in vitro cytokine production in acute ischaemic stroke

5.1 Introduction

Accumulating evidence supports a role for inflammation in the development of, and responses to, cerebral ischaemia, as discussed in chapter 1. Cytokines such as IL-1, TNF-α and IL-6 appear to be crucial mediators of the local and systemic inflammatory responses to cerebral ischaemia, yet much remains unknown about their complex interactions in the setting of clinical stroke. Extensive evidence supports roles for both IL-1 and TNF in acute experimental ischaemic CNS injury (for reviews see Rothwell, 2003; Hallenbeck, 2002), but a role for cytokines and soluble cytokine receptors in patients with acute stroke has yet to be clearly identified.

Intrathecal production of IL-1β may occur in stroke patients (Tarkowski *et al.*, 1995), although no rise in circulating IL-1β concentrations has been found (Fassbender *et al.*, 1994a; Tarkowski *et al.*, 1995; Grau *et al.*, 2001b). Increased IL-1β mRNA expression in peripheral mononuclear cells has been reported in acute stroke, and it correlated moderately with the degree of neurological impairment (Kostulas *et al.*, 1999), but peripheral IL-1β protein production appears to be no different from controls (Grau *et al.*, 2001b). Plasma concentrations of IL-1ra have been found to be elevated in patients recruited at a delay of 4 ± 2 days from acute ischaemic stroke, decline gradually, but remain elevated compared to healthy controls for up to 1 year (Beamer *et al.*, 1995, 1998). TNF-α has been shown to be upregulated in post-mortem brain tissue (Tomimoto *et al.*, 1996, Sairanen *et al.*, 2001), and CSF TNF-α concentrations were elevated in patients with acute ischaemic stroke (Vila *et al.*, 2000a; Zaremba *et al.*, 2001). Elevated circulating concentrations of TNF-α have also been found in acute ischaemic stroke patients (Intiso *et al.*, 1997; Carlstedt *et al.*, 1997; Vila *et al.*, 2000a; Zaremba *et al.*, 2001), with one exception where no rise was seen (Fassbender *et al.*, 1994a). There is a consensus that circulating IL-6 concentrations are increased in acute stroke patients (Fassbender *et al.*, 1994a; Beamer *et al.*, 1995; Tarkowski *et al.*, 1995; Kim *et al.*, 1996; Castellanos *et al.*, 2002; Emsley *et al*, 2003; Acalovschi *et al.*, 2003). Circulating IL-6 correlates with CT infarct volume (Fassbender *et al.*, 1994a; Vila *et al.*, 2000a; Perini *et al.*,

2001; Smith *et al.*, 2004), and clinical outcome (Fassbender *et al.*, 1994a; Clark *et al.*, 1998; Perini *et al.*, 2001), although not all studies support the relationship between IL-6 and outcome (Tarkowski *et al.*, 1995; Ferrarese *et al.*, 1999). LPS has been found to increase the release of IL-6 and TNF-α, but not of IL-1β, from stimulated peripheral whole blood from acute stroke patients to a greater degree than from controls (Ferrarese *et al.*, 1999; Grau *et al.*, 2001b). Low concentrations of circulating IL-10, relative to controls, have been reported in acute ischaemic stroke (Perini *et al.*, 2001), and this was associated with neurological worsening (Vila *et al.*, 2003).

Chapter 3 reported on a prospective study to evaluate the relationship between inflammation and stroke, in ischaemic stroke patients recruited early (within 12 h) after onset of symptoms and sampled at time points up to 1 year and control subjects individually matched for age, sex and degree of atherosclerosis (Emsley *et al.*, 2003; Smith *et al.*, 2004). This has shown some important relationships between various more established markers (CRP, IL-6 and leucocyte counts) and outcome. Here I report data for some of the more speculative mediators and markers, including a study of the capacity of peripheral blood to produce cytokines. Data concerning the relationships with radiological and clinical outcome are to be reported elsewhere (CJ Smith, MD thesis to be submitted; Emsley *et al.*, manuscript in preparation).

5.2 Materials and Methods

5.2.1 Patients, control subjects, radiology and assessment of atherosclerosis

The study was approved by the LREC and methodology for clinical evaluation was as described previously (chapter 3; Emsley *et al.*, 2003). Briefly, patients over 18 years of age presenting at Hope Hospital, Salford, within 12 hours of onset of symptoms of acute stroke, were eligible where either written informed consent or assent from a relative was available. Control subjects with no history of stroke or TIA or clinically evident infection necessitating medical treatment were matched for age (± 5 years), sex and degree of atherosclerosis. Assessment

of patients included history and clinical examination, blood pressure (BP), pulse and aural temperature (Braun Thermoscan LF20 digital thermometer) at all time points, NIHSS score (Lyden *et al.*, 1994) (on admission, at 5 to 7 days, 3 months and 12 months), and OCSP classification (Bamford *et al.*, 1991). The occurrence of infections or other events likely to provoke a marked inflammatory response in the 6 weeks prior to each assessment was recorded using available clinical, radiological and laboratory data.

CT brain scans were performed on an IGE CT Pace Plus 3rd generation CT scanner within 24 h of admission, in order to exclude patients with primary intracerebral haemorrhage or stroke mimic from further analysis. ABPI was used to classify degree of atherosclerosis where carotid duplex data were unavailable. Patients and control subjects were divided into two groups, one without significant atherosclerosis (< 50% carotid artery stenosis bilaterally, or lowest ABPI ≥ 0.92), and one with significant atherosclerosis ($\geq$ 50% carotid artery stenosis on at least one side, or lowest ABPI < 0.92).

5.2.2 Blood and urine samples

Venous blood samples were taken from patients at baseline, the next 09:00 time point where admission was before 07:00 or after 11:00, 24 hours after admission, plus 5 to 7 days, 3 months and 12 months at 09:00. Blood was also drawn from resting control subjects at 09:00 and at a time matched to the patient's time of admission if this was before 07:00 or after 11:00. Blood was collected into tubes containing pyrogen-free heparin, and wrapped in cool-gel packs. At 1 hour after collection, blood was centrifuged at 2000g for 30 minutes at 4°C. Plasma was separated, frozen and stored at -70°C until analysis. Urine was collected at the same time points into foil-wrapped sterile containers, wrapped in cool-gel packs, frozen and stored at -70°C until analysis.

5.2.3 Whole-blood stimulation

2 ml blood was mixed with 2 ml Roswell Park Memorial Institute (RPMI) medium and a further 2 ml blood was mixed with 2 ml RPMI containing LPS from *E.Coli* serotype 0128:B12 (Sigma, Gillingham, UK) at 200 ng/ml. Three x

1 ml volumes of each mixture were transferred to wells in a sterile microtitre plate. This plate was incubated for 24 hours at 37°C in 5% CO_2. The contents of each well were transferred into microcentrifuge tubes and centrifuged at 1200 rpm for 10 mins and the supernatant separated, frozen and stored at -70°C until analysis. Cytokine production has been corrected for monocyte count and expressed as ng/ml per million monocytes.

5.2.4 Enzyme-linked immunosorbent assays

Plasma (or supernatant) IL-1β, IL-1ra, sIL-1-RII, TNF-α, sTNF-RI, sTNF-RII, IL-10, IL-6 and leptin were measured using sandwich ELISAs. Analytes were captured on 96-well microplates (Costar, Cambridge, MA) with analyte-specific monoclonal antibodies (sIL-1-RII: cat no. MAB663; R&D Systems, Abingdon, UK, TNF-α: supplied by S. Poole, NIBSC, sTNF-RI: cat no. MAB625; R&D Systems, sTNF-RII: cat no. 58.177.08; Biosource, Nivelles, Belgium, leptin: cat no. MAB398; R&D Systems) and secondarily bound with biotinylated antibodies (sIL-1-RII: cat no. BAF263; R&D Systems, TNF-α: supplied by S. Poole, NIBSC, sTNF-RI: cat no. BAF225; R&D Systems, sTNF-RII: cat no. 58.177.02; Biosource, leptin: cat no. BAM398; R&D Systems) followed by streptavidin-HRP (cat no. 43-4323; Zymed Laboratories Inc., CA, USA) and developed with orthophenylene-diamine (Sigma, Poole, UK). Working standards (sIL-1-RII: cat no. 263-2R; R&D Systems, TNF-α: supplied by S. Poole, NIBSC, sTNF-RI: cat no. 225-B1; R&D Systems, sTNF-RII: cat no. 58.177.10; Biosource, leptin: cat no. 398-LP; R&D Systems) were calibrated against international standards where available as described in chapters 3 and 4. Minimum sensitivities were 86 pg/ml (sIL-1-RII), 7 pg/ml (TNF-α), 20 pg/ml (sTNF-RI), 100 pg/ml (sTNF-RII), < 50 pg/ml (leptin). Inter-assay coefficients of variation, determined in the appropriate working range, were 12% at 1.2 ng/ml, 12% at 750 pg/ml and 20% at 145 pg/ml (sIL-1-RII), 11% at 42 pg/ml, 13% at 13 pg/ml and 23% at 10 pg/ml (TNF-α), 7% at 200 pg/ml, 11% at 90 pg/ml and 36% at 23 pg/ml (sTNF-RI), 7% at 850 pg/ml and 9% at 300 pg/ml (sTNF-RII), 22% at 67.5 ng/ml, 24% at 26.3 ng/ml and 28% at 5.4 ng/ml (leptin). Reagents for the TNF-α assay were provided by S. Poole (NIBSC). The methods for IL-1β, IL-1ra and IL-6 were as described in chapters 3 and 4, and those for IL-10 are described elsewhere (V

Wickramasinghe, MSc thesis). Cytokine production data were corrected for monocyte count.

5.2.5 High performance liquid chromatography

The urinary neopterin/creatinine ratio was determined by reverse-phase high performance liquid chromatography (HPLC) at ambient temperature using a 4.6 x 100 mm analytical column packed with 3 μm particles of Waters Spherisorb ODS2, preceded by 4.6 x 10 mm guard cartridge packed with a generic C18 material. Elution was with a gradient of 15 mmol/l potassium phosphate buffer, pH 6.4, at 0.8 ml/min for 6 min followed by the addition of 6% acetonitrile at 0.8 ml/min for a further 3 minutes. Neopterin was measured fluorimetrically (excitation 353 nm, emission 438 nm) and the ratio was calculated relative to creatinine determined by ultraviolet absorbance at 235 nm. Urine samples were diluted 1/10 in 15 mmol/l potassium phosphate buffer, pH 6.4, containing 0.2% disodium EDTA prior to injection of 20 μl on to the column. Urinary neopterin was related to urinary creatinine to account for physiological variation in urine volumes. Inter-assay coefficients of variation for the urinary neopterin/creatinine ratio were 11.5% at 259, 23.4% at 178 and 19.8% at 101.

5.2.6 Statistical analysis

The pre-specified, primary outcome measures were peak plasma IL-1ra and maximum IL-1β production during the first week after ischaemic stroke versus CT cerebral infarct volume at 5 to 7 days and mRS at 3 months. Maximum or minimum values during the first week were analysed only for those patients surviving to the 5-7 day assessment. Post-hoc evaluation of the data resulted in a change in the IL-1β parameter to minimum production because of the observed trough rather than a peak. Secondary analyses were plasma IL-1ra, IL-1β, sIL-1-RII, TNF-α, sTNF-RI, sTNF-RII, IL-10, leptin and urinary neopterin, and IL-1β, IL-6 and TNF-α production (a) at all time points compared with controls, (b) peak cytokine in the first week versus CT infarct volume at 5 to 7 days and clinical outcome at 3 and 12 months, and (c) versus presence of infection. Log-transformed cytokine, cytokine receptor, leptin, urinary neopterin, and cytokine production data were compared using paired *t*-tests. Correlations were assessed

using the Spearman Rank correlation coefficient. Mann-Whitney U or Wilcoxon signed ranks tests were used where other comparisons were made of non-parametric data. The sample size of 36 patient-control subject pairs had 80% power at the 5% significance level to detect a difference of 0.67 standard deviation (SD) in primary analyses between control subjects and patients. All calculations were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL).

5.3 Results

Forty-three patients were recruited between April 2000 and January 2001. Six patients with primary intracerebral haemorrhage and one patient for whom no matched control could be found, were excluded. The thirty-six remaining patients included in the analysis were recruited at a median interval of 4.75 h (range 1.5 h to 11.75 h) from onset of symptoms of acute stroke. Fourteen patients died by 12 months: certified causes of death were index stroke (8), recurrent stroke (1), pulmonary embolism (1), left ventricular failure secondary to myocardial infarction (1) and sepsis (3). Infections and other events associated with an inflammatory response occurring in the 6-week period prior to each assessment were as described previously (chapter 3).

5.3.1 Plasma cytokines and soluble receptors

Plasma cytokine and soluble receptor concentrations are shown in table 5.1. The median plasma IL-1ra concentration was significantly elevated at admission, and remained elevated until 5-7 days (fig 5.1, table 5.1). Plasma IL-1β and sIL-1-RII concentrations were not significantly different from controls at any time point (fig 5.1, table 5.1). Plasma sTNF-RI concentration was significantly elevated at 5-7 days in patients without evidence of infection (fig 5.1, table 5.1). Plasma TNF-α and sTNF-RII concentrations followed a similar pattern and peak plasma TNF-α, sTNF-RI and sTNF-RII concentrations all showed strong correlation with each other (TNF-α – sTNF-RI, $r = 0.64$, $p < 0.001$; TNF-α – sTNF-RII, $r = 0.77$, $p < 0.001$; sTNF-RI – sTNF-RII, $r = 0.83$, $p < 0.001$), but TNF-α and sTNF-RII did not differ significantly from controls at any time point (fig 5.1,

table 5.1). Considerable variability in circulating concentrations was seen between individual patients (fig 5.2).

As mentioned earlier, data pertaining to outcome are to be reported elsewhere (CJ Smith, MD thesis to be submitted; Emsley *et al.*, manuscript in preparation), but in summary, peak IL-1ra concentration in the first week showed modest correlation with infarct volume ($r = 0.45$, $p = 0.018$), and admission NIHSS score ($r = 0.51$, $p = 0.003$), but did not correlate significantly with any other measures of outcome. Peak plasma sTNF-RI concentration in the first week correlated strongly with infarct volume ($r = 0.62$, $p = 0.001$), mRS at 3 months ($r = 0.59$, $p < 0.001$) and 1 year ($r = 0.57$, $p = 0.001$), and BI at 3 months ($r = -0.58$, $p = 0.001$) and 1 year ($r = -0.61$, $p < 0.001$).

Table 5.1 *Circulating plasma cytokine and cytokine receptor concentrations*

	Control subjects		Patients				
	09:00	Admission matched†	Admission	24 hours	5-7 days	3 months	12 months
	n = 36	*n* = 36	*n* = 36	*n* = 35	*n* = 33	*n* = 24	*n* = 18
IL-1β (pg/ml)	6.5 (6.5, 97.5)	6.5 (6.5, 97.5)	6.5 (6.5, 69.5)	6.5 (6.5, 74.1)	6.5 (6.5, 65.9)	6.5 (6.5, 65.4)‡	6.5 (6.5, 61.7)
sIL-1-RII (pg/ml)	6.6 (4.4, 20.9)	6.5 (4.4, 15.9)	7.4 (5.1, 15.7)	7.3 (4.2, 16.7)	6.7 (4.1, 17.8)	7.2 (3.4, 10.6)‡	6.4 (4.5, 10.6)
IL-1ra (pg/ml)	352 (352, 886)	352 (352, 666)	415** (352, 1375)	390 (352, 3280)‡	424 (352, 1518)	352 (352, 833)‡	352 (352, 738)
TNF-α (pg/ml)	15.5 (8.9, 52.3)	14.1 (5.7, 38.2)	15.9 (4.0, 39.6)	16.6 (6.0, 47.1)	18.6 (9.2, 43.6)	18.3 (7.1, 59.0)‡	18.0 (10.8, 52.2)
sTNF-RI (pg/ml)	1.3 (0.7, 3.5)	1.3 (0.7, 3.3)	1.2 (0.6, 39.8)	1.3 (0.8, 44.5)	1.5 (0.9, 25.9)	1.4 (1.0, 40.4)‡	1.3 (0.8, 4.2)
sTNF-RII (pg/ml)	4.8 (2.4, 11.5)	4.5 (2.0, 15.4)	4.3 (1.5, 68.7)	4.3 (2.1, 69.3)	5.3 (3.2, 52.7)	5.7 (2.6, 75.6)‡	4.9 (2.6, 12.7)
IL-10 (pg/ml)	0.6 (0.6, 38.0)	0.6 (0.6, 45.3)	0.6 (0.6, 106.9)	0.6 (0.6, 83.4)	0.7 (0.6, 96.0)	0.6 (0.6, 48.1)	0.6 (0.6, 45.5)

**$p \leq 0.001$ (relative to controls) *n* = number of observations
data shown as median (min, max)
†admission matched data includes 09:00 values if admission was between 07:00 and 11:00
‡ 1 missing case

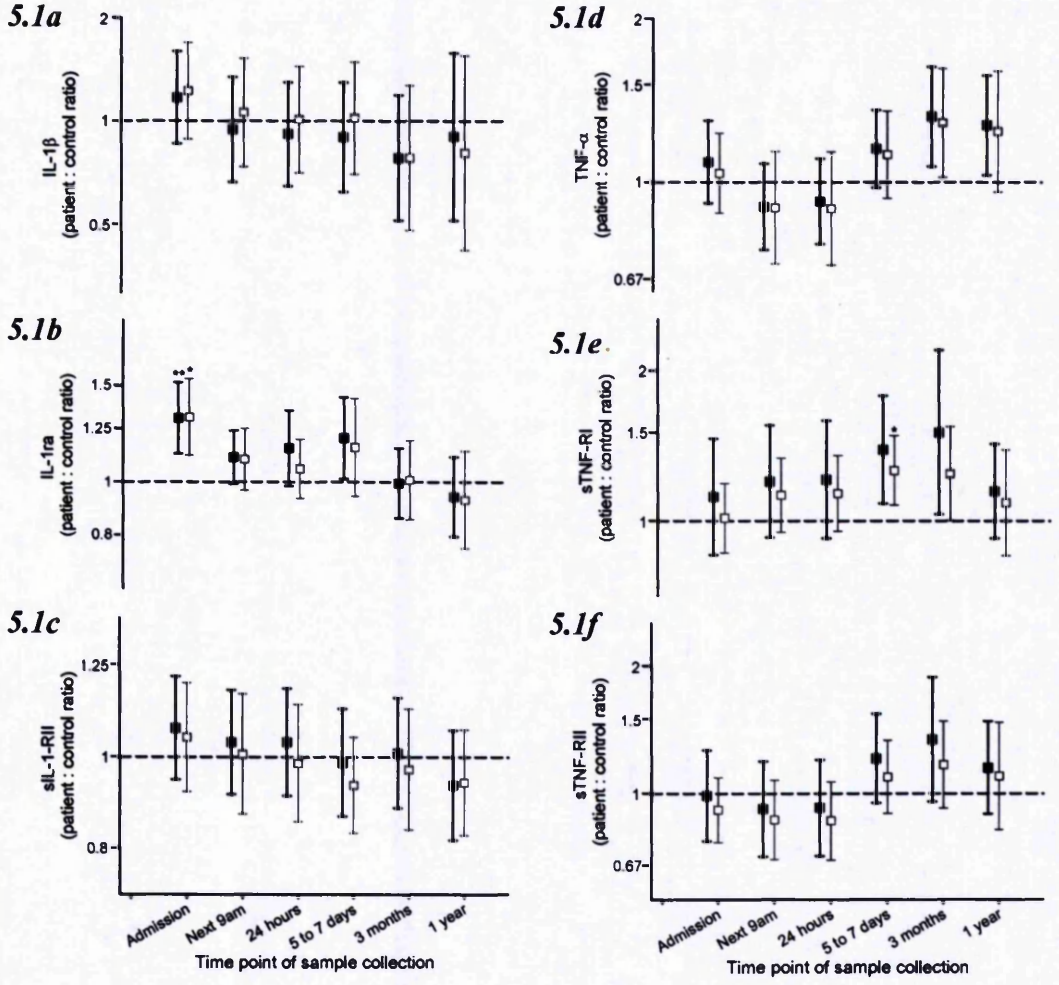


Figure 5.1a-f *Plasma cytokine kinetics in all patients and patients without infection (in the 6 weeks preceding assessment). Patient values are expressed as multiples (ratios) of their corresponding controls (with 95% CIs) to account for skewed distributions. The 24h time point includes "next 9am" data where admission was between 07:00 and 11:00. *p ≤0.01 **p ≤0.001 (relative to controls)*

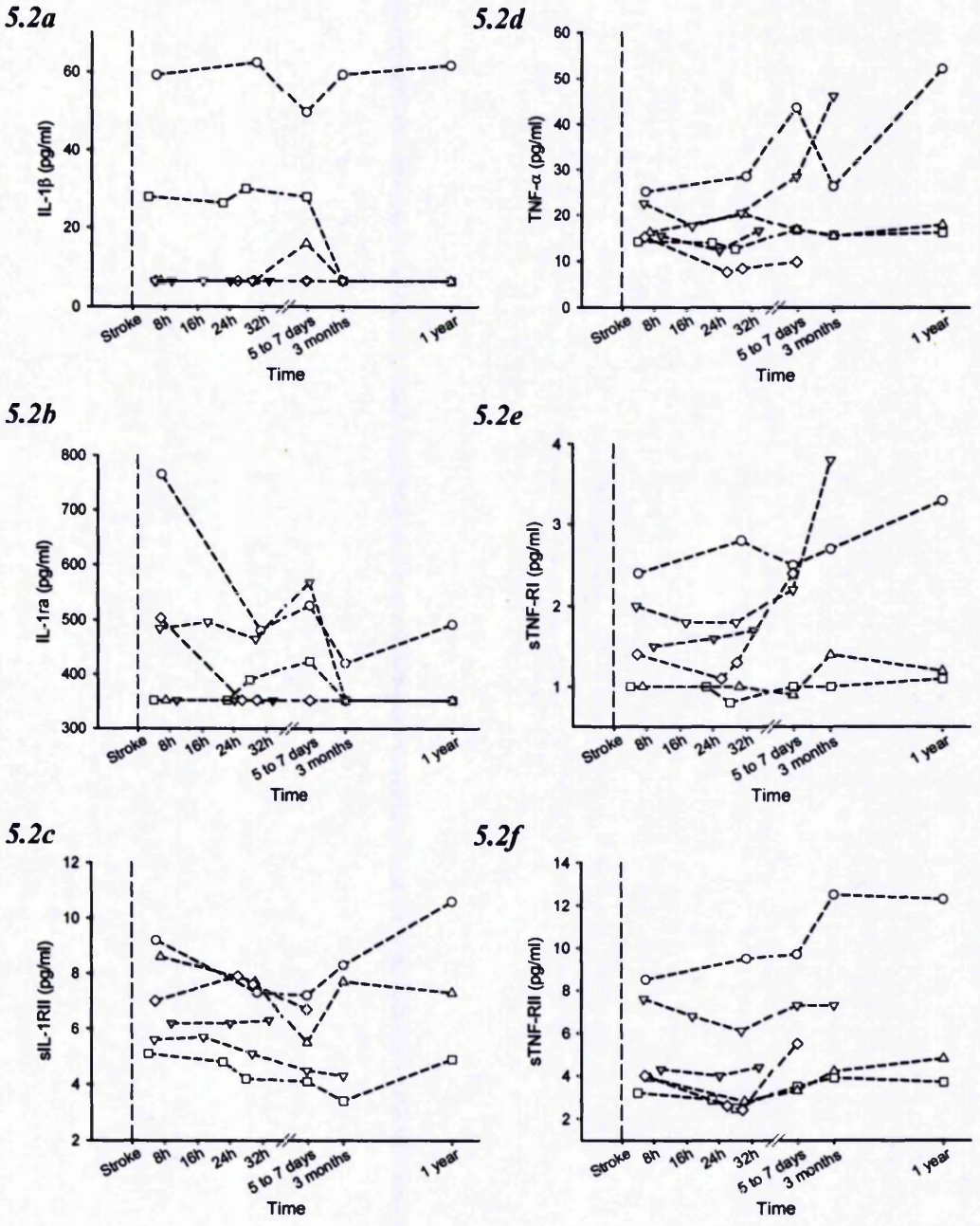


Figure 5.2a-f *Variability in cytokine and cytokine receptor concentrations between individual patients. Every sixth patient included in the analysis is shown, for illustrative purposes only. The same six patients as for figure 3.4 are shown.*

Admission NIHSS score (OCSP classification) for each patient are shown to the right. Infections / other events associated with an inflammatory response occurring in these patients are detailed in chapter 3.

□ 12 (LACS)
○ 16 (PACS)
△ 12 (PACS)
▽ 22 (TACS)
◇ 15 (TACS)
▽ 9 (PACS)

5.3.2 Peripheral blood cytokine production

Monocyte count-corrected *in vitro* IL-1β and TNF-α production was significantly reduced relative to controls between admission and 5-7 days (fig 5.3, table 5.2). Monocyte count-corrected *in vitro* IL-6 production was also significantly reduced on admission, at the next 09:00 time-point and at 5-7 days. A similar pattern was seen for corrected *in vitro* IL-1β and TNF-α production in patients without evidence of infection, although the reduction was not significant at 24 h, whilst IL-6 production was significantly reduced only on admission. Considerable inter-individual variability was observed when cytokine production was expressed either as a total amount or after correction for monocyte count (figure 5.4).

Plasma cortisol concentration (data presented in chapter 3) was strongly inversely correlated with corrected cytokine production between admission and 5-7 days (table 5.3). No correlations were seen at 3 months, 1 year, or in control subjects.

Whole-blood samples incubated with RPMI medium in the absence of lipopolysaccharide exhibited absent or very little (low pg/ml concentrations) *in vitro* IL-1β, IL-6 or TNF-α production (data not shown).

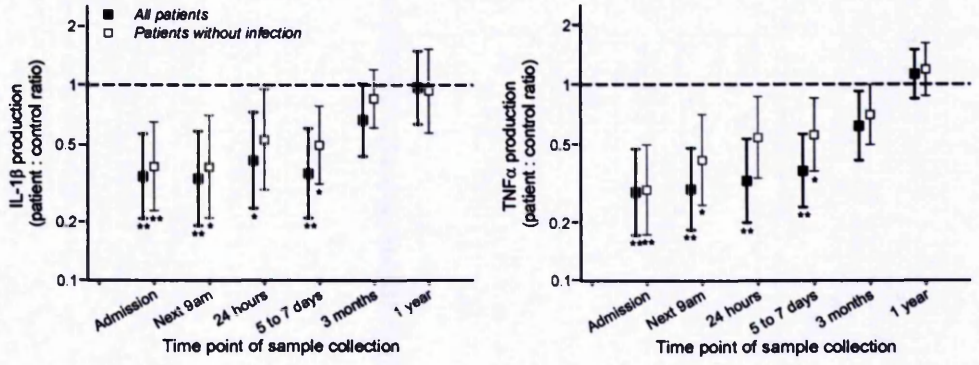
Detailed outcome data are not reported here, but in summary, minimum *in vitro* IL-1β and IL-6 production in the first week correlated strongly with admission NIHSS score and mRS and BI scores at 3 and 12 months. Weaker correlations were also seen between minimum *in vitro* TNF-α production and clinical outcome. Minimum *in vitro* cytokine production was significantly lower in non-survivors than in survivors at 12 months (IL-1β, $p \leq 0.001$; IL-6, $p \leq 0.001$; TNF-α, $p = 0.005$).

Table 5.2 *In vitro cytokine production following whole-blood stimulation*

	Control subjects		Patients				
	09:00	Admission matched†	Admission	24 hours	5-7 days	3 months	12 months
	n = 36	*n* = 36	*n* = 36	*n* = 35	*n* = 33	*n* = 24	*n* = 18
LPS-stimulated IL-1β (ng / million monocytes)	25.1 (7.3, 66.5)	26.1 (7.3, 66.5)	11.8** (0.5, 131.6)‡	14.3* (0.1, 53.3)§	13.3** (0.11, 53.9)‡	20.5 (1.43, 59.2)‡	22.1 (9.0, 127.8)§
LPS-stimulated IL-6 (ng / million monocytes)	190 (130, 359)	191 (123, 359)‡	138* (28, 632)‡	166 (13, 437)§	158** (23, 315)‡	203 (85, 349)‡	203 (105, 541)§
LPS-stimulated TNF-α (ng / million monocytes)	9.22 (1.57, 35.65)	9.80 (1.51, 35.65)‡	2.49** (0.11, 30.32)‡	2.62** (0.12, 18.60)‖	3.46** (0.29, 20.31)‡	5.25 (0.64, 35.30)‡	9.89 (3.14, 33.81)§

*p ≤ 0.01 **p ≤ 0.001 (relative to control subjects) *n* = number of observations
data shown as median (min, max)
†admission matched data includes 09:00 values if admission was between 07:00 and 11:00
‡ 1 missing case; § 2 missing cases; ‖ 3 missing cases

5.3a ***5.3b***



5.3c

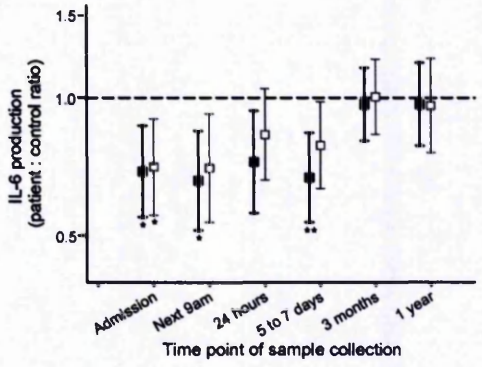


Figure 5.3a-c *LPS-stimulated whole-blood cytokine production kinetics in all patients and patients without infection (in the 6 weeks preceding assessment). Patient values are expressed as multiples (ratios) of their corresponding control subjects (with 95% CIs) to account for skewed distributions. The 24h time point includes "next 9am" data where admission was between 07:00 and 11:00.*
**p ≤0.01 **p ≤0.001*
(relative to controls)

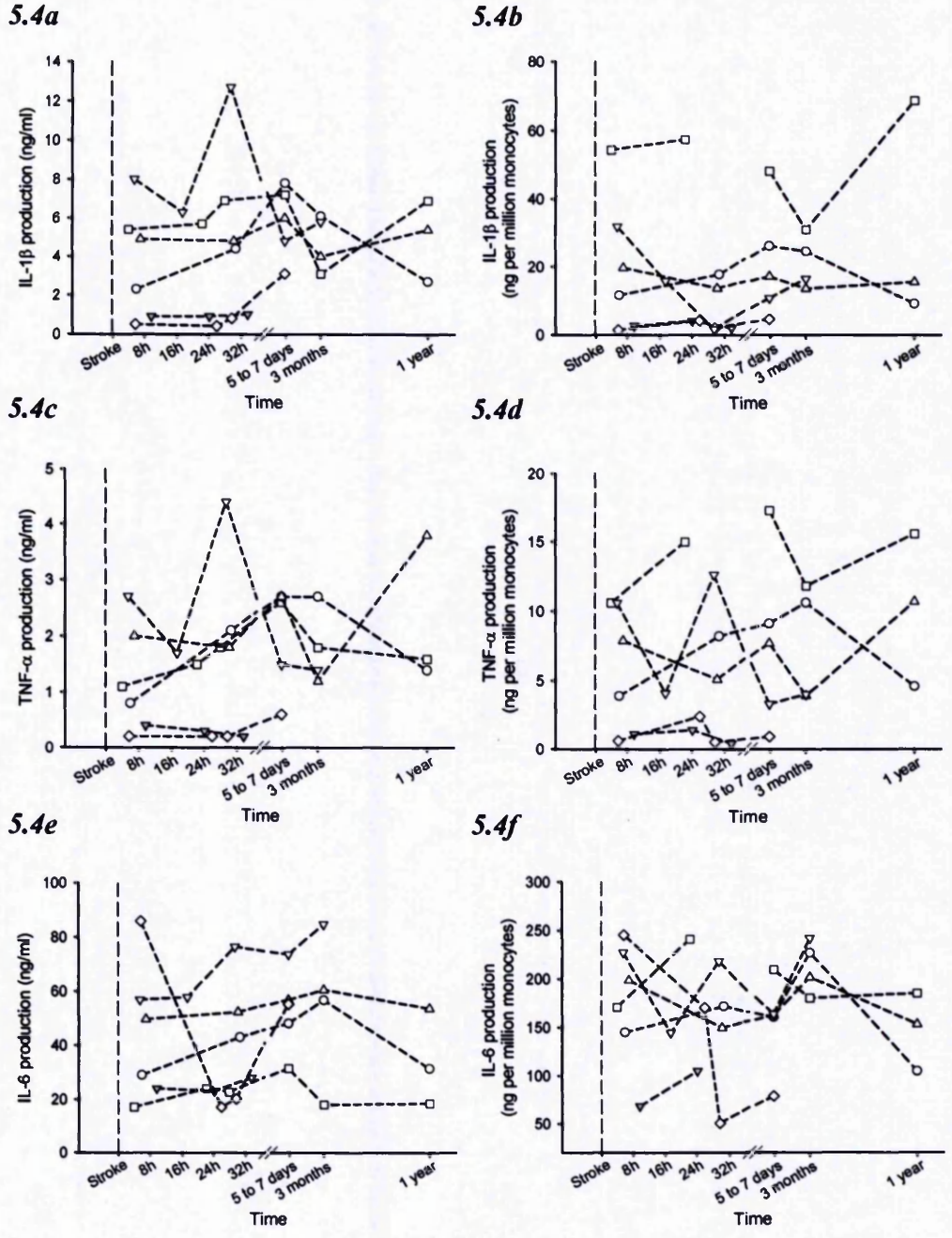


Figure 5.4a-f *Variability in LPS-stimulated whole blood cytokine production between individual patients. Every sixth patient included in the analysis is shown, for illustrative purposes only. IL-1β, TNF-α and IL-6 production (ng/ml) are shown in 5.4a, c and e; IL-1β, TNF-α and IL-6 production (ng per million monocytes) are shown in 5.4 b, d and f.*

For admission NIHSS score, OCSP classification, and data concerning infections / other events associated with an inflammatory response, refer to the legend for figure 5.2. The same six patients are shown here.

Table 5.3 *Correlation matrix of plasma cortisol concentration versus in vitro cytokine production*

	Control subjects		Patients				
	09:00	Admission matched†	Admission	24 hours	5-7 days	3 months	12 months
IL-1β production	-0.11	0.18	-0.57**	-0.65**	-0.58**	-0.05	-0.35
IL-6 production	-0.04	-0.02	-0.59**	-0.64**	-0.48*	0.13	-0.09
TNF-α production	-0.33	-0.06	-0.72**	-0.67**	-0.68**	-0.10	0.17

data shown are Spearman Rank correlation coefficients
*$p \leq 0.01$ **$p \leq 0.001$
†admission matched data includes 09:00 values if admission was between 07:00 and 11:00

5.3.3 Plasma leptin and urinary neopterin

Urinary neopterin was significantly raised in patients at 5-7 days (median 354 μmol / mol creatinine) compared to controls (median 254 μmol / mol creatinine) ($p = 0.002$), but no significant difference from controls was seen at any other time point. A similar elevation occurred at 5-7 days in patients with evidence of infection excluded. Peak urinary neopterin correlated significantly with peak TNF-α concentration ($r = 0.6$, $p < 0.001$). Plasma leptin concentration showed no significant difference from controls at any time point.

5.3.4 Atherosclerosis

Seventeen patients and control subjects were classified as having significant atherosclerosis. A significantly higher urinary neopterin concentration was seen in control subjects with significant atherosclerosis (median 309 μmol / mol creatinine) than those without (median 244 μmol / mol creatinine) ($p < 0.01$). IL-10 concentration was lower in control subjects with atherosclerosis (median 0.6 pg/ml) than those without (1.0 pg/ml) ($p = 0.02$). No differences in other plasma cytokines, leptin, soluble receptors or *in vitro* cytokine production were seen between those individuals (including patients and controls) with or without significant atherosclerosis at any time-point.

5.4 Discussion

This study provides further evidence of the modification of plasma concentrations of cytokines, soluble receptors and *in vitro* cytokine production in patients recruited early after ischaemic stroke and sampled serially up to 1 year. These data provide new insights into the importance of cytokine pathways in the pathophysiology of stroke, and in addition demonstrate for the first time, to the best of my knowledge, that potential for cytokine induction strongly correlates with stroke severity and clinical outcome.

Particular strengths of the present study are the very early recruitment of patients after onset of symptoms of acute stroke (median < 5 h), and further assessments within the first 24 h of admission. In studies where later sampling time points have been used it is difficult to distinguish the effects of the stroke itself from complicating infections or other events. However, I recognise that cytokine responses seen at 3 months and 1 year reflect the fact that patients with the most severe strokes were amongst the non-survivors at the later time points (data not shown). This study also took account of potential diurnal variation in inflammatory markers by collecting blood samples at 09:00 wherever possible (Perdiz *et al.*, 1996). Control subjects were also individually matched for degree of atherosclerosis in addition to age and sex because all of these variables are known to influence peripheral inflammatory markers including IL-6 and neopterin (Erren *et al.*, 1999). The rationale for carotid artery stenosis and ABPI thresholds for 'significant atherosclerosis' used in the present study was guided by the available evidence, as discussed previously in chapter 3. The sample size is reported here is relatively small, but this potential criticism may be offset, to an extent, by the care taken to control for other factors as outlined above.

The present data demonstrate variation between concentrations of various plasma cytokines and soluble receptors. The elevation in plasma IL-1ra concentration on admission in patients with and without evidence of infection, at a much earlier stage after stroke onset than previously reported (Beamer *et al.*, 1995), suggests early activation of anti-inflammatory components of the cytokine system. The

absence of significant changes in plasma IL-1β or sIL-1-RII concentrations is in agreement with other work (Fassbender *et al.*, 1994a; Tarkowski *et al.*, 1995). Leptin was studied here because it may mediate neuroimmune responses dependent on IL-1 (Luheshi *et al.*, 1999). Although high leptin levels were recently found to be associated with stroke (Söderberg *et al.*, 2003), the present data do not support a clear change in leptin concentration following stroke.

A significant elevation was observed in sTNF-RI concentration at 5-7 days but no significant changes were seen in TNF-α or sTNF-RII. Interestingly, sTNF-RI has a significant association with clinical cardiovascular disease (Cesari *et al.*, 2003). A more robust relationship with stroke severity and outcome may reflect a longer half-life compared to cytokines themselves. These findings with respect to TNF-α are consistent with one study (Fassbender *et al.*, 1994a), but contrast with others (Intiso *et al.*, 1997; Carlstedt *et al.*, 1997; Vila *et al.*, 2000a; Zaremba *et al.*, 2001), where data suggest elevations of plasma and cerebrospinal fluid TNF-α concentration may occur after stroke and correlate with cerebral infarct volume. The fact that elevated serum sTNF-RI and sTNF-RII concentrations have been found in carotid atherosclerosis (Elkind *et al.*, 2002) may explain why the present data differ from those who used healthy controls. However, a trend was seen in TNF-α, sTNF-RI and sTNF-RII towards elevation at 5-7 days and 3 months compared to controls and, given the strong correlations between their peak concentrations, their release appears to have been proportionate to one another. The 5-7 day elevation in sTNF-RI is consistent with earlier observations of elevated sTNF-RI concentration in stroke patients with nosocomial infection (Fassbender *et al.*, 1997).

Although IL-10 concentrations did not differ significantly from controls, a trend towards lower values was noted in control subjects with significant atherosclerosis compared to those without, which may suggest that IL-10 is downregulated in both atherosclerosis and following stroke, and certainly this interpretation would be consistent with previous work (Perini *et al.*, 2001). In this respect, data suggesting that IL-10 deficiency results in increased

atherosclerosis, thrombosis and increased LDL cholesterol in apolipoprotein E-deficient mice are interesting (Caligiuri *et al.*, 2003).

Neopterin in plasma or urine is a sensitive marker of monocyte and macrophage activation and has previously been shown to be raised in the circulation of individuals with atherosclerosis (Tatzber *et al.*, 1991). Plasma neopterin concentration has been reported to be increased at 1 year follow-up after cerebral ischaemia (Anwaar *et al.*, 1999), and during the first week after stroke compared to age- and sex-matched controls (Grau *et al.*, 2001c). In the present study urinary neopterin was indeed raised in controls with significant atherosclerosis compared to those without. Furthermore, in the present patient population relative to atherosclerosis-matched controls, urinary neopterin was increased at 5-7 days, perhaps reflecting greater peripheral macrophage activation at this time following stroke in excess of activation associated with atherosclerosis alone.

Measurement and interpretation of cytokines and other factors can be difficult in plasma (Hopkins, 1995) and I have taken care to avoid many of the potential pitfalls with these assays. As with most other clinical studies, I have focused on plasma cytokine and soluble receptor concentrations both because of the relative ease of obtaining blood, and also partly because in patients with severe stroke, lumbar puncture adds risk of cerebral herniation, and it is thus difficult to obtain cerebrospinal fluid, especially at repeated time points. However, I cannot be certain whether cytokines in peripheral blood from patients with acute stroke are derived from cerebral sources or from the periphery. The available data suggest that there is probably a combination of both CNS (Tarkowski *et al.*, 1995; Zaremba *et al.*, 2001; Sairanen *et al.*, 2001) and peripheral sources (Fassbender *et al.*, 1994a; Beamer *et al.*, 1995, 1998; Ferrarese *et al.*, 1999; Perini *et al.*, 2001; Vila *et al.*, 2000a).

Increasingly, *in vitro* cytokine production is used as a measure of individuals' potential for pro- and/or anti-inflammatory cytokines to be induced. Cytokine production has been corrected for monocyte count in the present work because despite the suggestion that neutrophils may produce modest amounts of cytokines, monocytes are the principal source of cytokines in blood (Xing and

atherosclerosis, thrombosis and increased LDL cholesterol in apolipoprotein E-deficient mice are interesting (Caligiuri *et al.*, 2003).

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Remick, 2003). I have demonstrated a significant reduction in *in vitro* cytokine production following stroke, relative to controls. Detailed clinical outcome data are to be reported separately, but significant inverse correlations were seen between IL-1, IL-6 and TNF-α production and worse mRS and BI scores at 3 months and 1 year, as well as increased mortality in patients with lower levels of IL-1β, IL-6 and TNF-α production. These data differ from previous reports of increased IL-6 and TNF-α release from LPS stimulated whole blood after acute stroke (Ferrarese *et al.*, 1999; Grau *et al.*, 2001b). The apparently conflicting data might have various explanations, including cytokine production kinetics being dependent on the duration of LPS-exposure, since the latter studies incubated samples for only 4 h, or perhaps the duration of exposure to cortisol (see below). Alternatively, it may again reflect differences between healthy and atherosclerosis-matched control subjects. The lack of whole-blood activation in the absence of LPS-stimulation in the present study (and the virtual absence of peripheral blood cytokine mRNA, data not shown) suggests that plasma cytokines are not being produced by peripheral blood cells, but reflect inflammation in the brain or peripheral tissues, whilst peripheral blood reflects the intrinsic capacity to respond to a stimulus such as LPS.

Stroke-induced anti-inflammatory feedback may represent a possible explanation for reduced cytokine production. An extensive early cortisol elevation was noted in these patients (chapter 3). Elevated cortisol concentrations were therefore present in the whole-blood cultures from the samples collected early after the stroke. Interestingly, strong inverse correlations were seen between plasma cortisol concentration and LPS-stimulated cytokine production. These inverse correlations were maintained until 5-7 days, but were not present at 3 months or 1 year, nor was any similar correlation present in the control subjects. No similar correlations were seen between plasma cortisol concentrations and plasma cytokine concentration. This apparent effect of elevated cortisol on cytokine production post-stroke is, to my knowledge, a new observation.

It is unclear from the present data why individuals with lower cytokine inducibility, whether pre-determined prior to the stroke or consequent upon the

stroke, appear to have poorer outcome and higher mortality. Inter-individual differences in cytokine production *in vitro* may be genetically controlled by polymorphic variants in cytokine genes (Warlé *et al.*, 2003). It might also be possible that larger lesion size is associated with lower cytokine production and therefore poorer outcome, or lower cytokine production confers less protection against infections (recognised clinically or not) which contribute to poor outcome and/or death. Cytokines do play a key role in host defence against infection, and it has been suggested that an innate anti- or pro-inflammatory cytokine profile may influence outcome from infectious disease (Westendorp *et al.*, 1997). Inevitably the observations relating to cortisol raise the controversial question of corticosteroids as a potential therapy for stroke. Whether they confer benefit or harm is not clearly established (Davis and Donnan, 2004), but given their potential for side-effects such as immunosuppression and infection, any further impairment of cytokine production which might occur in the presence of exogenous corticosteroids may be disadvantageous.

Conventional vascular risk factors such as hypertension are also known to influence peripheral inflammatory markers and may increase LPS-induced production of cytokines in the experimental setting (Hallenbeck *et al.*, 1991). Previously recognised hypertension was more prevalent in the present patient population compared to controls, and this may have influenced the results reported here, although given the above observation might be expected to augment rather than reduce LPS-stimulated cytokine production. Statins, antiplatelet agents and β-blockers were more prevalent amongst the present patient population, and these drugs might have inhibited some cytokine responses (and thus tend to lessen any differences seen between patients and control subjects). Such environmental, as well as genetic factors, may account in part for the variation in inflammatory response and differences in risk of development of cerebral ischaemia between individuals.

In conclusion, the present data provide new insights into the importance of cytokine pathways in the pathophysiology of ischaemic stroke. This study confirms the association between peripheral inflammatory markers and stroke or

atherosclerosis and indicates that the association between stroke and atherosclerosis may have resulted in an overinterpretation in previous studies of the association between cytokines and the stroke event itself. These data also show, for the first time that, compared to age-, sex-, and atherosclerosis-matched control subjects, induced cytokine production in blood is reduced after acute ischaemic stroke. Low levels of production are significantly correlated with worse outcome and mortality. Further study is warranted in order to explore ways of exploiting inter-individual differences to identify vulnerable subjects and target future therapies.

CHAPTER 6

Design of a phase II randomised controlled trial of interleukin-1 receptor antagonist in acute stroke

6.1 Introduction

As discussed in chapter 1, considerable efforts have been devoted in recent years to the development of treatments for acute stroke aimed at restoring cerebral perfusion (thrombolysis) or at limiting neuronal damage (neuroprotection). The term 'neuroprotection' encompasses a diverse range of potential therapies, including modulators of the excitatory amino acid system, modulators of calcium influx, metabolic activators, anti-oedema agents, inhibitors of leucocyte adhesion, free radical scavengers and other agents. In excess of 100 clinical trials have addressed the safety and efficacy of over 50 neuroprotective agents (Liebeskind and Kasner, 2001; Gladstone *et al.*, 2002), but there has been a disappointing failure to translate results seen in animal models to humans (Muir, 2002). Various problems, usually attributable to either unsuitability of the pre-clinical model or suboptimal clinical trial design, have hampered the translation of experimentally promising treatments into clinical practice. A standardised approach has been advocated for pre-clinical and clinical methodology in future clinical trials of neuroprotective agents (STAIR, 1999; STAIR-II, 2001; Fisher, 2003). IL-1 has already been discussed in detail. In summary, the IL-1 cytokine system is a particularly attractive therapeutic target in stroke. Although IL-1 appears to play a minimal if any role in normal brain function, IL-1 is strongly implicated in ischaemic brain damage, whilst IL-1ra limits injury in all forms of cerebral ischaemia tested to date. More extensive preclinical data exist for IL-1ra than probably for any other potential neuroprotective agent that has entered the clinical arena. This chapter describes the design of the first phase II randomised controlled trial of rhIL-1ra in patients with acute stroke.

6.2 Objectives and clinical hypothesis

6.2.1 Primary objective

The primary objective was to compare the safety profile of rhIL-1ra with placebo in patients with acute stroke, as determined by serious adverse events and major deterioration in stroke deficit (by > 4 points on the NIHSS), within the first 72 h after the initiation of test treatment.

6.2.2 Secondary objective

To perform an exploratory efficacy analysis based on comparative 3-month survival curves, changes in neurological impairment as determined by NIHSS score and changes in disability as determined by BI and mRS scores at 3 months, changes in biological markers (including WBC count, ESR, CRP and IL-6), and CT cerebral infarct volume at 5-7 days. Withdrawals within 72 h after the initiation of test treatment, and the reasons for them were noted but not analysed as a specific outcome.

6.2.3 Clinical hypothesis

Treatment with rhIL-1ra (100 mg iv loading dose, and 2 mg/kg/h iv infusion for 72 h) is safe and well tolerated in patients with acute stroke.

6.3 Recombinant human IL-1ra: pre-clinical and clinical studies

Pre-clinical and clinical studies of IL-1 in the context of experimental models of cerebral ischaemia and clinical stroke have been described in chapter 1. This section summarises the data on rhIL-1ra pertaining to animal studies of toxicology and phase I studies designed to determine the metabolic and pharmacological actions in humans, together with data on side effects and drug metabolism. Phase II and phase III studies, conducted for other indications, are also mentioned.

6.3.1 Animal studies

Data on toxicology were provided by Amgen. The effects of rhIL-1ra following single intravenous (iv) administered doses in rats (1.5, 15, 150, 720 mg/kg) and Cyanomolgus monkeys (1.5, 15, 150 mg/kg) showed no pharmacotoxic signs, body weight changes, macroscopic or microscopic changes attributable to rhIL-1ra. Daily iv administration of rhIL-1ra for 14 days to these monkeys did not result in any systemic toxicity at doses up to 30 mg/kg/day. Perivascular inflammation at the injection site was noted in the rat only. The no-observable-effect level (NOEL) for systemic toxicity after iv administration was determined to be $\geq$ 30 mg/kg/day. For local injection site toxicity, the NOEL was

10 mg/kg/day for rats and 30 mg/kg/day for monkeys. rhIL-1ra was administered to monkeys by continuous infusion at 150 mg/kg/day for 1 week with no treatment-related effects noted. No effects of rhIL-1ra on fertility and early development, embryo-foetal development, or perinatal and postnatal development were observed in the rat or the rabbit following subcutaneous (sc) administration of rhIL-1ra at dosages of up to 200 mg/kg/day. Mutagenicity studies with rhIL-1ra were negative. Two-year carcinogenicity studies have not been performed, but since rhIL-1ra is a recombinant human protein, it is thought unlikely to be a direct DNA damaging agent. rhIL-1ra could have indirect effects on tumour growth via immunomodulation, but no evidence of immunosuppression was seen in preclinical toxicology studies in rats or monkeys. Additionally, rhIL-1ra had no effect on specific immune functions in these animals and it is unlikely that rhIL-1ra impairs the immune system's ability to recognize and kill tumour cells.

The proposed dose in the current trial was an iv loading dose of 100 mg, followed by 2 mg/kg/h for 72 h. This approximates to the maximal dose of 100 mg loading and 133 mg/h for 72 hours studied in earlier sepsis studies (Fisher *et al.*, 1994b). The dose in this trial is also 4 times lower than the no-adverse-effect level for systemic toxicity determined in a 4-week toxicity study (200 mg/kg/day sc) and 3 times lower than a similar 1-week toxicity study (150 mg/kg/day iv), in Rhesus monkeys (data provided by Amgen).

6.3.2 Clinical experience

rhIL-1ra has been administered iv to healthy volunteers, to patients with renal and hepatic dysfunction, and to patients suffering from severe sepsis. The safety and pharmacokinetic profile was investigated in 14 healthy subjects at doses ranging from 1 – 10 mg/kg, given as a 3 h infusion, with and without concurrently administered endotoxin. rhIL-1ra did not induce any detectable clinical effects on its own, nor did it inhibit endotoxin induced symptoms, fever, or tachycardia. However, it did reverse endotoxin-induced neutrophilia and inhibited the actions of endotoxin on peripheral blood mononuclear cell proliferation in an *ex vivo* assay (data provided by Amgen). Mean peak plasma

levels were dose-dependent and reached > 29 μg/ml in the 10 mg/kg group. rhIL-1ra distributed in plasma with a steady-state distribution volume of 12.2 ± 2.7 litres and plasma clearance was 169 ± 12 ml/min. The terminal half-life was 102 ± 18 min (Granowitz *et al.*, 1993).

The mean plasma clearance values following iv bolus administration (1 mg/kg) in patients with hepatic dysfunction were 30% lower (95.1 ml/min), and in patients with chronic renal failure receiving dialysis were 86% lower (18.5 ml/min) than those of healthy volunteers. A positive correlation between individual plasma clearance values and creatinine clearance values has been found, suggesting that the kidney plays the major role in the elimination of rhIL-1ra after iv administration (Yang *et al.*, 2003). The decrease in elimination of iv rhIL-1ra in patients with hepatic dysfunction is consistent with a slight reduction of renal function (as determined by creatinine clearance) in these patients (data provided by Amgen).

The therapeutic potential of rhIL-1ra has been convincingly demonstrated in patients with rheumatoid arthritis, where it has recently been licensed (Bresnihan *et al.*, 1998; Schwetz, 2002). It has also been the subject of three trials involving approximately 1900 patients suffering from severe sepsis (Fisher *et al.*, 1994a; Fisher *et al.*, 1994b; Opal *et al.*, 1997). The highest dose groups in the sepsis studies included over 600 patients who were treated with a regime of rhIL-1ra comparable to that employed in the current trial (100 mg bolus followed by 2 mg/kg/h infusion for 72 h). In all three trials, the adverse event profile in all treatment groups (active and placebo) was comparable and typical of such a severely ill population; no serious adverse events could be clearly attributed to iv rhIL-1ra when administered by this dose regime.

Although no specific safety concerns have been raised for rhIL-1ra when administered by the regime employed in the current trial (100mg iv loading dose, and 2mg/kg/h iv infusion for 72 h), the product has not been investigated in acute stroke. For this reason, the main emphasis in this trial was on the assessment of safety. Nevertheless, the use of efficacy scales of proven consistency and

sensitivity (NIHSS, BI and mRS) provided the opportunity to perform exploratory analyses of efficacy.

6.4 *Experimental plan*

6.4.1 *Study design*

This was a phase II randomised, double-blind, placebo-controlled study in patients with acute stroke. Patients presenting with a preliminary diagnosis of acute stroke (via the Accident and Emergency department, by General Practitioner, or in-patients referred by staff in the relevant department), were notified to the Acute Stroke Research Team. The investigators conducted appropriate screening, and if the eligibility criteria were satisfied, consent or assent was obtained as appropriate. A schematic representation of the study design is shown in figure 6.1.

Patients with clinically diagnosed acute stroke of any aetiology (excluding SAH) were eligible for inclusion in the study. Cortical stroke was defined by clinical classification, and on the basis of CT evidence, as PICH or infarction. Clinical classification alone was used if no lesion was seen on CT. If the stroke was determined to be a PICH or lacunar infarction on the basis of CT, such patients continued to be evaluated for safety, but were excluded from the exploratory analysis of efficacy.

Individual patients received treatment for 72 hours. The total duration of the study from baseline assessment and randomisation to final assessment was 3 months. It was anticipated that the overall duration of the study would be 24 months from start of enrollment to end of study for the last patient.

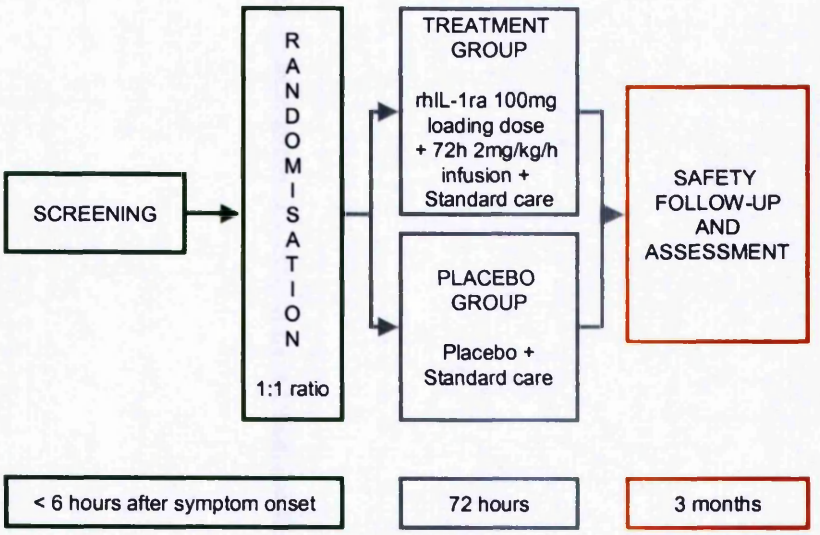


Figure 6.1 *Schematic diagram showing study design*

6.4.2 *Patient selection, enrolment and randomisation*

A screening log was maintained of all potential study candidates that included limited information about the potential candidate (initials, age, sex, race), date, and outcome of the screening process (eg, "enrolled into study", "reason for ineligibility" [if available], or "refused to participate"). Eligibility criteria are summarized in table 6.1.

Table 6.1 *Eligibility criteria*

Inclusion criteria	*Exclusion criteria*
• Aged at least 18 years	• Clinically significant, concurrent medical condition that could affect the evaluation of tolerability, safety, or efficacy in this study
• Treatment started within 6 h of symptom onset	• Rapid clinical improvement prior to randomisation
• Written informed consent from patient or written assent from carer	• NIHSS score ≤ 4
	• mRS ≥ 4 for the 4 week period preceding stroke
	• Previous inclusion in the current study
	• Patients receiving, or who have received any investigational drug within the previous 30 days (or patients currently using an investigational device)
	• Pregnancy or breast-feeding

All patients (or their carer) were required to sign and date the LREC approved informed consent form before any study procedures were undertaken. Patients were considered enrolled once the informed consent (or assent) form had been signed.

Treatment group assignment was performed by an independent, third party randomisation service, available 24 hours a day, 365 days a year. The service used a dedicated randomisation telephone number. This connected to a computerised randomisation system. The caller was asked to confirm patient eligibility and prompted to enter data required to stratify the randomisation. The caller used a security code number to access the system. The patient was allocated either 'treatment' or 'placebo'. The caller was given a 'blinded' box number. A list of placebo and active box numbers was supplied to the randomisation service by Amgen. The randomisation list comprised random permuted blocks within strata:

Age at randomisation:	2 levels	$< 70, \geq 70$ years
NIHSS score:	3 levels	4 – 9, 10 – 20, 21 – 24
Time since onset:	2 levels	< 4 hours, ≥ 4 hours

Patients meeting all eligibility criteria received their randomly assigned treatment allocation immediately upon completion of baseline tests and procedures. Treatment randomisation followed a 1:1, rhIL-1ra : placebo ratio based on a central randomisation assignment schedule prepared in advance of the trial commencing.

6.5 Study procedures

6.5.1 Test treatment, packaging and formulation

Anakinra (or Kineret®), r-metHuIL-1ra, or rhIL-1ra, is a recombinant form of the human interleukin-1 receptor antagonist. It is a recombinant protein consisting of 153 amino acids with a molecular weight of 17.3 kilodaltons (kDa) and, except for the addition of one N-terminal methionine, is identical to the naturally occurring, non-glycosylated form of human IL-1ra. It is produced by

recombinant DNA technology using *E. coli* fermentation and isolated and purified to obtain a pharmaceutical grade material.

rhIL-1ra is manufactured and packaged by Amgen, and provided as a clear, colourless liquid supplied in 3 ml glass vials containing 1.0 ml of 100 mg/ml rhIL-1ra. The formulation consists of 10 millimolar (mM) sodium citrate, 140mM sodium chloride, 0.5mM ethylene dichlorotetraacetic acid (EDTA), and 0.1% (w/w) polysorbate-80 (Tween® 80), pH 6.5. Test treatment (rhIL-1ra or placebo) was provided in boxes identified by lot number, and each vial was labelled r-metHuIL-1ra 100 mg or placebo. Vials labels stated the contents as r-metHuIL-1ra 100 mg or placebo for iv administration, the volume and storage requirements.

6.5.2 Storage, preparation and administration

The recommended storage temperature for rhIL-1ra is 2°C – 8°C. Test treatment was therefore stored at 2°C – 8°C in a secured refrigerator with a temperature alarm system in an area with restricted access. The actual storage conditions during the study period were recorded automatically using continuous temperature recordings. Any vial that became frozen was not used. Test treatment was allowed to come to room temperature before injection and was well mixed by gentle inversion only. Doses were administered in predefined volumes from a 100 mg/ml formulation.

To prepare the test treatment, the contents of the appropriate number of vials were added, using aseptic technique, to 500 ml of normal saline. The appropriate number of vials for the patient according to weight is shown in table 6.2. For placebo patients, an equivalent number of 1.0 ml vials of vehicle were added to 500 ml of normal saline. Thus the total volume (rhIL-1ra or placebo vehicle plus normal saline) infused every 12 hours was approximately 500 ml. Study medication was infused using a volumetric infusion pump at a rate of approximately 42.0 ml per hour. Any residual solution remaining at the end of 12 hours was infused before initiating the subsequent 12 hour infusion. The total

infusion time was approximately 72 hours, and the nominal rhIL-1ra dose administered in that time period was 9,700 mg for a 70 kg patient.

***Table 6.2** Weight table for test treatment dosage*

Weight range (kg)	Number of vials (100mg) per 12h
46 – 54	12
55 – 62	14
63 – 71	16
72 – 79	18
80 – 87	20
88 – 96	22
97 – 105	24

At study initiation and as needed thereafter, test treatment was shipped to Hope Hospital. The investigators checked the amount and condition of the drug and recorded these data. At the end of the study, or as directed, all test treatment supplies, including unused, partially used, or empty containers, were destroyed. Individual investigational drug accountability records were kept for each patient receiving test treatment. In addition, separate master log investigational drug accountability records were also maintained for each shipment of study drug.

All patients received a loading dose of either rhIL-1ra 100 mg (1.0 ml volume) or placebo 1.0 ml by intravenous loading dose over approximately 60 seconds. The rhIL-1ra or placebo loading dose was diluted with normal saline to a total volume of 10 ml and injected directly into a venous access site. A 72-hour 2 mg/kg/h continuous intravenous infusion of rhIL-1ra or placebo, administered by the investigators, commenced immediately after administration of the loading dose.

The baseline weight of the patient was used to determine the volume of test treatment to be administered throughout the trial. The weight was estimated or gathered from a reliable informant if necessary. The patients' drug compliance (ie volume administered) was recorded in the CRF.

Patients entering the study with decreased renal function (defined as a serum creatinine concentration >177 μmol/l), or developing decreased renal function during the test treatment infusion, had their assigned infusion rate decreased by 50% (the initial loading bolus of rhIL-1ra 100mg or placebo was administered to all patients). In the event of the serum creatinine falling below 177 μmol/l, the patient was returned to the original assigned infusion rate. Discontinuation of drug treatment due to withdrawal of consent, or for any adverse event considered sufficient to warrant withdrawal, was documented in the CRF. Throughout the study, the physician in charge of the patient's care was able to prescribe any concomitant medications or treatments deemed necessary to provide adequate supportive care. Details of such concomitant medications were recorded in the CRF.

The investigators were responsible for ensuring the safe storage and location of the randomisation code-break, and that access was readily available to the relevant staff in case of an emergency (see figure 6.2). A patient's treatment assignment would be unblinded only if essential for the further management of the patient. Unblinding for any other reason was considered a protocol deviation.

6.5.3 Other study procedures

All study procedures to be undertaken at each time-point are shown in table 6.3. On the day of enrolment (day 0) an initial assessment at the screening stage comprised a medical history and brief clinical assessment. Written informed consent was then obtained. Prior to treatment initiation, the NIHSS score and pre-stroke mRS were required, together with the patient's weight. Following randomization, the test treatment was prepared according to a standard procedure (see section 6.4.2). Immediately prior to the initiation of test treatment, blood samples were collected. The remaining data were then obtained and clinical investigations performed, including a more detailed medical history and clinical examination, OCSP classification, BI, aural temperature, electrocardiogram (ECG) and chest X-ray (CXR). A CT brain scan was performed within the first 24 hours of enrolment.

Blood samples were collected for a range of laboratory tests. FBC with differential WBC count, urea and electrolytes (U+Es), liver function tests (LFTs), bone profile, glucose, and ESR were analysed as described in chapter 2. The methodology used for determination of plasma CRP, IL-6, and IL-1ra concentrations has already been described in chapters 3 and 5. Patients also underwent an assessment of peripheral vascular disease (PVD) as described in chapter 2.

Assessments were performed on each of day 1, day 5 to 7, and 3 months at 09:00. These comprised brief general physical examination, measurement of pulse, blood pressure, and aural temperature. Blood was also collected, for FBC with differential, U+Es, LFTs, bone profile, ESR, CRP, IL-6 and IL-1ra concentration. In patients with ischaemic stroke, a second CT brain scan was performed at days 5 to 7. The final assessment was carried out at 3 months ± 2 weeks. Patients not recruited between 07:00 and 11:00 underwent an additional assessment at 24 hours after the time of randomisation. At this time point the same assessment and blood tests were undertaken as for day 1, excepting that routine clinical chemistry (U+Es, LFTs, bone profile) was not performed. On each of days 2, 3 and 4, patients were assessed at 09:00, when pulse, blood pressure, and aural temperature were recorded. In addition, blood was collected for FBC with differential, U+Es, LFTs, bone profile, CRP, ESR and IL-1ra concentration. NIHSS score was recorded after the end of the test treatment infusion, and again at 5 to 7 days and 3 months. mRS and BI was also recorded at 3 months.

Table 6.3 *Study schedule*

	Initial assessment and consent	Day 0	Day 1	24 hours*	Day 2	Day 3	Day 4	Day 5-7	3 months**
Clinical assessments									
Medical and drug history	•								•
Clinical examination	•		•	•	•	•	•	•	•
Height & weight		•							
Pulse and blood pressure	•		•	•	•	•	•	•	•
Aural temperature		•	•	•	•	•	•	•	•
Stroke scales									
NIHSS		•				•†	•†	•	•
mRS		•							•
BI		•							•
Laboratory tests									
Full blood count		•	•	•	•	•	•	•	•
Urea and electrolytes		•	•		•	•	•	•	•
Liver function tests		•	•		•	•	•	•	•
Bone profile		•	•		•	•	•	•	•
Glucose		•							
ESR		•	•	•				•	•
CRP		•	•	•	•	•	•	•	•
IL-6		•	•	•	•	•	•	•	•
IL-1ra		•	•	•	•	•	•	•	•
Other									
Chest X-ray		•							
CT brain scan		•‡						•	
Electrocardiogram		•							

* Assessment at 24 hours after randomisation where randomisation was not between 07:00 and 11:00

** 3 months ± 2 weeks

† NIHSS score was recorded at the next 09:00 after the end of test treatment infusion (day 3 or day 4)

‡ CT brain scan performed within 24 hours of randomisation

6.6 Adverse events

6.6.1 Definitions

Adverse events

An adverse event is any undesirable medical experience occurring to a patient, during a clinical trial, whether or not considered related to the test treatment (CPMP Working Party on Efficacy of Medicinal Products, 1990). Therefore,

adverse events are test treatment emergent signs or symptoms. Elective hospitalisations for pretreatment conditions (e.g., elective cosmetic procedures) are not adverse events. Abnormal laboratory values should not be reported as adverse events; however, any clinical consequences of the abnormality should be reported as adverse events.

Serious adverse events

A serious adverse event is defined by regulatory agencies as one that suggests a significant hazard or side effect, regardless of the investigators' opinion on the relationship to test treatment. This includes, but may not be limited to, any event that (at any dose):

- is fatal
- is life-threatening (places the patient at immediate risk of death)
- requires in-patient hospitalization or prolongation of existing hospitalization
- is a persistent or significant disability/incapacity
- is a congenital anomaly/birth defect (Medical Research Council, 1998).

Important medical events that may not be immediately life threatening or result in death or hospitalization, but may jeopardize the patient or require intervention to prevent one of the outcomes listed above, or result in urgent investigation, may be considered serious.

6.6.2 Reporting procedures for all adverse events

All serious and non-serious adverse events were recorded up to three months after the initiation of test treatment. This process followed a standard operating procedure (SOP) (figure 6.2).

Adverse events

All adverse events occurring during the study, whether or not attributed to the test treatment, observed by the investigators or reported by the patient, were recorded in the CRF. Attribution of all adverse events was made by the investigators. All adverse events were reported to the independent Data Monitoring Committeee (DMC). It was left to the investigators' clinical

judgment whether or not an adverse event was of sufficient severity to require that the patient should be withdrawn from test treatment. A patient might also voluntarily withdraw from test treatment due to what he or she perceived to be an intolerable adverse event. If either of the above occurred, the patient was required to undergo an end-of-study assessment and be given appropriate care under medical supervision until symptoms ceased or the condition became stable. The following attributes needed to be assigned where known: description; dates of onset and resolution; severity; assessment of relatedness to study drug, other suspect drug, or device; and action taken.

Serious adverse events

All serious adverse events were reported to the DMC, Medicines and Healthcare products Regulatory Agency (MHRA) (formerly the Medicines Control Agency) and Amgen. All deaths occurring during test treatment were reported to the DMC, MHRA and Amgen. These include deaths within 30 days of the end of test treatment infusion and deaths up to the last formal follow-up at 3 months. For all deaths, available post-mortem reports and relevant medical reports were sent to the MHRA. The investigators were required to notify the LREC of serious adverse events occurring at the site in accordance with local procedures.

The severity of serious adverse events was assessed on the scale shown below, with appropriate clinical definitions. The relationship of adverse events to the study medication was assessed by means of the question: 'Is there a reasonable possibility that the event may have been caused by the study medication?'

1 = MILD - aware of sign or symptom, but easily tolerated

2 = MODERATE - discomfort enough to cause interference with usual activity

3 = SEVERE – incapacitating, with inability to work or do usual activity

4 = LIFE-THREATENING - Refers to an event in which the patient was, in the view of the Investigator, at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe

5 = FATAL

All serious adverse events were reported to the MHRA within fifteen calendar days of discovery or notification of the event and a copy will be sent to Amgen. Initial serious adverse event information and all amendments or additions were recorded on a Serious Adverse Event form (see appendix) and sent to the MHRA and Amgen.

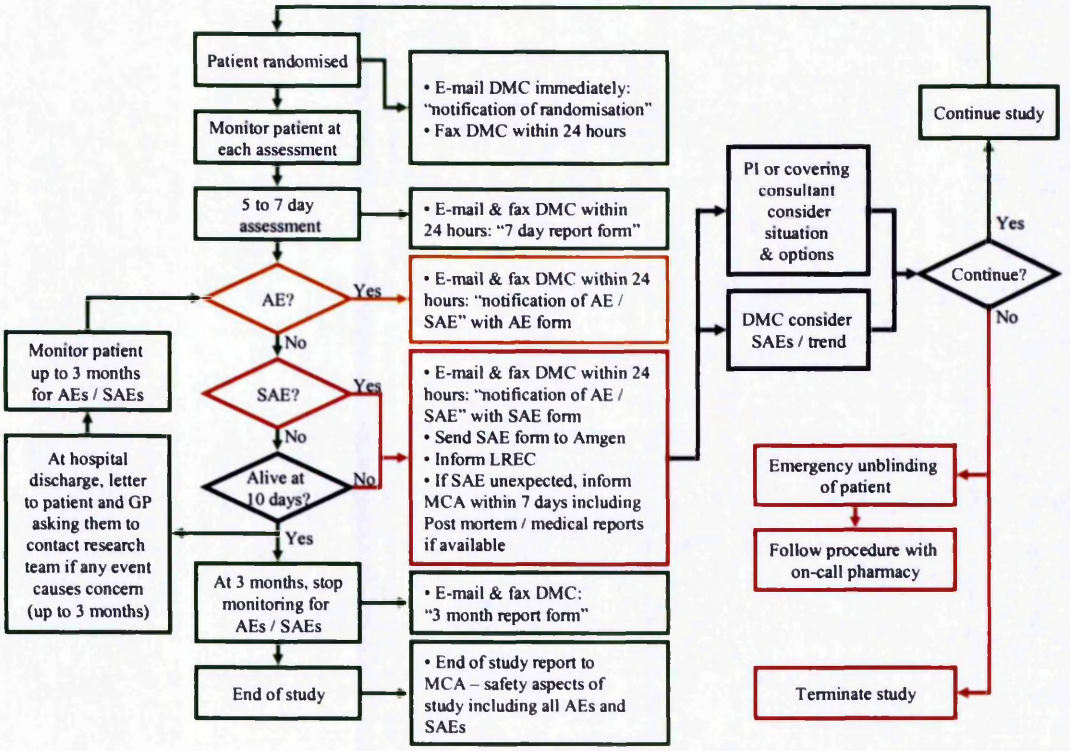


***Figure 6.2** Standard operating procedure for adverse events*

6.7 Statistical considerations

This was primarily a safety study, but exploratory information about efficacy was also collected as a secondary analysis to guide possible phase III studies in the future. The outcome measures are summarised below.

6.7.1 Primary and secondary outcome measures

Primary outcome measures

These were (1) serious adverse events in the first 72 h after the initiation of test treatment and (2) deterioration in NIHSS score > 4 points in the first 72 h after the initiation of test treatment.

Secondary outcome measures

Secondary outcome measures were (1) adverse events, (2) markers of biological activity (including WBC count, ESR, CRP and IL-6), (3) CT brain infarct volume at 5 to 7 days, and (4) clinical outcomes (survival to 3 months, NIHSS, BI and mRS scores at 3 months). Mortality of 30% was estimated in the study population prior to the final 3-month assessment. Analyses of 3-month outcome was performed incorporating "death" within categorised scales.

6.7.2 Sample size considerations

A study size of 40 cortical infarcts per group was thought to be practically achievable within the timescale. Thirty acute stroke patients per month were anticipated, of whom approximately four would satisfy the eligibility criteria. It was proposed to commence recruitment at Hope Hospital and only to recruit other centres within the region as necessary to complete recruitment within the 24-month timescale. Allowing for non-consent rate of 20%, the target recruitment was expected to be achieved within 24 months. Patients withdrawn from the study for any reason would not be replaced but every reasonable effort would be made to record outcomes for these patients. All analyses were by the intention-to-treat principle wherever applicable. Analyses of adverse events were performed "per protocol".

6.7.3 Interim analysis and early stopping guidelines

Safety monitoring was undertaken by the independent DMC. No termination was considered on the grounds of efficacy. The DMC might have suspended or terminated recruitment on the grounds of safety, but would not extend the study. The DMC received blinded reports (see appendix 1) on each patient one week

after recruitment. The report included baseline characteristics, adverse events (serious and non-serious), and the investigators' assessment and explanation of whether clinical status was better or worse than anticipated. DMC members communicated by telephone and e-mail. The DMC were not expected to meet formally except in the event of consideration of termination. No statistical interim analysis was planned, and was only to be conducted at the request of the DMC.

6.7.4 Planned methods of analysis

Safety analyses

Analysis of serious adverse events was primary. It was anticipated that these would be rare. Analysis using Fisher's 'exact' test was planned. Deterioration in NIHSS score would be categorised blind to allocation and compared using Fisher's 'exact' test. Withdrawal during infusion would also be compared using Fisher's 'exact' test. Occurrence of all adverse events would be tabulated by body system, preferred term and severity, using the medical dictionary for regulatory activities (MedDRA) terminology. CT infarct volume at 5-7 days would be compared by 95% confidence interval for the difference in group means. Survival to 3 months would be compared using the Kaplan-Meier method with log-rank test.

Efficacy analyses

All efficacy analysis were secondary and exploratory. Descriptive statistics would be presented for the various outcome scales at 3 months. No inferential analysis was planned.

6.8 Regulatory and ethical obligations

6.8.1 Licence exemption

An appropriate exemption from licence (DDX) was granted by the MHRA for the use of rhIL-1ra in this trial.

6.8.2 *Declaration of Helsinki*

The study was conducted in full conformity with the current revision of the Declaration of Helsinki (2000), and account was also taken of the International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines (1996) and the MHRA regulations.

6.8.3 *Informed consent*

Before a patient's participation in the trial, it was the investigators' responsibility to obtain written informed consent from the patient, or written assent from the patient's carer after adequate explanation of the aims, methods, anticipated benefits, and potential hazards of the study and before any protocol-specific screening procedures or test treatment were administered. The acquisition of informed consent was documented in the patient's medical records, and the informed consent form was signed and personally dated by the patient or carer, and by the person conducting the informed consent discussion. The original signed informed consent form was retained in accordance with institutional policy, and a copy of the signed consent form was provided to the patient or carer. The date that informed consent was given was recorded in the patient's medical notes.

Patients had the right to withdraw from the study at any time and for any reason without prejudice to their subsequent medical care. Other reasons for removal of patients might have included an administrative decision, ineligibility, protocol deviation, patient non-compliance or the occurrence of an adverse event (AE) or serious adverse event (SAE), dependent on individual circumstances. In the event of a patient's (or representative's) request or decision to withdraw, all efforts were made to complete and report the observations as thoroughly as possible up to the date of withdrawal. All information was recorded in the CRF.

6.8.4 *Local Research Ethics Committee*

A copy of the protocol, proposed patient information sheet, informed consent form, and other written patient information was submitted to the LREC for written approval. The investigators submitted and, where necessary, obtained

approval from the LREC for any subsequent protocol amendments and changes to the informed consent document. The investigators were to notify the LREC of deviations from the protocol or serious adverse events occurring at the site in accordance with local procedures. The investigators were responsible for obtaining annual LREC approval/renewal throughout the duration of the study.

6.9 *Administrative and legal obligations*

6.9.1 *Study documentation and storage*

A list was maintained of appropriately qualified persons to whom trial duties were delegated. The investigators maintained a comprehensive and centralized filing system of all study-related (essential) documentation, suitable for inspection at any time by representatives from applicable regulatory authorities. Elements included patient files containing completed CRFs, informed consents, and supporting copies of source documentation (eg hospital records, laboratory reports, ECGs and CT brain scans), study files containing the protocol with all amendments, Investigator's Brochure, copies of pre-study documentation, all correspondence to and from the LREC, MCA, DMC, randomization service, and all test treatment-related correspondence. All essential documentation were to be retained by the institution for the same period of time required for medical records retention.

6.9.2 *Data collection*

All CRFs were typed or filled out legibly with a black or blue ball-point pen. Corrections (initialled and dated) to paper forms were made by a single line stroke through the error with insertion of the correction above or beside the error. The investigators signed and dated the indicated places on the CRF.

6.10 Discussion

This chapter summarises the rationale and design of a phase II randomised controlled trial of rhIL-1ra in patients with acute stroke. It has considered the study objectives, pre-clinical and clinical experience of rhIL-1ra, experimental plan and study procedures, adverse event procedures, statistical issues, and the regulatory, ethical, administrative and legal obligations. A few specific points are worthy of further discussion.

The population under study is intended to be representative of patients typically presenting within 6 h of onset of symptoms of acute stroke. The intention was to recruit predominantly patients with cortical infarcts, because these are more likely to benefit from neuroprotection than patients with other stroke sub-types (Muir, 2002). Although patients with lacunar infarcts or PICH (other than SAH) were to be included in the analyses of safety and biological activity, only patients with cortical infarcts were to be included in the pre-specified exploratory analysis of efficacy.

Although not originally designed for this purpose, the NIHSS score is predictive of clinical outcome (Adams *et al.*, 1999); patients with very mild strokes (NIHSS score ≤ 4) and those with very severe damage (NIHSS score ≥ 20) are less likely to benefit from treatment with a neuroprotective agent. Hence, the eligibility criteria for the present study specifically exclude patients with an NIHSS score ≤ 4. No upper NIHSS score cut-point was specified because the catch-all exclusion criterion 'clinically significant, concurrent medical condition that could affect the evaluation of tolerability, safety, or efficacy in this study' was preferred.

The medical dictionary for regulatory activities (MedDRA) is a unified standard terminology for recording and reporting adverse drug event data (Brown *et al.*, 1999). The ICH has agreed upon its structure and content. It is a hierarchical terminology, with terms derived from several sources, including WHO adverse reaction terminology (WHO-ART) and International Classification of Diseases (ICD) 9. Although some potential problems have been identified (Brown, 2003),

this system was applied to adverse events in the current trial because it is becoming widely adopted by agencies such as the MHRA, and it is generally regarded as an improvement on the previous situation where multiple terminologies were applied.

Although it has been suggested that 50% of UK patients with suspected stroke present to hospital within six hours of symptom onset (Harraf *et al.*, 2002), an analysis of patient eligibility for thrombolytic therapy in Canada showed that the majority of patients are unable to receive rt-PA for acute ischaemic stroke because they do not reach hospital soon enough (Barber *et al.*, 2001). Clearly, late presentation was always a potential limiting factor in the recruitment of adequate numbers of acute stroke patients to the planned study. Ethical approval was sought for the inclusion of patients unable to give written informed consent or where assent was unavailable on the basis that this was an acute intervention with a short 'therapeutic window' of 6 hours from the onset of symptoms (Medical Research Council, 1998). However, this was not granted on the basis that this was primarily a safety study. The involvement of other centres in the region was reserved as an option during the conduct of the trial depending on the rate of patient recruitment, although logistical considerations ultimately prevented this from happening.

The main advantage of conducting the study in a single centre lies in the ability to be in control of a careful, exploratory investigation of biological activity and efficacy in addition to the detailed evaluation of safety. Recommendations for clinical trial evaluation of acute stroke therapies accept that phase II studies usually address a hypothesis of biological activity but are not powered to determine conclusive efficacy (STAIR-II, 2001). This study was a close collaborative effort between basic and clinical science, enabling the investigation of basic mechanisms underlying the rationale for IL-1ra action and inflammatory mechanisms associated with stroke, by monitoring plasma levels of IL-1ra and examining any differences in cytokines or inflammatory markers between individuals treated with rhIL-1ra or placebo. It would have been difficult to conduct the study on a multi-centre basis owing to its complexity and the care required with blood sampling and processing.

CHAPTER 7

A randomised phase II study of interleukin-1 receptor antagonist in patients with acute stroke

7.1 Introduction

This chapter reports the results of the first phase II study of rhIL-1ra in patients with acute stroke, and has been prepared to comply with the revised CONSORT statement for reporting randomised trials (Altman *et al.*, 2001).

Numerous clinical stroke trials of neuroprotective agents have failed to show efficacy or have been halted due to adverse effects. Although these failures might imply that the experimental model is not predictive of events occurring during clinical stroke, only limited preclinical data existed for many of the agents tested. Any new agent should demonstrate efficacy in several different types of experimental cerebral ischaemia, even when the compound is administered several hours after the onset of ischaemia (STAIR, 1999). Other anti-inflammatory approaches have tended to target very specific processes. For example, anti-neutrophil approaches are of benefit in experimental transient ischaemia but less so in permanent ischaemia (Jiang *et al.*, 1998). In contrast, IL-1ra limits injury in all forms of cerebral ischaemia tested to date and the preclinical data for IL-1ra are probably more extensive than for any other potential neuroprotective agent that has entered the clinical arena.

Extensive toxicity studies have revealed no adverse effects of rhIL-1ra in animals or humans (data provided by Amgen). Subcutaneous rhIL-1ra is safe and effective in rheumatoid arthritis patients and is now licensed for this indication (Cohen & Rubbert, 2003). The therapeutic potential of intravenous rhIL-1ra has also been investigated in severe sepsis where adverse event (AE) profiles in the treatment groups (active and placebo) were comparable and no serious adverse events (SAEs) could be attributed to rhIL-1ra (Opal *et al.*, 1997).

This was the first randomised, double-blind, placebo-controlled study of rhIL-1ra in patients with acute stroke. The primary aim of this study was to assess the safety of rhIL-1ra in acute stroke patients. Secondary aims of this study were to obtain information on biological activity and efficacy, based on 3-month survival, well validated stroke scales, a range of biological markers and CT cerebral infarct volume.

7.2 Methods

7.2.1 Hypothesis, location and selection of patients

The primary hypothesis tested was that intravenous rhIL-1ra is safe and well tolerated in patients with acute stroke. Secondary objectives were to test whether rhIL-1ra has biological activity, and to perform an exploratory analysis of efficacy. The trial was conducted at Salford Royal Hospitals NHS Trust, an 875-bed acute teaching hospital trust in the UK. The study received approval from the LREC, and was registered at inception on the UK National Research Register (N0217086513). Patients with a provisional diagnosis of acute stroke were screened for eligibility. Inclusion criteria were (1) age $\geq$ 18 years, (2) written informed consent / assent, and (3) within 6 h of onset of symptoms. Exclusion criteria were (1) clinically significant concurrent medical condition affecting evaluation of tolerability, safety or efficacy, (2) rapid clinical improvement, (3) NIHSS score $\leq$ 4, (4) pre-stroke mRS $\geq$ 4, (5) previous inclusion in the current study, (6) investigational drug or device within the previous 30 days and (7) pregnancy or breast-feeding. Very mild strokes were excluded as they tend to have excellent clinical outcomes (Adams *et al.*, 1999).

7.2.2 Randomisation, treatment and study procedures

Initial assessment included brief medical history, clinical examination, NIHSS score (Lyden *et al.*, 1994) and mRS (van Swieten *et al.*, 1988) prior to randomisation. Patients were randomised to rhIL-1ra or matching placebo, in addition to standard treatment. Treatment group assignment was performed by an independent, interactive voice-response service (BioCall, Nottingham, UK). Restricted block randomisation balanced the groups for age (< 70, $\geq$ 70 years), baseline severity (NIHSS score 4 – 9, 10 – 20, $\geq$ 21), and time since onset (< 4, $\geq$ 4 h). The randomisation system allowed for the unblinding of treatment assignment in an emergency, but this facility was not used during the study. After randomisation and baseline blood sampling, test treatment was initiated as soon as possible. Detailed history, examination, aural temperature, BI (Mahoney and Barthel, 1965) and OCSP classification (Bamford *et al.*, 1991) were also recorded. Video training was used for NIHSS scoring (Lyden *et al.*, 1994). CT brain scans were performed within 24 h of admission and at 5-7 days in those patients with

ischaemic stroke for determination of cerebral infarct volume (Gavin *et al.*, 2004). Other study procedures are detailed in chapter 6. Anakinra (Kineret®; Amgen Inc., Thousand Oaks, CA) is the recombinant human interleukin-1 receptor antagonist, r-metHuIL-1ra. Test treatment was supplied as a clear, colourless liquid in 3 ml glass vials containing 1.0 ml of 100 mg/ml rhIL-1ra or matching placebo and stored at 2°C – 8°C. Test treatment was administered intravenously by a 100 mg loading dose over 60 seconds, followed by consecutive 2 mg/kg/hour infusions over 72 h. Venous blood samples were taken at baseline, the next 09:00 time point where admission was before 07:00 or after 11:00, 24 hours after admission, then on days 2, 3, 4, 5 to 7, 3 months and 12 months at 09:00. Samples were collected into serum gel tubes, fluoride oxalate/EDTA and EDTA-containing tubes (Sarstedt, UK) for serum biochemical profile and plasma glucose (Roche Integra 700 analyser), ESR (Starrsed III analyser), and FBC and differential WBC count (Coulter Gen-S analyser). Blood for CRP, IL-6 and IL-1ra concentrations was collected into tubes containing pyrogen-free heparin, and wrapped in cool-gel packs. One hour after collection, blood was centrifuged at 2000g for 30 minutes at 4°C. Plasma was separated, frozen and stored at -70°C until analysis. CRP, IL-6 and IL-1ra concentrations were measured using enzyme-linked immunosorbent assays as described previously in chapters 3 and 4.

7.2.3 Outcome measures, sample size and statistical analysis

The primary objective was to compare the safety profile of rhIL-1ra with placebo in patients with acute stroke. Primary outcome measures were, in the first 72 hours after the initiation of test treatment, (1) serious adverse events (SAE) and (2) major increase in stroke severity (increase in NIHSS score > 4 points). Secondary outcome measures were adverse events, markers of biological activity (including WBC count, ESR, CRP and interleukin-6), CT brain infarct volume at 5 to 7 days, and clinical outcomes (survival to 3 months, NIHSS, BI and mRS scores at 3 months). AEs and SAEs were recorded to 3 months, and classified using the medical dictionary for regulatory activities (MedDRA). AEs were reported to an independent data monitoring committee (DMC), comprising a stroke physician, clinical pharmacologist and medical statistician. SAEs were reported to the DMC, Medicine and Healthcare Product Regulatory Authority (MHRA), Amgen and the

LREC. The protocol prepared prior to the study stated that clinical outcome would be assessed as a secondary outcome in patients with cortical infarcts only, in order to compare a more homogenous group.

The intention was to recruit 50 patients per group, to have 40 cortical infarcts per group and rule out common, unanticipated adverse events in acute stroke and provide pilot data for planning phase III trials of efficacy. Analysis was planned to be by intention to treat, with additional 'per protocol' analysis of safety outcomes. In practice, all randomised patients received treatment as allocated. Analysis of serious adverse events and deterioration in NIHSS score used Fisher's 'exact' test. CT infarct volume at 5-7 days was compared by 95% confidence interval for the difference in group means, and survival to 3 months was compared using the Kaplan-Meier method with log-rank test. All efficacy analysis was secondary and exploratory.

7.3 Results

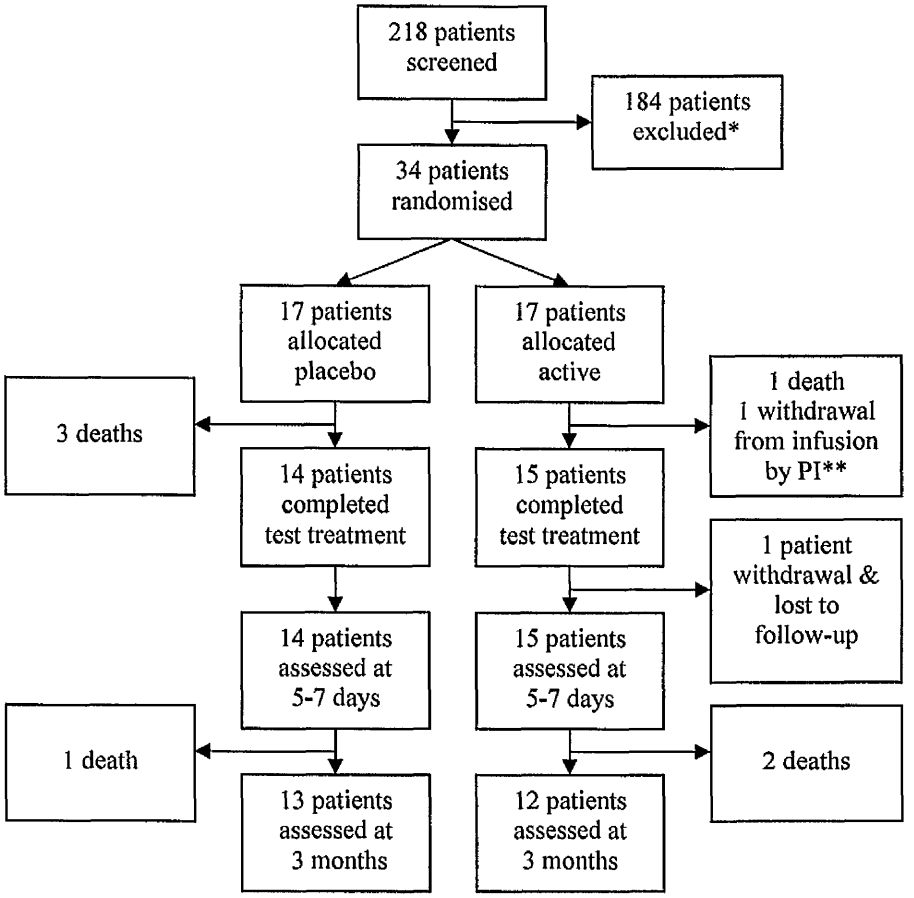
7.3.1 Treatment assignment, patient characteristics and rhIL-1ra infusion kinetics

Of 218 patients screened between February 2001 and July 2003, 184 (84%) were excluded (figure 7.1). Reasons for exclusion were: duration of symptoms > 6 hours or time of onset not reliably known ($n = 66$), mild stroke (NIHSS score ≤ 4) or rapid clinical improvement ($n = 32$), suspected non-stroke diagnosis ($n = 29$), no consent or assent ($n = 15$), significant concurrent medical condition ($n = 15$), significant pre-existing disability (mRS ≥ 4) ($n = 11$) and others ($n = 16$). Thirty-four patients were randomised, 17 in each group. The study was time-limited and the decision to terminate was taken blind to the study data.

By design, the median age (72 years), baseline NIHSS score (13) and time since onset (3.6 h) were similar in the two groups (tables 7.1). Conventional stroke risk factors (hypertension, smoking, diabetes mellitus, total cholesterol and atrial fibrillation), ethnic origin, and medications at baseline were also similar in the two groups. All patients were treated within 6.25 h, and 19 (56%) within 4 h of onset of symptoms. Overall, 29 (85%) patients had ischaemic stroke, and 24 had cortical

infarcts (71%). Of 17 patients who received rhIL-1ra, 12 (71%) had ischaemic stroke and 10 (59%) had cortical infarcts (table 7.1). All 5 patients (29%) with PICH were in the rhIL-1ra group. Demography and baseline characteristics for patients with cortical infarcts are shown in table 7.2.

***Figure 7.1** Trial profile*



* Reasons for exclusion detailed in text
** Principal Investigator

Table 7.1 *Demography and baseline characteristics: all patients*

		All patients (n = 34)	Placebo (n = 17)	Active (n = 17)
Demographics				
Age*, years		72 (61 to 77)	74 (62 to 78)	71 (56 to 78)
	< 70 years	15 (44%)	8 (47%)	7 (41%)
	≥ 70 years	19 (56%)	9 (53%)	10 (59%)
Male		17 (50%)	7 (41%)	10 (59%)
Caucasian		32 (94%)	17 (100%)	15 (88%)
BMI** (kg/m^2) mean (SD)		26 (3)	26 (4)	26 (3)
Test treatment initiation				
Onset to treatment*, h		3.6 (2.8 to 5.0)	3.6 (2.6 to 4.9)	3.6 (2.8 to 5.0)
	< 4 h	19 (56%)	10 (59%)	9 (53%)
	≥ 4 h	15 (44%)	7 (41%)	8 (47%)
Pre-stroke disability				
	BI*	100 (100 to 100)	100 (100 to 100)	100 (100 to 100)
	mRS*	0 (0 to 1)	0 (0 to 1)	0 (0 to 1)
Stroke severity and subtype				
Baseline NIHSS score*		13 (11 to 18)	13 (11 to 18)	13 (10 to 19)
	4 – 9	6 (17.6%)	3 (17.6%)	3 (17.6%)
	10 – 20	23 (67.6%)	12 (70.6%)	11 (64.7%)
	≥ 21	5 (14.7%)	2 (11.8%)	3 (17.6%)
Ischaemic		29 (85.3%)	17 (100%)	12 (70.6%)
OCSP classification	TACS	15 (44.1%)	10 (58.8%)	5 (29.4%)
	PACS	9 (26.5%)	4 (23.5%)	5 (29.4%)
	LACS	4 (11.8%)	2 (11.8%)	2 (11.8%)
	POCS	1 (2.9%)	1 (5.9%)	0 (0%)
Haemorrhagic	PICH	5 (14.7%)	0 (0%)	5 (29.4%)
Risk factors				
Atrial fibrillation		6 (17.6%)	3 (17.6%)	3 (17.6%)
Hypertension		18 (52.9%)	9 (52.9%)	9 (52.9%)
Diabetes mellitus		2 (5.9%)	0 (0%)	2 (11.8%)
Current smoker		12 (35.3%)	7 (41.2%)	5 (29.4%)
Ex-smoker		6 (17.6%)	2 (11.8%)	4 (23.5%)
Total cholesterol* (mmol/l)		5.4 (4.6 to 6.1)	5.4 (4.9 to 6.1)	5.2 (4.6 to 6.0)
Previous stroke/TIA		6 (17.6%)	4 (23.5%)	2 (11.8%)
Ischaemic heart disease		9 (26.5%)	4 (23.5%)	5 (29.4%)

*data shown as median (interquartile range) **body mass index

***Table 7.2** Demography and baseline characteristics: patients with cortical infarcts*

	All patients with cortical infarcts (n = 24)	Placebo (n = 14)	Active (n = 10)
Demographics			
Age*, years	70 (62 to 78)	71 (62 to 80)	74 (58 to 77)
< 70 years	10 (41.7%)	7 (50.0%)	3 (30.0%)
≥ 70 years	14 (58.3%)	7 (50.0%)	7 (70.0%)
Male	12 (50.0%)	6 (42.9%)	6 (60.0%)
Caucasian	24 (100.0%)	14 (100.0%)	10 (100.0%)
BMI** (kg/m^2) mean (SD)	25 (3)	25 (3)	25 (3)
Test treatment initiation			
Onset to treatment*, h	3.6 (2.8 to 5.0)	3.6 (2.7 to 4.5)	4.0 (2.9 to 5.3)
< 4 h	13 (54.2%)	8 (57.1%)	5 (50.0%)
≥ 4 h	11 (45.8%)	6 (42.9%)	5 (50.0%)
Stroke severity and subtype			
Baseline NIHSS score*	15 (11 to 19)	14 (11 to 19)	16 (10 to 20)
4 – 9	2 (8.3%)	1 (7.1%)	1 (10.0%)
10 – 20	18 (75.0%)	11 (78.6%)	7 (70.0%)
≥ 21	4 (16.7%)	2 (14.3%)	2 (20.0%)
OCSP classification TACS	15 (62.5%)	10 (71.4%)	5 (50.0%)
OCSP classification PACS	9 (37.5%)	4 (28.6%)	5 (50.0%)
Risk factors			
Atrial fibrillation	6 (25.0%)	3 (21.4%)	3 (30.0%)
Hypertension	15 (62.5%)	8 (57.1%)	7 (70.0%)
Diabetes mellitus	2 (8.3%)	0 (0.0%)	2 (20.0%)
Current smoker	10 (41.7%)	6 (42.9%)	4 (40.0%)
Ex-smoker	5 (20.8%)	2 (14.3%)	3 (30.0%)
Total cholesterol* (mmol/l)	5.2 (4.6 to 5.7)	5.2 (4.8 to 5.9)	5.1 (4.6 to 6.1)
Previous stroke/TIA	6 (25.0%)	4 (28.6%)	2 (20.0%)
Ischaemic heart disease	8 (33.3%)	4 (28.6%)	4 (40.0%)

*data shown as median (interquartile range) **body mass index

The test treatment was very well tolerated, being completed in 29 (85%) of patients, with a median (interquartile range, IQR) duration of 72.3 h (72.1 to 72.6 h). During the test treatment infusion, the median (IQR) IL-1ra plasma concentration was between 370 pg/ml (302 to 561 pg/ml) and 617 pg/ml (451 to 705 pg/ml) in the patients receiving placebo, and between 28 μg/ml (21 to 38 μg/ml) and 30 μg/ml (25 to 36 μg/ml) in those receiving rhIL-1ra. After the test treatment infusion the median (IQR) plasma IL-1ra concentration in patients who received rhIL-1ra declined to 402 ng/ml (130 to 632 ng/ml) by day 4, 20 ng/ml (6 to 37 ng/ml) by day 5-7, and 428 pg/ml (271 to 575 pg/ml) by 3 months. Four patients died prior to completion of the test treatment infusion, and one patient was withdrawn at the investigators' discretion prior to neurosurgery for evacuation of haematoma. One patient withdrew from the active group after completing the test treatment. This patient was independently mobile at discharge, but 3-month outcome is unknown.

7.3.2 Safety analysis

In the first 72 hours there were three SAEs in three patients in the placebo group (3 deaths) and two SAEs in two patients in the active group (1 death, 1 neurological deterioration secondary to PICH) ($p = 1.0$) (table 7.3). None was attributed to test treatment. No other patients experienced an increase of more than 4 points in NIHSS score. There were 14 non-serious AEs in 10 patients in the placebo group and 9 non-serious AEs in 9 patients in the active group by 72 hours ($p = 1.0$). Again, none was attributed to test treatment.

By 3 months, 5 SAEs occurred in each group: 4 deaths and 1 recurrent stroke in the placebo group (in 5 patients); 3 deaths, 1 neurological deterioration secondary to PICH, and 1 recurrent stroke in the active group (in 5 patients) ($p = 1.0$). All deaths were certified as being due to the index stroke, and the death rate was similar between the two groups ($p = 0.65$). Other than the 7 deaths, no other patients experienced an increase of more than 4 points in NIHSS score. Patient survival to 3 months was similar in the two groups. There were 31 non-serious adverse events in both the placebo group (in 12 patients) and in the active group (in 13 patients) by 3 months ($p = 1.0$). Again, none was attributed to test

treatment. A summary of adverse events occurring in patients with cortical infarcts is shown in table 7.4. The most frequently occurring adverse events were infections (tables 7.5, 7.6, 7.7), but these were less common in patients with cortical infarcts (9 events in the placebo group, 6 in the active group) than in all patients (9 events in the placebo group, 16 in the active group). No test treatment emergent changes of clinical significance were observed in any haematological or biochemical laboratory data.

Table 7.3 *Serious adverse events (SAEs) and non-serious adverse events (AEs): all patients*

		All patients ($n = 34$)	Placebo ($n = 17$)	Active ($n = 17$)
SAEs*	by 72h	5 (0.15)	3 (0.18)	2 (0.12)
	by 3 months	10 (0.29)	5 (0.29)	5 (0.29)
AEs*	by 72h	19 (0.56)	13 (0.76)	6 (0.35)
	by 3 months	62 (1.82)	31 (1.82)	31 (1.82)
Death / increase in NIHSS score by > 4 points	by 72h	5 (14.7%)	3 (17.6%)	2 (11.8%)
	by 3 months	7 (20.6%)	4 (23.5%)	3 (17.6%)

*event rate per patient in parentheses

Table 7.4 *Serious adverse events (SAEs) and non-serious adverse events (AEs): patients with cortical infarcts*

		All patients with cortical infarcts ($n = 24$)	Placebo ($n = 14$)	Active ($n = 10$)
SAEs*	by 72h	4 (0.17)	3 (0.21)	1 (0.10)
	by 3 months	7 (0.29)	4 (0.29)	3 (0.30)
AEs*	by 72h	14 (0.58)	12 (0.86)	2 (0.20)
	by 3 months	40 (1.67)	28 (2.00)	12 (1.20)
Death / increase in NIHSS score by > 4 points	by 72h	4 (16.7%)	3 (21.4%)	1 (10.0%)
	by 3 months	6 (25.0%)	4 (28.6%)	2 (20.0%)

*event rate per patient in parentheses

Table 7.5 *Summary of SAEs and AEs by MedDRA system organ class: all patients*

All adverse events by system organ class	Placebo		Active	
	AE	SAE	AE	SAE
Nervous system disorders	3 (9.7%)	4 (80.0%)	0 (0.0%)	4 (80.0%)
General disorders and administration site conditions	0 (0.0%)	1 (20.0%)	1 (3.2%)	1 (20.0%)
Infections and infestations	9 (29.0%)	0 (0.0%)	16 (51.6%)	0 (0.0%)
Cardiac disorders	4 (12.9%)	0 (0.0%)	2 (6.5%)	0 (0.0%)
Respiratory, thoracic and mediastinal disorders	4 (12.9%)	0 (0.0%)	4 (12.9%)	0 (0.0%)
Gastrointestinal disorders	3 (9.7%)	0 (0.0%)	2 (6.5%)	0 (0.0%)
Injury, poisoning and procedural complications	3 (9.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Musculoskeletal and connective tissue disorders	3 (9.7%)	0 (0.0%)	1 (3.2%)	0 (0.0%)
Reproductive system and breast disorders	1 (3.2%)	0 (0.0%)	1 (3.2%)	0 (0.0%)
Skin and subcutaneous tissue disorders	1 (3.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Blood and lymphatic system disorders	0 (0.0%)	0 (0.0%)	1 (3.2%)	0 (0.0%)
Immune system disorders	0 (0.0%)	0 (0.0%)	1 (3.2%)	0 (0.0%)
Investigations	0 (0.0%)	0 (0.0%)	1 (3.2%)	0 (0.0%)
Psychiatric disorders	0 (0.0%)	0 (0.0%)	1 (3.2%)	0 (0.0%)

***Table 7.6** MedDRA classification of SAEs and AEs: placebo group*

Subject	*Event*	*System organ class*	*Preferred term*	*Severity**
1	1	Musculoskeletal and connective tissue disorders	Tenosynovitis	o
4	1	Gastrointestinal disorders	Gastrointestinal haemorrhage	o
	2	Infections and infestations	Localised infection (gastrostomy site)	o
	3	Reproductive system and breast disorders / Infections and infestations	Epididymitis / Orchitis	o
9	1	Nervous system disorders	Cerebrovascular accident	•
10	1	Infections and infestations	Pneumonia	o
	2	Cardiac disorders	Atrial fibrillation	o
	3	General disorders and administration site conditions	Brain death	•
14	1	Infections and infestations	Lower respiratory tract infection	o
	2	Cardiac disorders	Atrial fibrillation	o
	3	Skin and subcutaneous tissue disorders	Rash	o
	4	Infections and infestations	Localised infection (jejunostomy site)	o
	5	Gastrointestinal disorders	Gastrointestinal haemorrhage	o
	6	Musculoskeletal and connective tissue disorders	Arthralgia	o
	7	Respiratory, thoracic and mediastinal disorders	Pneumonia aspiration	o
16	1	Respiratory, thoracic and mediastinal disorders	Pneumonia aspiration / Pulmonary oedema	o
	2	Infections and infestations	Urinary tract infection	o
	3	Respiratory, thoracic and mediastinal disorders	Pulmonary oedema	o
	4	Nervous system disorders	Haemorrhagic transformation stroke	o
	5	Infections and infestations	Oropharyngeal candidiasis	o
18	1	Nervous system disorders	Headache / Dizziness	o
	2	Respiratory, thoracic and mediastinal disorders	Chronic obstructive airways disease exacerbated	o
20	1	Musculoskeletal and connective tissue disorders	Back pain / Shoulder blade pain	o
	2	Nervous system disorders	Carpal tunnel syndrome	o
	3	Injury, poisoning and procedural complications	Soft tissue injury	o
22	1	Infections and infestations	Pneumonia	o
	2	Cardiac disorders / Respiratory, thoracic and mediastinal disorders	Atrial fibrillation / Pulmonary oedema	o
23	1	Nervous system disorders	Neurodegenerative disorder or neurological disorder not otherwise specified	•
25	1	Injury, poisoning and procedural complications	Fall / Head injury	o
	2	Gastrointestinal disorders	Gastrointestinal haemorrhage	o
	3	Nervous system disorders	Neurodegenerative disorder or neurological disorder not otherwise specified	•
26	1	Injury, poisoning and procedural complications	Thermal burns	o
33	1	Cardiac disorders	Atrial fibrillation	o
	2	Infections and infestations	Urinary tract infection bacterial	o
	3	Nervous system disorders	Neurodegenerative disorder or neurological disorder not otherwise specified / Brain oedema	•
34	1	Infections and infestations	Lower respiratory tract infection	o

* o Non-serious adverse event

• Serious adverse event

***Table** 7.7 MedDRA classification of SAEs and AEs: active group*

Subject	*Event*	*System organ class*	*Preferred term*	*Severity**
2	1	Infections and infestations	Urinary tract infection	o
	2	Infections and infestations	Lung infection pseudomonal	o
	3	Infections and infestations	Enterococcal infection	o
3	1	Infections and infestations	Urinary tract infection	o
6	1	Respiratory, thoracic and mediastinal disorders	Pneumonia aspiration	o
	2	Infections and infestations	Lower respiratory tract infection	o
8	1	Investigations	Chest X-ray abnormal	o
12	1	Infections and infestations	Wound infection	o
	2	Respiratory, thoracic and mediastinal disorders	Pneumonia aspiration	o
15	1	Immune system disorders	Acquired hypogammaglobulinaemia	o
17	1	Infections and infestations	Urosepsis	o
	2	Infections and infestations	Oral candidiasis	o
	3	General disorders and administration site conditions	Acute phase reaction	o
	4	Reproductive system and breast disorders	Ovarian cyst	o
19	1	Nervous system disorders	Neurodegenerative disorder or neurological disorder not otherwise specified	•
	2	Infections and infestations	Lower respiratory tract infection	o
	3	Blood and lymphatic system disorders	Anaemia	o
21	1	Psychiatric disorders	Alcohol withdrawal syndrome	o
24	1	Respiratory, thoracic and mediastinal disorders	Pulmonary embolism	o
	2	Musculoskeletal and connective tissue disorders	Arthralgia	o
27	1	Nervous system disorders	Neurodegenerative disorder or neurological disorder not otherwise specified / Haemorrhagic transformation stroke	•
28	1	Gastrointestinal disorders	Gastrointestinal haemorrhage	o
	2	Nervous system disorders	Neurodegenerative disorder or neurological disorder not otherwise specified	•
	3	Infections and infestations	Clostridium colitis	o
29	1	Infections and infestations	Urinary tract infection bacterial	o
	2	Respiratory, thoracic and mediastinal disorders	Pneumonia aspiration	o
	3	Infections and infestations	Clostridium colitis	o
	4	General disorders and administration site conditions	General physical health deterioration	•
30	1	Nervous system disorders	Cerebrovascular accident	•
31	1	Cardiac disorders	Angina pectoris	o
	2	Gastrointestinal disorders	Diarrhoea	o
	3	Infections and infestations	Urinary tract infection bacterial	o
	4	Infections and infestations	Pneumonia	o
	5	Cardiac disorders	Pericarditis	o
	6	Infections and infestations	Clostridium colitis	o
	7	Infections and infestations	Clostridium colitis	o

* o Non-serious adverse event

• Serious adverse event

7.3.3 *Analysis of biological activity*

Peripheral total WBC counts and neutrophil counts were lower in the rhIL-1ra-treated compared with placebo-treated patients after the initiation of test treatment (figures 7.2a and 7.2b). Median total WBC count was up to 27% lower, and median neutrophil count up to 45% lower, in the rhIL-1ra-treated compared with placebo treated patients. Similar trends were observed in plasma CRP and IL-6 concentrations (figures 7.2c and 7.2d). Individualised data plots for the placebo and active groups are shown in figure 7.3a-f and reveal considerable inter-individual variability.

Figure 7.2a

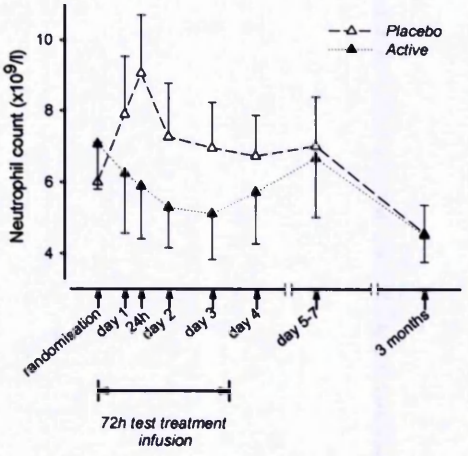


Figure 7.2c

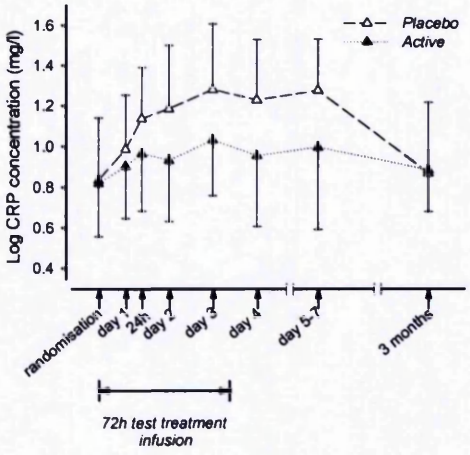


Figure 7.2b

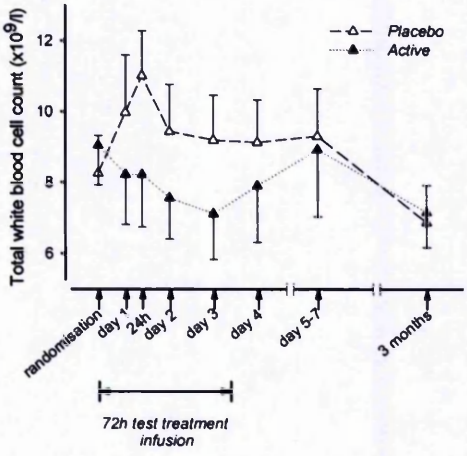


Figure 7.2d

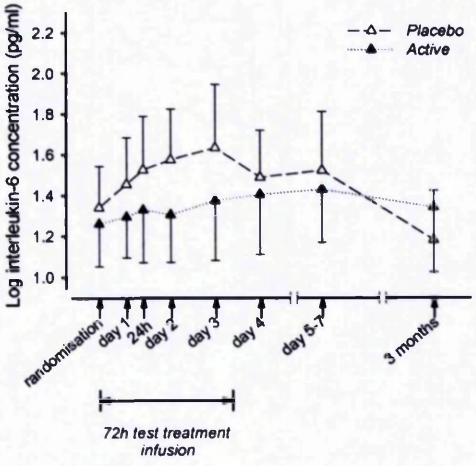


Figure 7.2 *(a) Neutrophil count. (b) Total white cell count. (c) Log CRP concentration. (d) Log interleukin-6 concentration. In each of (a) – (d), data are shown as median (with positive or negative 95% confidence intervals).*

Figure 7.3a

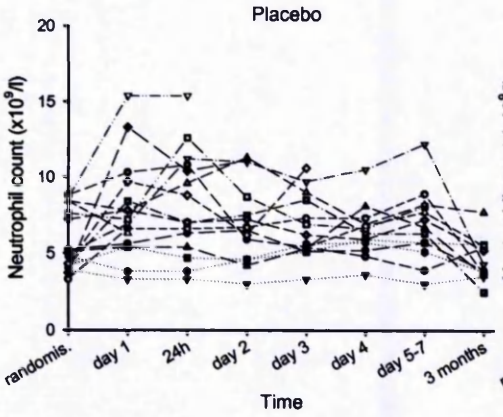


Figure 7.3b

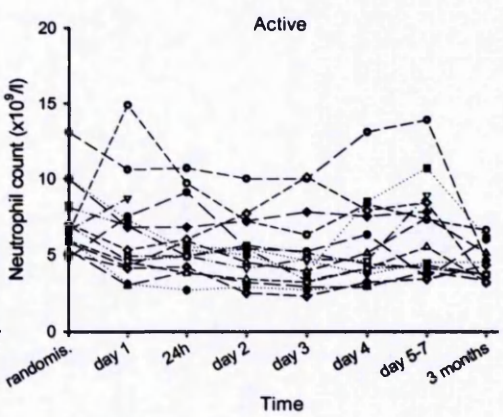


Figure 7.3c

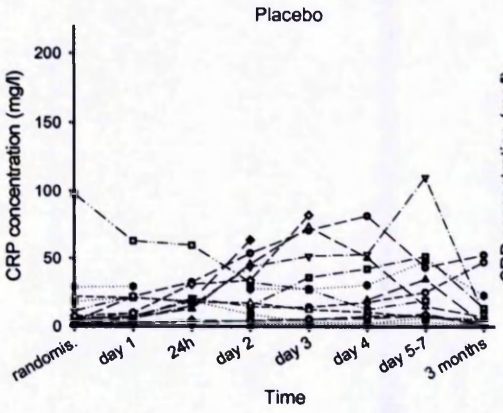


Figure 7.3d

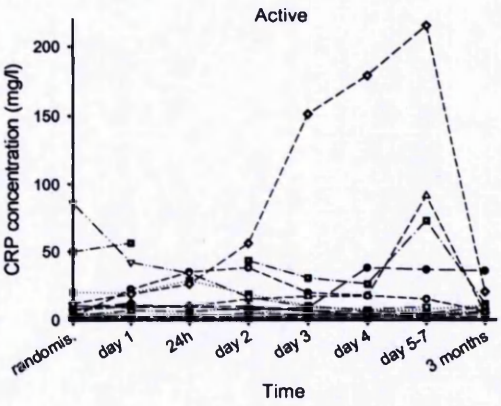


Figure 7.3e

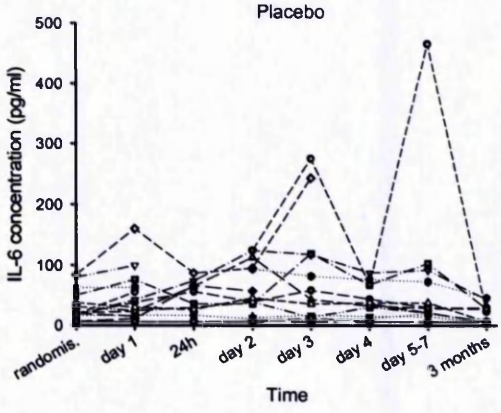


Figure 7.3f

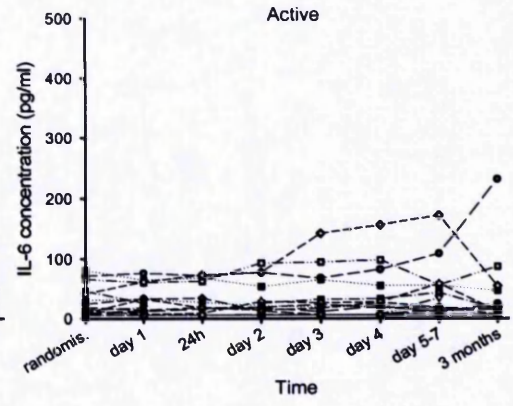


Figure 7.3 *Plots of individual patient data against time in placebo and active groups respectively for neutrophil count (7.3a and 7.3b), CRP concentration (7.3c and 7.3d) and IL-6 concentration (7.3e and 7.3f).*

Mean log CRP concentration was up to 28% lower, and mean log IL-6 concentration up to 23% lower, in the rhIL-1ra-treated compared with placebo treated patients. No differences between the groups were seen in ESR or aural temperature. CT brain infarct volume was similar in the two groups (p = 0.6), mean difference (95% CIs) 4.5 cm^3 (-61.7, 70.7) (table 7.8).

7.3.4 Exploratory efficacy analysis

When all patients were considered, NIHSS scores were better in the active group compared with placebo at 72 hours and at day 5 to 7 (figure 7.4a, table 7.8). At 3 months, more patients receiving rhIL-1ra (29.4%) had a BI of 95 – 100 than those receiving placebo (17.6%) (figure 7.4c, table 7.8). Similarly, 23.5% of patients receiving rhIL-1ra had a modified Rankin scale of 0 – 1 at 3 months compared with 17.6% of those receiving placebo (figure 7.4d, table 7.8). 3-month survival is shown in figure 7.4e. Baseline NIHSS and pre-stroke BI and mRS scores for all patients are shown in table 7.1; secondary outcome data for all patients is shown in table 7.8. Because cortical infarcts are more likely to benefit than other stroke sub-types from neuroprotection (Muir, 2002), analyses of efficacy were pre-specified to include only cortical infarcts. In patients with cortical infarcts, median baseline NIHSS score (IQR) was 14 (11 to 19) and 16 (10 to 20) in the placebo and active groups respectively; pre-stroke median BI (IQR) was 100 (100 to 100) in both groups, and pre-stroke median mRS (IQR) was 1 (0 to 1) and 0 (0 to 0) in the placebo and active groups respectively (table 7.9). NIHSS scores were lower in the rhIL-1ra-treated group compared with placebo at 72 hours and at day 5 to 7, in patients with cortical infarcts (figure 7.4b, table 7.9). In patients with cortical infarcts, there was a median reduction of 4 points in NIHSS score at 3 months in rhIL-1ra treated patients, compared with a change of 1 point in those receiving placebo. At 3 months, a greater proportion of patients receiving rhIL-1ra (30%) had a BI of 95 – 100 than those receiving placebo (14%) (figure 7.4c, table 7.9). Similarly, 30% of patients receiving rhIL-1ra had a mRS of 0 – 1 at 3 months compared with 7% of those receiving placebo (figure 7.4d, table 7.9). A BI of 95 – 100 or a mRS score of 0 – 1 both represent favourable outcomes with minimal or no disability. Survival to 3 months is shown in figure 7.4f.

Table 7.8 *Secondary outcome measures: all patients*

Secondary outcome	All patients ($n = 34$)	Placebo ($n = 17$)	Active ($n = 17$)
*NIHSS at 72h**	10 (5 to 13)	11 (6 to 15)	10 (4 to 13)
0 – 1	3 (8.8%)	0 (0.0%)	3 (17.6%)
2 – 9	11 (32.4%)	6 (35.3%)	5 (29.4%)
10 – 20	13 (38.3%)	7 (41.2%)	6 (35.3%)
≥ 21	7 (20.6%)	4 (23.5%)	3 (17.6%)
*NIHSS at 5-7 days**	12 (6 to 19)	14 (7 to 22)	11 (4 to 17)
0 – 1	2 (5.9%)	0 (0.0%)	2 (11.8%)
2 – 9	12 (35.3%)	7 (41.2%)	5 (29.4%)
10 – 20	12 (35.3%)	6 (35.3%)	6 (35.3%)
≥ 21	7 (20.6%)	4 (23.5%)	3 (17.6%)
*NIHSS at 3 months**	8 (3 to 20)	8 (4 to 31)	7 (3 to 20)
0 – 1	2 (5.9%)	0 (0.0%)	2 (11.8%)
2 – 9	16 (47.1%)	9 (26.5%)	7 (41.2%)
10 – 20	7 (20.6%)	4 (23.5%)	3 (17.6%)
≥ 21	7 (20.6%)	4 (23.5%)	3 (17.6%)
*Barthel Index at 3 months**	40 (0 to 93)	40 (0 to 90)	45 (3 to 100)
95 – 100	8 (23.5%)	3 (17.6%)	5 (29.4%)
55 – 90	7 (20.6%)	5 (29.4%)	2 (11.8%)
0 – 50	11 (32.4%)	5 (29.4%)	6 (35.3%)
Dead	7 (20.6%)	4 (23.5%)	3 (17.6%)
*mRS at 3 months**	4 (2 to 5)	4 (2 to 6)	4 (1 to 5)
0 – 1	7 (20.6%)	3 (17.6%)	4 (23.5%)
2 – 3	8 (23.5%)	5 (29.4%)	3 (17.6%)
4 – 5	11 (32.4%)	5 (29.4%)	6 (35.3%)
Dead	7 (20.6%)	4 (23.5%)	3 (17.6%)
Infarct volume at 5-7 days(cm^3)*	28.3 (7.0 to 97.6) ($n = 21$)	29.4 (3.6 to 114.6) ($n = 12$)	26.6 (10.2 to 95.0) ($n = 9$)

*data shown as median (interquartile range)

Table 7.9 *Secondary outcome measures: patients with cortical infarcts*

Secondary outcome	All patients with cortical infarcts ($n = 24$)	Placebo ($n = 14$)	Active ($n = 10$)
*NIHSS at 72h**	13 (6 to 20)	14 (6 to 26)	10 (3 to 17)
0 – 1	2 (8.3%)	0 (0.0%)	2 (20.0%)
2 – 9	7 (29.2%)	4 (28.6%)	3 (30.0%)
10 – 20	9 (37.5%)	6 (42.9%)	3 (30.0%)
≥ 21	6 (25.0%)	4 (28.6%)	2 (20.0%)
*NIHSS at 5-7 days**	14 (6 to 23)	15 (7 to 29)	10 (4 to 21)
0 – 1	1 (4.2%)	0 (0.0%)	1 (10.0%)
2 – 9	7 (29.2%)	4 (28.6%)	3 (30.0%)
10 – 20	9 (37.5%)	6 (42.9%)	3 (30.0%)
≥ 21	6 (25.0%)	4 (28.6%)	2 (20.0%)
*NIHSS at 3 months**	12 (3 to 42)	13 (5 to 42)	12 (3 to 31)
0 – 1	1 (4.2%)	0 (0.0%)	1 (10.0%)
2 – 9	9 (37.5%)	6 (42.9%)	3 (30.0%)
10 – 20	7 (29.2%)	4 (28.6%)	3 (30.0%)
≥ 21	6 (25.0%)	4 (28.6%)	2 (20.0%)
*Barthel Index at 3 months**	25 (0 to 90)	20 (0 to 86)	25 (0 to 100)
95 – 100	5 (20.8%)	2 (14.3%)	3 (30.0%)
55 – 90	4 (16.7%)	3 (21.4%)	1 (10.0%)
0 – 50	7 (29.2%)	5 (35.7%)	2 (20.0%)
Dead	7 (29.2%)	4 (28.6%)	3 (30.0%)
*mRS at 3 months**	4 (2 to 6)	5 (2 to 6)	4 (1 to 6)
0 – 1	4 (16.7%)	1 (7.1%)	3 (30.0%)
2 – 3	5 (20.8%)	4 (28.6%)	1 (10.0%)
4 – 5	8 (33.3%)	5 (35.7%)	3 (30.0%)
Dead	6 (25.0%)	4 (28.6%)	2 (20.0%)

*data shown as median (interquartile range)

Figure 7.4a

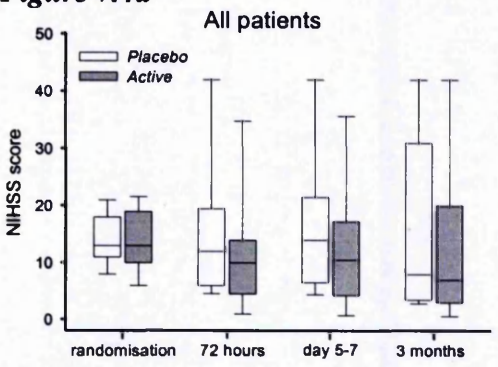


Figure 7.4b

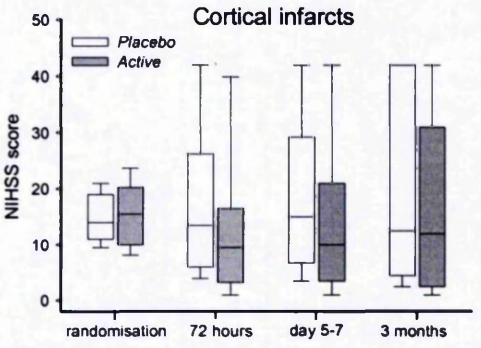


Figure 7.4c

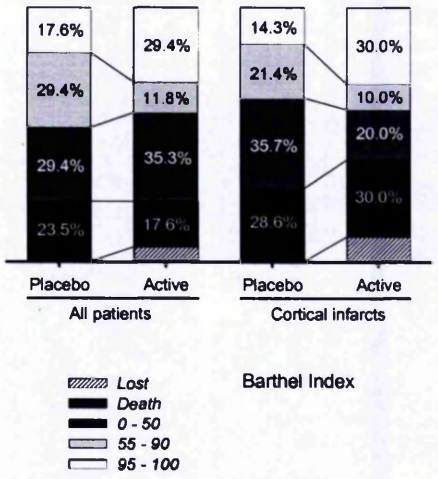


Figure 7.4d

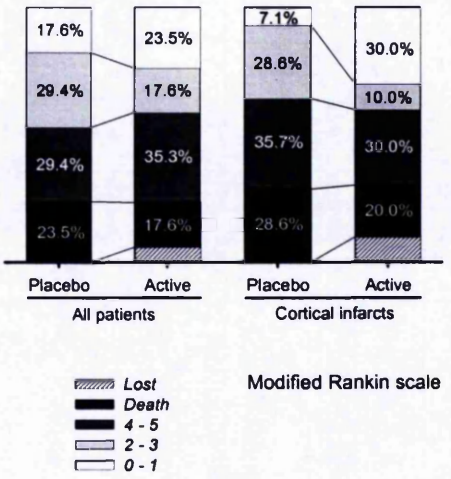


Figure 7.4e

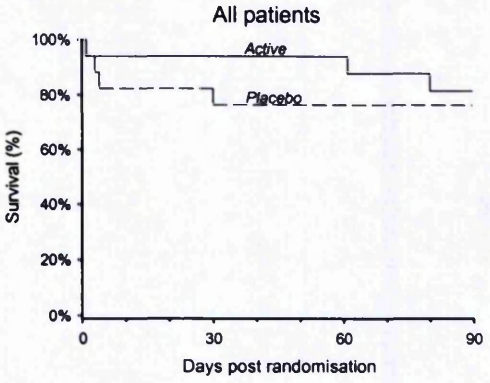


Figure 7.4f

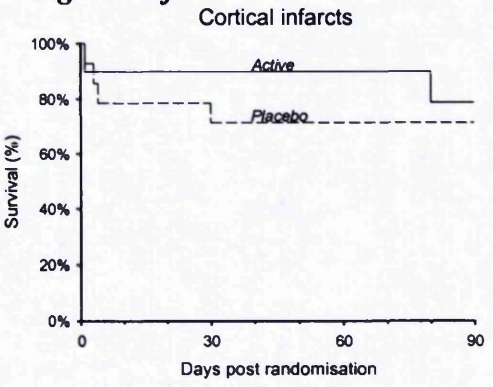


Figure 7.4 *Clinical outcome: Boxplot of NIHSS score (boxes denote medians and interquartile ranges; whiskers denote 5th and 95th centiles) for all patients (a) and cortical infarcts (b) (excluding patients with other stroke subtypes); Barthel index at 3 months (c); modified Rankin scale at 3 months (d); Kaplan-Meier survival curves showing cumulative survival (%) to 3 months by treatment group for all patients (e) and cortical infarcts (f).*

7.4 Discussion

This study suggests that intravenous rhIL-1ra is safe and well tolerated in patients with acute stroke. No deaths or adverse events were attributed to test treatment. Biological activity is suggested by a reduction in total white cell count, neutrophil count, and plasma CRP and IL-6 concentrations in patients receiving rhIL-1ra compared with placebo. Exploratory efficacy analysis indicates a greater proportion of patients receiving rhIL-1ra with minimal or no disability at 3 months compared with placebo.

The single-centre study design gave us the opportunity to conduct a careful, exploratory investigation of biological activity and efficacy in addition to the detailed evaluation of safety. Delayed presentation or uncertain time of onset were the commonest factors limiting recruitment, and this is not without precedent in acute stroke (Barber *et al.*, 2001). Although the time window of 6 hours used here is short, experimental models suggest that benefit will be seen in acute stroke only within a few hours of the onset of cerebral ischaemia. Efficacy has now been demonstrated up to at least 3 hours after initiation of cerebral ischaemia using temporary middle cerebral artery occlusion in rats (Mulcahy *et al.*, 2003), so the median (interquartile range) interval to treatment here of 3.6 hours (2.8 to 5.0) is biologically appropriate.

Administration over 72 hours was based on the profile of biologically active IL-1 in experimental stroke models (Rothwell, 2003) and the time course of emerging injury in patients with acute stroke (Heiss, 2000). This duration of treatment has been suggested to be optimal for ischaemic stroke (Dyker & Lees, 1998). Continuous infusion was required owing to the very short half-life of rhIL-1ra (approx. 3 mins) in plasma (Kim *et al.*, 1995). The dose regime of 100mg bolus followed by 2 mg/kg/h infusion for 72 h was chosen as this is the largest known to have been tolerated previously (Opal *et al.*, 1997) and this regime provided mean (SD) plasma levels of IL-1ra of 25 (13) μg/ml (Fisher *et al.*, 1994b), very similar to those seen in the present study. Importantly, these plasma concentrations are

significantly higher than those achieved in rat models (≥ 100 ng/ml) where rhIL-1ra was an effective treatment (Martin *et al.*, 1994).

These findings are important because rhIL-1ra is a potential neuroprotective agent for stroke that has demonstrated biological activity relevant to the disease process and its clinical outcome. Cellular inflammation is important in the evolution of pathology in cerebral ischaemia both experimentally and clinically (Kochanek and Hallenbeck, 1992; Price *et al.*, 2003), while raised peripheral WBC counts correlate with clinical stroke risk (Prentice *et al.*, 1982), and WBC counts are elevated early after stroke and are associated with poor clinical outcome (Pozzilli *et al.*, 1985a; Emsley *et al.*, 2003). Inhibition of leucocyte trafficking into the central nervous system has been proposed as a therapeutic strategy (Price *et al.*, 2003). Reduced total WBC and neutrophil counts in patients receiving rhIL-1ra in this trial are thus probably of pathophysiological importance in stroke. Evidence for modified cytokine expression in patients with acute stroke derives from studies of plasma, CSF and post-mortem brain tissue (Emsley and Tyrrell, 2002). Inflammation is likely to predispose to stroke (Lindsberg and Grau, 2003), and high circulating levels of inflammatory markers (which may reflect local tissue levels of IL-1), in particular interleukin-6 and CRP, are predictive of poor clinical outcome (Smith *et al.*, 2004), and therefore the effects on plasma IL-6 and CRP concentrations in rhIL-1ra-treated patients are also likely to be relevant.

IL-1 has an important role in host defence against infection (Arend, 2002), and rhIL-1ra has been associated with an increased incidence of serious infections (2%) versus placebo (1%) (data provided by Amgen). Infection was the commonest system organ class of adverse events in this study, representing 29% of AEs in placebo-treated and 52% of AEs in all patients receiving rhIL-1ra. A lower infection rate was seen in patients with cortical infarcts compared with all patients receiving rhIL-1ra. The infectious episodes were predominantly bacterial and typical of infections often seen in patients with acute stroke (Langhorne *et al.*, 2000), rather than atypical, opportunistic, fungal or viral infections, and none was classified as serious. Despite the lower neutrophil count in rhIL-1ra-treated patients, neutropaenia (neutrophil count < $1x10^9$/l) did not occur in any patient during this study. No specific safety issues were raised by this trial, either in

patients with ischaemic stroke or in those with PICH. Despite the smaller than planned sample size, there should be confidence about safety, given the present data, previous data in other conditions, and no theoretical difference in risk in acute stroke.

All measures of clinical outcome were more favourable in the rhIL-1ra-treated group, but these analyses were secondary and exploratory. It is encouraging that 3-month scores on the BI and mRS consistent with minimal or no disability were recorded in a larger proportion of rhIL-1ra-treated than placebo-treated patients, and that this difference was even greater in the patients with cortical infarcts, an effect which has been predicted from pre-clinical studies (Muir, 2002).

This study suggests that rhIL-1ra is safe and well tolerated in acute stroke. In addition, rhIL-1ra exhibited biological activity that is relevant to the pathophysiology and clinical outcome of ischaemic stroke. Further studies are now required in order confirm safety and tolerability, and to test whether the improved clinical outcome measures in rhIL-1ra-treated patients in the present study are maintained.

CHAPTER 8

Summary, conclusions, and future studies

8.1 Introduction

This chapter summarises the contents and outlines the conclusions of this thesis. In addition, the implications of this work for future studies are considered.

8.2 Summary of thesis

8.2.1 Chapter 1

CNS inflammation is important in the pathophysiologal processes occurring after the onset of cerebral ischaemia in ischaemic stroke. In addition, inflammation in the CNS or in the periphery may be a risk factor for the initial development of cerebral ischaemia. Peripheral infection and inflammatory processes are likely to be important in this respect. It appears that inflammation may be important both before, in predisposing to a stroke, and afterwards, where it is an integral part of the mechanisms of cerebral injury and/or repair. Inflammation is mediated by both molecular components, including cytokines, and cellular components, such as leucocytes and microglia, many of which possess pro- and/or anti-inflammatory properties, with harmful and/or beneficial effects. Classic acute phase reactants and body temperature are also modified in stroke, and may be useful both in the prediction of events and outcome, and as therapeutic targets. IL-1 is implicated in cerebral ischaemia in rodents, and IL-1ra limits injury in all forms of experimental ischaemic cerebral injury tested to date. The IL-1 cytokine system is therefore a particularly attractive therapeutic target in stroke.

The aim of this thesis was to test two main hypotheses, namely (1) peripheral inflammatory responses occur in patients with acute stroke, and cytokines, in particular IL-1, have a role in the regulation of these responses, and (2) recombinant human IL-1ra (rhIL-1ra) treatment is safe, well tolerated, and biologically active in patients with acute stroke.

8.2.2 Chapters 2 and 4

Methodology common to the two clinical studies from which work is reported in this thesis was reported in chapter 2, including the location of the research, the clinical services in which patients were managed, patient recruitment, ethical

approval, research assessments, sample collection and laboratory methods, imaging techniques, assessment of atherosclerosis and data storage. Strengths and weaknesses of the methodology used for this work are discussed in the context of results reported in chapters 3, 5 and 7.

The aims of the work reported in chapter 4 were to develop and evaluate sensitive and accurate ELISAs for (1) human IL-1β and (2) human IL-1ra in plasma. This chapter summarises the work undertaken to develop the assays, optimize their methodology and assess their performance. The final assay procedures are reported, together with an account of various problems encountered during their development and how these were overcome. In the IL-1β assay, analyte recovery at 20pg/ml was 77% (± 12%), and the overall minimum sensitivity achieved for the stroke study samples reported in chapter 5 was 7.9 pg/ml. In the IL-1ra assay, analyte recovery at 100 pg/ml was 107% (± 15%), and the overall minimum sensitivity for the stroke study samples was 93 pg/ml. Intra-assay and inter-assay precision data are also presented.

8.2.3 Chapters 3 and 5

The aim of the work reported in chapter 3 was to identify (1) the extent to which a peripheral inflammatory response is activated in patients following acute stroke, and (2) whether there was evidence for pre-existing peripheral inflammation. Thirty-six patients with ischaemic stroke within 12 hours of onset of symptoms had serial blood samples taken up to 12 months for analysis of markers of inflammation. Thirty-six control subjects, individually matched for age, sex and degree of atherosclerosis, were also studied. Median CRP was elevated, relative to controls, from admission until 3 months, the greatest elevation occurring at 5 to 7 days. Elevations were also seen in ESR and WBC count until 3 months. Median plasma IL-6 was also elevated, relative to controls, by 24 hours after onset of symptoms, and remained elevated at 5 to 7 days, but not at 3 months. Less marked elevations in these markers were seen in patients without evidence of infection except for IL-6, which was not increased in the absence of infection. These data provide evidence of an early and sustained peripheral inflammatory response to acute ischaemic stroke in patients with or without evidence of infection. The very early increase in concentrations of

inflammatory markers after stroke may either be induced by stroke itself, or may indicate a pre-existing inflammatory condition in stroke patients which may contribute to the development of stroke. Data from this work concerning the relationship between the inflammatory response and radiological and clinical outcome have been reported separately. These show significant correlations between peak plasma IL-6 concentration (and other peripheral inflammatory markers including CRP) in the first week of ischaemic stroke and both cerebral infarct volume at 5-7 days and long-term clinical outcome (Smith *et al.*, 2004).

The work reported in chapter 5 considered more speculative markers and mediators of inflammation. Plasma cytokine and soluble cytokine receptor concentrations and cytokine production by LPS-stimulated whole-blood were measured. On admission plasma IL-1 receptor antagonist (IL-1ra) concentrations were significantly elevated, relative to controls, but IL-1β, soluble IL-1 receptor type II, TNF-α, soluble TNF receptor types I and II (sTNF-RI, sTNF-RII), IL-10 and leptin were not. The plasma sTNF-RI concentration increased with a similar pattern to TNF-RII and TNF-α. Spontaneous production of these cytokines over 24 h, was minimal. These cytokines were powerfully induced in the presence of LPS, but their production by blood from patients on admission was significantly reduced relative to controls. Increased plasma IL-1ra in patients within 12 h of onset of stroke suggests very early, or pre-existing activation. sTNF-RI shows a similar, but more robust, relationship with stroke severity than TNF-α. Cytokine production by unstimulated blood is minimal, but induced cytokine production in patients is reduced relative to controls after acute ischaemic stroke. Inter-individual differences were seen and may identify vulnerable subjects and help to target future therapies. Data from this work concerning the relationships with radiological and clinical outcome are to be reported separately (CJ Smith, MD thesis to be submitted; Emsley *et al.*, manuscript in preparation). Peak plasma sTNF-RI concentration in the first week correlated strongly with both cerebral infarct volume at 5-7 days and clinical outcome at 3 and 12 months. Low levels of IL-1β, TNF-α and IL-6 production correlate significantly with worse clinical outcome.

8.2.4 Chapters 6 and 7

The cytokine IL-1 mediates ischaemic brain damage in rodents. The endogenous, highly selective, IL-1 receptor antagonist (IL-1ra) protects against ischaemic cerebral injury in a range of experimental settings, and IL-1ra causes a marked reduction of cell death when administered peripherally or at a delay of up to 3 hours in transient cerebral ischaemia. Chapter 6 describes the design of the first randomised, double-blind, placebo-controlled trial of recombinant human IL-1ra (rhIL-1ra) in patients with acute stroke. The clinical hypothesis was that treatment with rhIL-1ra (100 mg iv loading dose, and 2 mg/kg/h iv infusion for 72 h) would be safe and well tolerated in patients with acute stroke. The primary objective was to compare the safety profile of rhIL-1ra with placebo in patients with acute stroke. Secondary objectives were to perform an exploratory efficacy analysis based on comparative 3 month survival curves, changes in neurological impairment as determined by NIHSS score and changes in disability as determined by BI and mRS scores at 3 months, changes in biological markers (including WBC count, ESR, CRP and IL-6), and CT cerebral infarct volume at 5-7 days.

The results of this trial are reported in chapter 7, and they suggest that rhIL-1ra is safe, well tolerated, and biologically active, in a relatively small patient sample. No adverse events were attributed to study treatment among 34 patients randomised. Markers of biological activity, including neutrophil and total WBC counts, CRP and IL-6 concentrations, were lower in rhIL-1ra treated patients. Among patients with cortical infarcts clinical outcomes at 3 months in the rhIL-1ra treated group were better than in placebo treated. These findings are important because rhIL-1ra is both neuroprotective in preclinical models and appears safe when administered to patients. In addition, it has shown evidence of biological activity that is relevant to stroke outcome. These findings identify rhIL-1ra as a potential new therapeutic agent for acute stroke.

8.3 *Conclusions*

In conclusion, this work addressed two main hypotheses:

The first hypothesis is supported by the demonstration of an early and sustained peripheral inflammatory response to acute ischaemic stroke in patients with or without evidence of infection. The data support a role for cytokines, including IL-1, in the regulation of the observed peripheral inflammatory response.

Evidence has also been presented to support the second hypothesis, and suggests that rhIL-1ra treatment is safe, well tolerated, and biologically active in patients with acute stroke. rhIL-1ra is a potential new therapeutic agent for acute stroke.

8.4 Future studies

8.4.1 Studies in ischaemic brain injury

Research in progress is building on the findings of the studies from which work is reported in this thesis. These studies, summarised in figure 8.1, are examining whether (a) differences in inflammatory molecules and/or differences in genes coding for inflammatory mediators predispose to cerebral ischaemic events, (b) there is a relationship between peripheral and brain inflammation, and (c) therapeutic strategies limiting IL-1 actions will lead to benefit in ischaemic brain damage.

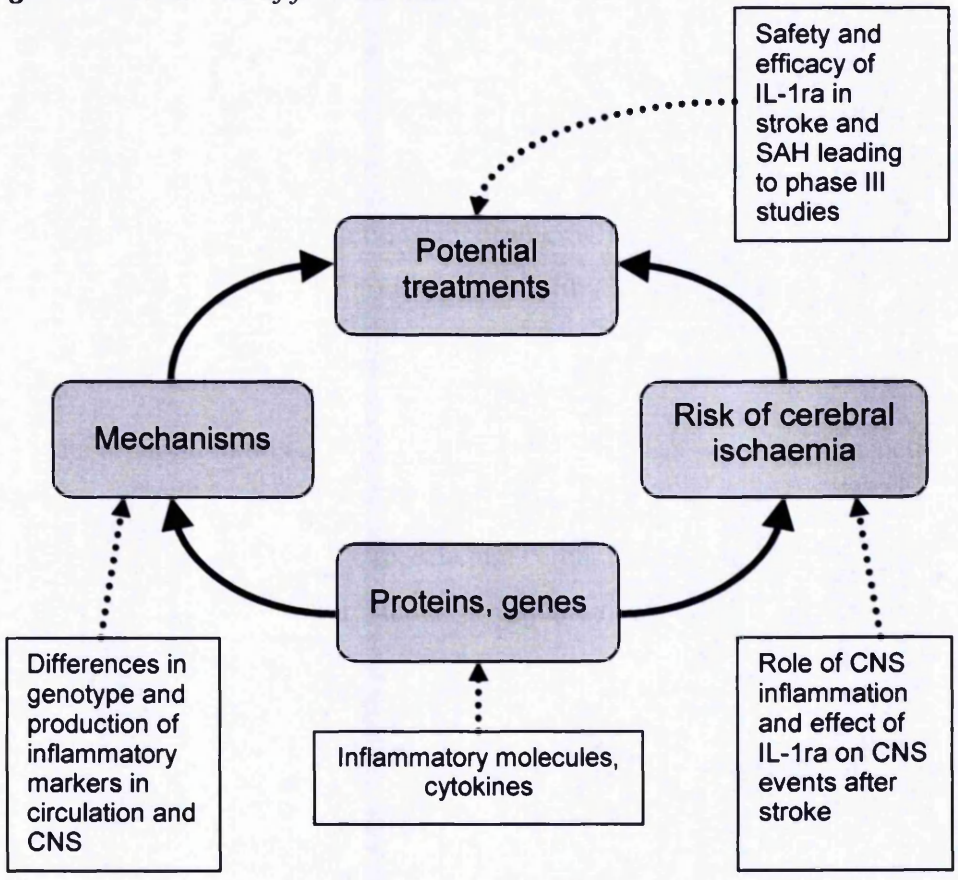
It is important to determine whether peripheral inflammatory markers are predictive of subsequent cerebral ischaemic events, as in the case of coronary events (Ridker, 2003). Such markers should be of clinical benefit as prognostic indicators, allowing the identification of individuals at high risk who may benefit from anti-inflammatory treatment. Polymorphisms in the IL-1 gene family have been associated with inflammatory diseases and recently have been suggested to be susceptibility factors for ischaemic stroke (Um *et al.,* 2003; Seripa *et al.*, 2003). Such cytokine polymorphisms merit further study to determine whether differences in genes coding for inflammatory mediators, such as the IL-1 gene family, predispose to cerebral ischaemic events.

The early, sustained increase in peripheral inflammatory markers reported in this thesis may represent a pre-existing inflammatory condition prior to stroke. There is a great deal of evidence to support cytokines, and in particular IL-1, as mediators of neuronal injury after experimental cerebral ischaemia. However, little data exists on the CNS events occurring early after cerebral ischaemia in patients, or brain events which may predispose to stroke in at risk patients. It is important to identify the relationship between brain and peripheral expression of inflammatory mediators, in order to assess their relevance to CNS events and to characterize those events. It is difficult to study the kinetics of CNS cytokine expression directly in stroke patients, but cerebral ischaemia occurs in other settings, such as in delayed cerebral ischaemia (DCI) after SAH where CSF is sometimes obtainable. It is also possible to use novel imaging techniques such as

[^{11}C]PK11195 PET to determine whether microglial activation is increased (ie evidence of CNS inflammation) in patients with asymptomatic carotid atherosclerosis with elevated concentrations of peripheral inflammatory markers.

The data reported in this thesis concerning rhIL-1ra as a potential therapeutic agent for acute stroke are encouraging. As discussed in chapter 1, extensive pre-clinical data exist for IL-1 in experimental cerebral ischaemia. Further pre-clinical studies of rhIL-1ra are underway, including the effects in aged animals and in white matter injury. Further clinical studies are also essential, including determination of the pharmacokinetics of rhIL-1ra in the CNS, the early effects of rhIL-1ra in stroke using MRI, and the feasibility of administration in patients with SAH. The longer term goal is to undertake phase III efficacy studies of IL-1ra in acute stroke, SAH and, potentially, other settings where inflammatory mechanisms contribute to neuronal injury from cerebral ischaemia, such as head injury.

Figure 8.1 *Schematic of future studies*



8.4.2 *Phase III study design*

A phase III study of rhIL-1ra in acute stroke would require an international, multi-centre design. The planning of such an efficacy trial typically involves knowledge of several basic characteristics, such as the effect size and its variability, so that sample size can be calculated to achieve a specified power when using a test at a given significance level. If these parameters are incorrectly estimated at the outset, the result can be an underpowered (or overpowered) trial. Recent years have seen the increasing use of more flexible trial designs. For example, interim analyses in group sequential trials permit the possibility of determination of efficacy (or safety) prior to the planned end of the study. In the recent trial of UK-279,276 in acute ischaemic stroke, an adaptive design efficiently examined the dose response and recommended early discontinuation (Krams *et al.*, 2003). Use of a sequential design has been examined for the GAIN International Study, and appears to be an attractive option for future stroke clinical trials (Bolland *et al.*, 2004). Key benefits from the use of this approach for a phase III study of rhIL-1ra would appear to be the automatic reaction of trial design should outcome proportions differ from anticipated values, or recruitment be slower than planned.

8.4.3 *Inflammation and carotid atherosclerosis*

Another important potential area for further investigation is the role of inflammation and inflammatory mediators in the development and stability of carotid atherosclerosis. As discussed earlier, atherosclerosis is now recognized as an inflammatory process. Novel imaging techniques such as [^{18}F]-fluorodeoxyglucose (^{18}FDG)-PET are beginning to provide new insights into the pathobiology of atherosclerotic plaque inflammation and plaque rupture (Rudd *et al.*, 2002). IL-1ra gene polymorphisms have recently been proposed as a susceptibility factor in the development of carotid atherosclerosis (Worrall *et al.*, 2003). Increased intima-media thickness (IMT) has been reported in the early phase of the carotid atherosclerosis, and elevated serum levels of inflammatory markers occur in subjects in the highest IMT quartile (Magyar *et al.*, 2003). It would thus be feasible to examine the roles of inflammatory molecules and

differences in inflammatory genes in carotid atherosclerosis, assessed using a technique such as IMT.

8.4.4 Inflammation and other neurological conditions

Research on the role of inflammation and cytokines in cerebral ischaemia is stimulating my interest in their potential role in other neurological conditions, such as certain forms of epilepsy. Febrile seizures (FS) are common in paediatric practice and are associated with hippocampal sclerosis (HS) and temporal lobe epilepsy (TLE). Cytokine genes including IL-1 are up-regulated in experimental seizures (Jankowsky and Patterson, 2001), IL-1β enhances seizure activity, whilst IL-1ra has been shown to be powerfully anticonvulsant (Vezzani *et al.*, 2000). When these observations are considered alongside the pyrogenic actions of IL-1, it is reasonable to hypothesise that altered regulation of either the production or biological effects of IL-1 may be a critical determinant of susceptibility to FS. Clinical studies suggest plasma and CSF cytokine concentrations change in FS (Haspolat *et al.*, 2002; Virta *et al.*, 2002a), and that specific IL-1 gene polymorphisms may increase the risk of FS (Virta *et al.*, 2002b). Research is currently planned to establish (a) whether IL-1 gene polymorphisms are associated with clinical FS, (b) whether circulating cytokine concentrations are modified in patients with FS, (c) whether cytokine levels are modified and whether IL-1 has a pathophysiological role in an experimental model of FS.

A case-control study is planned in which cases with FS and febrile age- and sex-matched control subjects would be recruited. IL-1 genotyping and multiple single nucleotide polymorphism (SNP) haplotype determinations will be undertaken. In addition, plasma and/or CSF concentrations of cytokines and cytokine receptors would be studied in a separate population of children with FS, compared with febrile controls, as part of an ongoing study of children with suspected CNS infections. An experimental model of FS would also be used to characterize changes in cytokine expression after induction of FS, and to establish whether administration of IL-1 or IL-1ra has any effect on the profile of FS.

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APPENDIX

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

CONFIDENTIAL

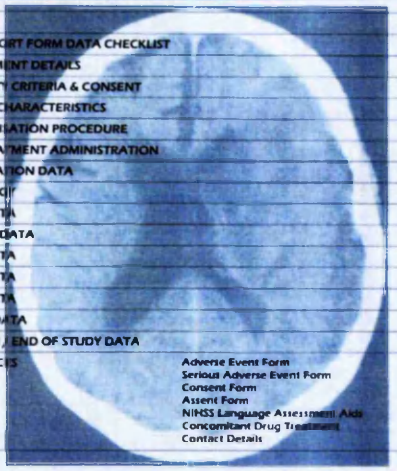
A RANDOMISED, DOUBLE-BLIND, PLACEBO-CONTROLLED PILOT STUDY TO INVESTIGATE THE SAFETY OF ANAKINRA [r-metHuIL-1ra] IN PATIENTS WITH ACUTE STROKE

Principal Investigator: Dr Pippa TYRRELL
Institution: Salford Royal Hospitals NHS Trust
Clinical Sciences Building
Hope Hospital
Salford
M6 8HD
Telephone & Fax: 0161 787 5586
E-Mail: ptyrrell@fs1.ho.man.ac.uk

Ageing

NATIONAL LOTTERY CHARITIES BOARD

THE UNIVERSITY of MANCHESTER



CASE REPORT FORM PAGE 1

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

CASE REPORT FORM DATA CHECKLIST

PRESENTATION DATA COMPLETE HE CS CL EB RD PB Date

RADIOLOGY – CT#1 FILM HE CS CL EB RD PB Date
CT#1 REPORT IN CRF HE CS CL EB RD PB Date
CXR REPORT IN CRF HE CS CL EB RD PB Date
ORIGINAL ECG IN CRF HE CS CL EB RD PB Date
CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
CSB LAB DATA IN CRF HE CS CL EB RD PB Date

DAY 1 DATA COMPLETE HE CS CL EB RD PB Date

CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
CSB LAB DATA IN CRF HE CS CL EB RD PB Date

24 HOUR DATA COMPLETE HE CS CL EB RD PB Date

CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
CSB LAB DATA IN CRF HE CS CL EB RD PB Date

DAY 2 DATA COMPLETE HE CS CL EB RD PB Date

CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
IL-1ra ASSAY RESULT IN CRF HE CS CL EB RD PB Date

DAY 3 DATA COMPLETE HE CS CL EB RD PB Date

CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
IL-1ra ASSAY RESULT IN CRF HE CS CL EB RD PB Date

CASE REPORT FORM PAGE 2

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

CASE REPORT FORM DATA CHECKLIST – cont'd

DAY 4 DATA COMPLETE HE CS CL EB RD PB Date

CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
IL-1ra ASSAY RESULT IN CRF HE CS CL EB RD PB Date

DAY 5-7 DATA COMPLETE HE CS CL EB RD PB Date

RADIOLOGY – CT#2 FILM HE CS CL EB RD PB Date
CT#2 REPORT IN CRF HE CS CL EB RD PB Date
CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
CSB LAB DATA IN CRF HE CS CL EB RD PB Date

3 MONTH / END OF STUDY DATA COMPLETE HE CS CL EB RD PB Date

CLINICAL LAB REPORTS IN CRF HE CS CL EB RD PB Date
CSB LAB DATA IN CRF HE CS CL EB RD PB Date

CASE REPORT FORM PAGE 3

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

RECRUITMENT DETAILS HE CS CL EB Date

Patient Name _____ Hospital Number
Patient Number Date of Recruitment
Date of Birth Date of Onset
Time of Onset : Hours post Stroke : hrs

ELIGIBILITY CRITERIA

INCLUSION CRITERIA	Y	N
Is the patient aged 18 years or over?		
Is there written informed consent or		
Onset of symptoms of acute stroke (excluding		

§ details of significant concurrent medical condition(s)

EXCLUSION CRITERIA	Y	N
Clinically significant concurrent		
Rapid clinical improvement?		
NIHSS score ≤ 4?		
mRS score ≥ 4 in the 4 weeks		
Previously included in current study?		
Receiving/received investigational		
Breast-feeding?		
Positive pregnancy test?		

CONSENT/ASSENT HE CS CL EB RD PB Date Time :

Refer to appendix for consent and assent forms

PATIENT CHARACTERISTICS

Sex Male Female Age ____ years
Race Caucasian Hispanic Black
Oriental Other
Handedness Right-handed Left-handed
Height ____ cm Estimated Measured
Weight ____ kg Estimated Measured
BMI m/kg²
Smoker Yes No Pack Years

RANDOMISATION PROCEDURE **Telephone 0115 9930416**

Ensure that you have Site Number and Access Code available. Press # after every response

Date of birth (dd/mm/yyyy) Male 1 Female 2
NIHSS
Date of onset (dd/mm/yyyy)
Time of onset (24hr clock) :
Allocated treatment boxes (3)
BX BX BX

BioCel

CASE REPORT FORM PAGE 4

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

TEST TREATMENT ADMINISTRATION

Weight range (kg)	Number of vials (100mg) per 12h (n)
46-54	12
55-62	14
63-71	16
72-79	18
80-87	20
88-96	22
97-105	24

Initial bolus dose of 100mg in 1.0ml administered iv over 60 seconds
Then iv infusion of 2mg/kg/h over next 72h (six continuous 12h infusions)

Each 12h infusion contains n vials of anakinra or placebo in a total volume of 500ml normal saline administered via infusion pump at 42ml/h

NB If serum creatinine>177µmol/l reduce infusion rate to 1mg/kg/h, ie halve number of vials in the infusion

Bolus (100mg) Date given HE CS CL
Time given : EB

Infusion No.		Planned times	Actual times	Made up by	Started by	Serum creat.	No. of vials	Box Number(s)
1	Start date & time	:	:	HE	HE	<177µmol/l		
				CS	CS	Yes		
	End date & time			CL	CL	No		
		:	:	EB	EB	Reduce to 1mg/kg/h		
2	Start date & time	:	:	HE	HE	<177µmol/l		
				CS	CS	Yes		
	End date & time			CL	CL	No		
		:	:	EB	EB	Reduce to 1mg/kg/h		
3	Start date & time	:	:	HE	HE	<177µmol/l		
				CS	CS	Yes		
	End date & time			CL	CL	No		
		:	:	EB	EB	Reduce to 1mg/kg/h		
4	Start date & time	:	:	HE	HE	<177µmol/l		
				CS	CS	Yes		
	End date & time			CL	CL	No		
		:	:	EB	EB	Reduce to 1mg/kg/h		
5	Start date & time	:	:	HE	HE	<177µmol/l		
				CS	CS	Yes		
	End date & time			CL	CL	No		
		:	:	EB	EB	Reduce to 1mg/kg/h		
6	Start date & time	:	:	HE	HE	<177µmol/l		
				CS	CS	Yes		
	End date & time			CL	CL	No		
		:	:	EB	EB	Reduce to 1mg/kg/h		

CASE REPORT FORM **PAGE 5**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

PRESENTATION DATA HE CS CL EB Date

MEDICAL, SURGICAL HISTORY & CONCOMITANT DISEASES

Allergic/Immunologic	No	Yes	Specify
Dermatological	No	Yes	Specify
Ophthalmological	No	Yes	Specify
ENT	No	Yes	Specify
Endocrine	No	Yes	Specify
Cardiovascular	No	Yes	Specify
Respiratory	No	Yes	Specify
Gastrointestinal	No	Yes	Specify
Genitourinary	No	Yes	Specify
Neurological	No	Yes	Specify
Musculoskeletal	No	Yes	Specify
Haematological	No	Yes	Specify
Psychiatric	No	Yes	Specify
Previous surgery	No	Yes	Specify
Other	No	Yes	Specify

Source of history (eg patient, spouse etc) _____

Concomitant Medications – Please enter in appendix

VITAL SIGNS

Pulse bpm Temperature .°C

Blood Pressure / mmHg

PHYSICAL EXAMINATION

			Specify any abnormality
Cardiovascular			
JVP	Normal	Abnormal	
Carotid Bruits	Absent	Present	
Heart Sounds	Normal	Abnormal	
Murmurs	Absent	Present	
Peripheral Pulses	Present	Absent	
Respiratory system	Normal	Abnormal	
Abdomen	Normal	Abnormal	
Musculoskeletal	Normal	Abnormal	

(excluding abnormalities attributable to present stroke)

Other (please specify) _____

CASE REPORT FORM **PAGE 6**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

PRESENTATION DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) **ITEMS 1 – 4**

ITEM	DESCRIPTION	SCORE
1a Level of consciousness: The investigator must choose a response, even if a full evaluation is prevented by such obstacles as an ET tube, language barrier, orotracheal trauma/bandages. A 3 is scored only if the patient makes no movement (other than reflexic posturing) in response to noxious stimulation	Alert, keenly responsive Not alert, but rousable by minor stimulation to obey, answer, or respond Not alert, requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped) Responds only with reflex motor or autonomic effects, or totally unresponsive flaccid, areflexic	0 1 2 3
1b LOC questions: The patient is asked the month and his/her age. The answer must be correct- there is no partial credit for being close. Aphasic and stuporous patients who do not comprehend the questions will score 2. Patients unable to speak because of endotracheal intubation, orotracheal trauma, severe dysarthria from any cause, language barrier or any other problem not secondary to aphasia are given a 1. It is important that only the initial answer be graded and the examiner not "help" the patient with verbal or non-verbal cues.	Answers both correctly Answers one correctly Answers neither correctly	0 1 2
1c LOC commands: The patient is asked to open and close the eyes, and then to grip and release the non-paretic hand. Substitute another one step command if the hands cannot be used. Credit is given if an unequivocal attempt is made but not completed due to weakness. If the patient does not respond to command, the task should be demonstrated to them and score the result (i.e. follows none, one or two commands). Patients with trauma, amputation or other physical impediments should be given suitable one-step commands. Only the first attempt is scored.	Performs both tasks correctly Performs one task correctly Performs neither task correctly	0 1 2
2 Best gaze: Only horizontal eye movements will be tested. Voluntary or reflexic (oculocephalic) eye movements will be scored but caloric testing is not done. If the patient has a conjugate deviation of the eyes that can be overcome by voluntary or reflexic activity the score will be 1. If a patient has an isolated peripheral nerve paresis (CN III, IV or VI) score a 1. Gaze is testable in all aphasic patients. Patients with ocular trauma, bandages and pre-existing blindness or other disorders of visual acuity or fields should be tested with reflexic movements and a choice made by the investigator. Establishing eye contact and then moving about the patient from side to side will occasionally clarify the presence of a partial gaze palsy.	Normal Partial gaze palsy; abnormal but not forced deviation Forced deviation/total gaze paresis not overcome by the oculocephalic manoeuvre	0 1 2
3 Visual: Visual fields (upper and lower quadrants) are tested by confrontation using finger counting or visual threat as appropriate. Patient must be encouraged but if they can look at the side of the moving fingers appropriately, this can be scored as normal. If there is unilateral blindness or enucleation, visual fields in the remaining eye are scored. Score 1 if only a clear cut asymmetry, including quadrantanopia is found. If the patient is blind from any cause, score 3. Double simultaneous stimulation is performed at this point. If there is extinction patient receives a 1 and the results are used to score question 11.	No visual loss Partial hemianopia Complete hemianopia Bilateral hemianopia (blind including cortical blindness)	0 1 2 3
4 Facial palsy: Ask, or use pantomime to encourage the patient to show teeth or raise eyebrows and close eyes. Score symmetry of grimace in response to noxious stimuli in the poorly responsive or non-responding patient. If facial trauma/bandages, orotracheal tube, tape or other physical barrier obscures the face, these should be removed to the extent possible.	Normal symmetrical movement Minor paralysis (flattened nasolabial fold, asymmetry on smiling) Partial paralysis (total or near total paralysis of lower face) Complete paralysis of on or both sides (absence of facial movements in the upper and lower face)	0 1 2 3

CASE REPORT FORM **PAGE 7**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

PRESENTATION DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) **ITEMS 5 – 9**

ITEM	DESCRIPTION	SCORE	
5 & 6 Motor arm and leg: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine) and the leg 30 degrees (always tested supine). Drift is scored if the arm falls before 10 seconds or the leg before 5 seconds. The aphasic patient is encouraged using urgency in the voice and pantomime but not noxious stimulation. Each limb is tested in turn, beginning with the non-paretic arm. Only in the case of amputation or joint fusion at the shoulder or the hip may the score be '9' and the examiner must clearly write the explanation for scoring as a '9'.	No drift, limb holds 90 (or 45) degrees for full 10 seconds. Drift. Limb holds 90 (or 45) degrees, but drifts down before full 10 seconds; does not hit bed or other support. Some effort against gravity, limb cannot maintain (if cued) 90 (or 45) degrees, drifts down to bed, but has some effort against gravity. No effort against gravity, limb falls. No movement Amputation, joint fusion explain:	0 1 2 3 4 9	R arm L arm R leg L leg Total
7 Limb ataxia: This test is aimed at finding evidence of a unilateral cerebellar lesion. Test with eyes open. In case of visual defect, ensure test is done in intact visual field. The finger-nose and heel-shin tests are done on both sides, and ataxia is only scored if present out of proportion to weakness. Ataxia is absent in the patient who cannot understand or who is paralysed. Only in case of amputation or joint fusion may the item be scored '9', and the examiner must clearly write the explanation for not scoring. In case of blindness, test by touching nose from extended arm position.	Absent. Present in one limb. Present in two limbs. If present, is ataxia in Right arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Right leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain	0 1 2	
8 Sensory: Sensation or grimace to pin prick when tested, or withdrawal from noxious stimuli in the obtunded or aphasic patient. Only sensory loss attributed to stroke is scored as abnormal and the examiner should test as many body areas as needed to accurately check for hemisensory loss. A score of 2 should only be given when a severe or total loss of sensation can be clearly demonstrated. Stuporous or aphasic patients will therefore probably score 1 or 0. The patient with brainstem stroke who has bilateral loss of sensation is scored 2. If the patient does not respond and is quadriplegic score 2. Patients in coma (item 1a = 3) are arbitrarily given a 2 on this item.	Normal, no sensory loss Mild to moderate sensory loss; patients feels pinprick is less sharp or dull on the affected side; or there is a loss of superficial pain with pinprick but patient is aware he/she is being touched Severe to total sensory loss; patient is not aware of being touched in the face, arm and leg.	0 1 2	
9 Best language: The patient is asked to describe what is happening in the attached picture, to name the items on the attached naming sheet and to read from the attached list of sentences. Comprehension is judged from responses here as well as to all the commands in the preceding general neurological exam. If visual loss interferes with tests ask the patient to identify objects placed in the hand, repeat, and produce speech. The intubated patient should be asked to write. The patient in coma (1a=3) will arbitrarily score 3 on this item. The examiner must choose a score in the patient with stupor or limited cooperation but a score of 3 should only be used if the patient is mute and follows no one step commands.	No aphasia, normal Mild to moderate aphasia; some obvious loss of fluency or facility of comprehension, without significant limitation of ideas or form of expression. Reduction of speech and/or comprehension, however, makes conversation about provided material difficult or impossible. For example in conversation about provided material examiner can identify picture or naming card from patients response Severe aphasia; all communication is through fragmentary expression; great need for inference, questioning and guessing by listener. Range of information that can be exchanged is limited; listener carries burden of communication. Examiner cannot identify materials provided from patients response Mute, global aphasia; no usable speech or auditory comprehension	0 1 2 3	

CASE REPORT FORM **PAGE 8**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

PRESENTATION DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) ITEMS 10 – 11

ITEM	DESCRIPTION	SCORE
10 Dysarthria: If patient is thought to be normal an adequate sample of speech must be obtained by asking patient to read or repeat words from the attached list. If the patient has severe aphasia, the clarity of articulation of spontaneous speech can be rated. Only if the patient is intubated or has other physical barrier to producing speech may the item be scored '9' and the examiner must clearly write an explanation for not scoring. Do not tell the patient why he/she is being tested.	Normal Mild to moderate; patient slurs at least some words and, at worst, can be understood with some difficulty. Severe; patients speech is so slurred as to be unintelligible in the absence of, or out of proportion to any dysphasia, or is mute/ aphasic. Intubated or other physical barrier, explain	0 1 2 9
11 Extinction and inattention (formerly neglect): Sufficient information to identify neglect may be obtained during the prior testing. If the patient has a severe visual loss preventing visual double simultaneous stimulation, and the cutaneous stimuli are normal, the score is normal. If the patient has aphasia but does appear to attend to both sides then the score is normal. The presence of visual spatial neglect or anosagnosia may also be taken as evidence of abnormality. Since the abnormality is scored only if present, the item is never untestable.	No abnormality Visual, tactile, auditory, spatial or personal inattention or extinction to bilateral simultaneous stimulation in one of the sensory modalities. Profound hemi-inattention or hemi-inattention to more than one modality. Does not recognize own hand or orients to only one side of space.	0 1 2
	TOTAL	

CASE REPORT FORM PAGE 9

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

PRESENTATION DATA

BARTHEL INDEX

Based on the 4 weeks prior to stroke

INDEX ITEM	SCORE	DESCRIPTION	
BOWELS		Incontinent or needs enemas Occasional incontinence (<once per week) Continent	0 5 10
BLADDER		Incontinent/unable to manage catheter Occasional accident (<once per day) Continent	0 5 10
GROOMING		Needs help with shaving, washing, hair or teeth Independent	0 5
TOILET USE		Dependent Needs some help Independent on, off, dressing and cleaning	0 5 10
FEEDING		Dependent Needs some help (eg with cutting, spreading) Independent if food provided within reach	0 5 10
TRANSFER (eg bed to chair)		Unable and no sitting balance Needs major help Needs minor help	0 5 10
MOBILITY		Unable Wheelchair independent indoors Walks with help or supervision	0 5 10
DRESSING		Dependent Needs some help Independent including fasteners	0 5 10
STAIRS		Unable Needs some help or supervision Independent up and down	0 5 10
BATHING		Dependent Independent in bath or shower	0 5
	TOTAL		

MODIFIED RANKIN SCALE

Based on the 4 weeks prior to stroke

DESCRIPTION	GRADE
No symptoms at all	0
No significant disability despite symptoms: able to carry out all usual duties and activities	1
Slight disability: unable to carry out all previous activities but able to look after own affairs	2
Moderate disability: Requiring some help, but able to walk without assistance	3
Moderately severe disability: unable to walk without assistance and unable to attend to own	4
Severe disability: bedridden, incontinent and requiring constant nursing care and attention	5

CASE REPORT FORM PAGE

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

PRESENTATION DATA

BASELINE STROKE DATA
List Neurological Deficits

SITE OF INSULT
- Right hemisphere
- Left hemisphere

OCSP CLASSIFICATION

TACS Total Anterior Circulation Syndrome
All of
Hemiplegia contralateral to the cerebral lesion
Hemianopia contralateral to the cerebral lesion
New disturbance of higher cerebral function (eg dysphasia, visuospatial disturbance)

PACS Partial Anterior Circulation Syndrome
Any of
Motor/sensory deficit + hemianopia
Motor/sensory deficit + new higher cerebral dysfunction
New higher cerebral dysfunction + hemianopia
Pure motor/sensory deficit less extensive than for LACS (eg monoparesis)
New higher cerebral dysfunction alone
When more than one type of deficit is present, they must all reflect damage in the same cerebral hemisphere

POCS Posterior Circulation Syndrome
Any of
Ipsilateral cranial nerve palsy (single or multiple) with contralateral motor and/or sensory deficit
Bilateral motor and/or sensory deficit
Disorder of conjugate eye movement (horizontal or vertical)
Cerebellar dysfunction without ipsilateral long tract deficit (as seen in ataxic hemiparesis)
Isolated hemianopia or cortical blindness
Cases where there is disturbance of higher cortical function alongside any of the above should be considered as POCS

LACS Lacunar Syndromes
Definition
Maximum deficit from a single vascular event
No visual field deficit
No new disturbance of higher cortical function
No sign of brainstem disturbance

Categories
Pure motor stroke (PMS), pure sensory stroke (PSS), Ataxic hemiparesis (AH) and sensorimotor stroke (SMS)
To be acceptable as a PMS, PSS or SMS, the relevant deficit must involve at least two out of three areas of the face, arm and leg, and, with particular reference to the arm, should involve the whole limb and not just the hand

CASE REPORT FORM PAGE 11

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

PRESENTATION DATA HE CS CL ED RD PB Date

PERIPHERAL VASCULAR DISEASE ASSESSMENT

Claudication Yes No Not known

Claudication distance (yards): <50, 51-200, 201-500, >500

Site			
Foot	Right	Left	
Calf	Right	Left	
Thigh	Right	Left	
Buttock	Right	Left	
Critical ischaemia	Rest pain	Right	Left
	Ulcer	Right	Left
	Gangrene	Right	Left

Time since onset

Pulses present

Carotid	Right	Left
Radial	Right	Left
Femoral	Right	Left
Popliteal	Right	Left
Dors Pedis	Right	Left
Post Tib	Right	Left

Doppler pressures (mmHg)

Right brachial	
Left brachial	
Right ankle	
Right ABPI	
Left ankle	
Left ABPI	

DEGREE OF ATHEROSCLEROSIS Significant Non-significant

CASE REPORT FORM PAGE 12

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

PRESENTATION DATA

HAEMATOLOGY AND BIOCHEMISTRY

Full blood count: Hb g/l; Wcc x10⁹; Neutrophils x10⁹; Lymphocytes x10⁹; Monocytes x10⁹; Eosinophils x10⁹; Basophils x10⁹; Platelets x10⁹; ESR mm/hr

Urea and electrolytes: Na mmol/l; K mmol/l; Urea mmol/l; Creatinine µmol/l

Liver function tests: Bilirubin µmol/l; Albumin g/l; Globulin g/l; ALP U/l; Total protein g/l; ALT g/l

Bone profile: Calcium mmol/l; Corr. Ca^{2+} mmol/l; Phosphate mmol/l; Glucose mmol/l; Total cholesterol mmol/l

BIOLOGICAL MARKERS

CRP mg/l; Plasma cortisol nmol/l; IL-1β pg/ml; IL-1β production pg/ml; IL-1sRI pg/ml; IL-1sRII pg/ml; IL-1ra pg/ml; IL-6 pg/ml; IL-6 production pg/ml

TNFa pg/ml; TNF-RI pg/ml; TNF-RII pg/ml; IL-1β mRNA; IL-6 mRNA; Leptin pg/ml; Urinary neopterin: creatinine ratio

ECG

Date; Time

Heart rate bpm

Rhythm: SR; AF; other

Specify abnormalities _____

CASE REPORT FORM **PAGE 13**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

RADIOLOGY

CHEST X-RAY

Within normal limits: Yes / No; Date

Specify abnormalities _____

CT BRAIN SCANS

SCAN #1 Date; Time; Time since stroke onset hrs

Report _____

SCAN #2 Date; Time; Days since stroke onset days

Report _____

Infarct volumes

Scan #1 cm^3

Scan #2 cm^3

Scan #1 used only if scan #2 is unavailable

CASE REPORT FORM **PAGE 14**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

DAY 1 DATA Hb; Cl; Cl; EB; BD; PB; Date

PATIENT STATUS

Alive; Dead; Date of death; Time

Specify primary cause of death _____

Withdrawn; Date of withdrawal; Time

Specify reason for withdrawal _____

Infusion stopped; Times stopped / restarted _____

Specify reason _____

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

CHECK INFUSION TIMES PAGE 5

VITAL SIGNS

Pulse bpm; Temperature °C

Blood Pressure / mmHg

HAEMATOLOGY AND BIOCHEMISTRY

Full blood count: Hb g/l; Wcc x10⁹; Neutrophils x10⁹; Lymphocytes x10⁹; Monocytes x10⁹; Eosinophils x10⁹; Basophils x10⁹; Platelets x10⁹; ESR mm/hr

Urea and electrolytes: Na mmol/l; K mmol/l; Urea mmol/l; Creatinine µmol/l

Liver function tests: Bilirubin µmol/l; Albumin g/l; Globulin g/l; ALP U/l; Total protein g/l; ALT g/l

Bone profile: Calcium mmol/l; Corr. Ca^{2+} mmol/l; Phosphate mmol/l; Glucose mmol/l

BIOLOGICAL MARKERS

CRP mg/l; Plasma cortisol nmol/l; IL-1β pg/ml; IL-1β production pg/ml; IL-1sRI pg/ml; IL-1sRII pg/ml; IL-1ra pg/ml; IL-6 pg/ml; IL-6 production pg/ml

TNFa pg/ml; TNF-RI pg/ml; TNF-RII pg/ml; IL-1β mRNA; IL-6 mRNA; Leptin pg/ml; Urinary neopterin: creatinine ratio

CASE REPORT FORM **PAGE 15**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

24 HOUR DATA Hb; Cl; Cl; EB; BD; PB; Date

PATIENT STATUS

Alive; Dead; Date of death; Time

Specify primary cause of death _____

Withdrawn; Date of withdrawal; Time

Specify reason for withdrawal _____

Infusion stopped; Times stopped / restarted _____

Specify reason _____

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

CHECK INFUSION TIMES PAGE 5

VITAL SIGNS

Pulse bpm; Temperature °C

Blood Pressure / mmHg

HAEMATOLOGY

Full blood count: Hb g/l; Wcc x10⁹; Neutrophils x10⁹; Lymphocytes x10⁹; Monocytes x10⁹; Eosinophils x10⁹; Basophils x10⁹; Platelets x10⁹; ESR mm/hr

BIOLOGICAL MARKERS

CRP mg/l; Plasma cortisol nmol/l; IL-1β pg/ml; IL-1β production pg/ml; IL-1sRI pg/ml; IL-1sRII pg/ml; IL-1ra pg/ml; IL-6 pg/ml; IL-6 production pg/ml

TNFa pg/ml; TNF-RI pg/m; TNF-RII pg/m; IL-1β mRNA; IL-6 mRNA; Leptin pg/m; Urinary neopterin: creatinine ratio

CASE REPORT FORM **PAGE 16**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

DAY 2 DATA HE CS CL EB RD PB Date

PATIENT STATUS

Alive Dead Date of death Time :

Specify primary cause of death

Withdrawn Date of withdrawal Time :

Specify reason for withdrawal

Infusion stopped Times stopped / restarted

Specify reason

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

CHECK INFUSION TIMES PAGE 5

VITAL SIGNS

Pulse bpm Temperature . °C

Blood Pressure / mmHg

HAEMATOLOGY, BIOCHEMISTRY and IL-1ra

Full blood count	Hb	g/l	Liver function tests	Bilirubin	µmol/l
	Wcc	x10⁹		Albumin	g/l
	Neutrophils	x10⁹		Globulin	g/l
	Lymphocytes	x10⁹		ALP	U/l
	Monocytes	x10⁹		Total protein	g/l
	Eosinophils	x10⁹		ALT	g/l
	Basophils	x10⁹	Bone profile	Calcium	mmol/l
	Platelets	x10⁹		Corr. Ca^{2+}	mmol/l
Urea and electrolytes	Na	mmol/l		Phosphate	mmol/l
	K	mmol/l		Glucose	mmol/l
	Urea	mmol/l		IL-1ra	pg/ml
	Creatinine	µmol/l			

CASE REPORT FORM **PAGE 17**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

DAY 3 DATA HE CS CL EB RD PB Date

PATIENT STATUS

Alive Dead Date of death Time :

Specify primary cause of death

Withdrawn Date of withdrawal Time :

Specify reason for withdrawal

Infusion stopped Times stopped / restarted

Specify reason

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

CHECK INFUSION TIMES PAGE 5

VITAL SIGNS

Pulse bpm Temperature . °C

Blood Pressure / mmHg

HAEMATOLOGY, BIOCHEMISTRY and IL-1ra

Full blood count	Hb	g/l	Liver function tests	Bilirubin	µmol/l
	Wcc	x10⁹		Albumin	g/l
	Neutrophils	x10⁹		Globulin	g/l
	Lymphocytes	x10⁹		ALP	U/l
	Monocytes	x10⁹		Total protein	g/l
	Eosinophils	x10⁹		ALT	g/l
	Basophils	x10⁹	Bone profile	Calcium	mmol/l
	Platelets	x10⁹		Corr. Ca^{2+}	mmol/l
Urea and electrolytes	Na	mmol/l		Phosphate	mmol/l
	K	mmol/l		Glucose	mmol/l
	Urea	mmol/l		IL-1ra	pg/ml
	Creatinine	µmol/l			

CASE REPORT FORM **PAGE 18**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

DAY 3/DAY 4 DATA*

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) **ITEMS 1 – 4**

ITEM	DESCRIPTION	SCORE
1a Level of consciousness: The investigator must choose a response, even if a full evaluation is prevented by such obstacles as an ET tube, language barrier, orotracheal trauma/bandages. A 3 is scored only if the patient makes no movement (other than reflexic posturing) in response to noxious stimulation	Alert, keenly responsive Not alert, but rousable by minor stimulation to obey, answer, or respond Not alert, requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped) Responds only with reflex motor or autonomic effects, or totally unresponsive flaccid, areflexic	0 1 2 3
1b LOC questions: The patient is asked the month and his/her age. The answer must be correct - there is no partial credit for being close. Aphasic and stuporous patients who do not comprehend the questions will score 2. Patients unable to speak because of endotracheal intubation, orotracheal trauma, severe dysarthria from any cause, language barrier or any other problem not secondary to aphasia are given a 1. It is important that only the initial answer be graded and the examiner not "help" the patient with verbal or non-verbal cues.	Answers both correctly Answers one correctly Answers neither correctly	0 1 2
1c LOC commands: The patient is asked to open and close the eyes, and then to grip and release the non-paretic hand. Substitute another one step command if the hands cannot be used. Credit is given if an unequivocal attempt is made but not completed due to weakness. If the patient does not respond to command, the task should be demonstrated to them and scored the result (i.e. follows none, one or two commands). Patients with trauma, amputation or other physical impediments should be given suitable one-step commands. Only the first attempt is scored.	Performs both tasks correctly Performs one task correctly Performs neither task correctly	0 1 2
2 Best gaze: Only horizontal eye movements will be tested. Voluntary or reflexic [oculocephalic] eye movements will be scored but caloric testing is not done. If the patient has a conjugate deviation of the eyes that can be overcome by voluntary or reflexic activity the score will be 1. If a patient has an isolated peripheral nerve paresis (CN III, IV or VI) score a 1. Gaze is testable in all aphasic patients. Patients with ocular trauma, bandages and pre-existing blindness or other disorders of visual acuity or fields should be tested with reflexic movements and a choice made by the investigator. Establishing eye contact and then moving about the patient from side to side will occasionally clarify the presence of a partial gaze palsy.	Normal Partial gaze palsy; abnormal but not forced deviation Forced deviation/total gaze paresis not overcome by the oculocephalic manoeuvre	0 1 2
3 Visual: Visual fields (upper and lower quadrants) are tested by confrontation using finger counting or visual threat as appropriate. Patient must be encouraged but if they can look at the side of the moving fingers appropriately, this can be scored as normal. If there is unilateral blindness or enucleation, visual fields in the remaining eye are scored. Score 1 if only a clear cut asymmetry, including quadrantanopia is found. If the patient is blind from any cause, score 3. Double simultaneous stimulation is performed at this point. If there is extinction patient receives a 1 and the results are used to score question 11.	No visual loss Partial hemianopia Complete hemianopia Bilateral hemianopia (blind including cortical blindness)	0 1 2 3
4 Facial palsy: Ask, or use pantomime to encourage the patient to show teeth or raise eyebrows and close eyes. Score symmetry of grimace in response to noxious stimuli in the poorly responsive or non-responding patient. If facial trauma/bandages, orotracheal tube, tape or other physical barrier obscures the face, these should be removed to the extent possible.	Normal symmetrical movement Minor paralysis (flattened nasolabial fold, asymmetry on smiling) Partial paralysis (total or near total paralysis of lower face) Complete paralysis of on or both sides (absence of facial movements in the upper and lower face)	0 1 2 3

CASE REPORT FORM **PAGE 19**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

DAY 3/DAY 4 DATA*

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) **ITEMS 5 – 9**

ITEM	DESCRIPTION	SCORE	
5 & 6 Motor arm and leg: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine) and the leg 30 degrees (always tested supine). Drift is scored if the arm falls before 10 seconds or the leg before 5 seconds. The aphasic patient is encouraged using urgency in the voice and pantomime but not noxious stimulation. Each limb is tested in turn, beginning with the non-paretic arm. Only in the case of amputation or joint fusion at the shoulder or the hip may the score be '9' and the examiner must clearly write the explanation for scoring as a '9'.	No drift, limb holds 90 (or 45) degrees for full 10 seconds. Drift, Limb holds 90 (or 45) degrees, but drifts down before full 10 seconds; does not hit bed or other support. Some effort against gravity, limb cannot maintain (if cued) 90 (or 45) degrees, drifts down to bed, but has some effort against gravity. No effort against gravity, limb falls. No movement Amputation, joint fusion explain:	0 1 2 3 4 9	R arm L arm R leg L leg Total
7 Limb ataxia: This test is aimed at finding evidence of a unilateral cerebellar lesion. Test with eyes open. In case of visual defect, ensure test is done in intact visual field. The finger-nose and heel-shin tests are done on both sides, and ataxia is only scored if present out of proportion to weakness. Ataxia is absent in the patient who cannot understand or who is paralysed. Only in case of amputation or joint fusion may the item be scored '9', and the examiner must clearly write the explanation for not scoring. In case of blindness, test by touching nose from extended arm position.	Absent. Present in one limb. Present in two limbs. If present, is ataxia in Right arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Right leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain	0 1 2	
8 Sensory: Sensation or grimace to pin prick when tested, or withdrawal from noxious stimuli in the obtunded or aphasic patient. Only sensory loss attributed to stroke is scored as abnormal and the examiner should test as many body areas as needed to accurately check for hemisensory loss. A score of 2 should only be given when a severe or total loss of sensation can be clearly demonstrated. Stuporous or aphasic patients will therefore probably score 1 or 0. The patient with brainstem stroke who has bilateral loss of sensation is scored 2. If the patient does not respond and is quadriplegic score 2. Patients in coma (item 1a = 3) are arbitrarily given a 2 on this item.	Normal, no sensory loss Mild to moderate sensory loss; patients feels pinprick is less sharp or dull on the affected side; or there is a loss of superficial pain with pinprick but patient is aware he/she is being touched Severe to total sensory loss; patient is not aware of being touched to the face, arm and leg.	0 1 2	
9 Best language: The patient is asked to describe what is happening in the attached picture, to name the items on the attached naming sheet and to read from the attached list of sentences. Comprehension is judged from responses here as well as to all the commands in the preceding general neurological exam. If visual loss interferes with tests ask the patient to identify objects placed in the hand, repeat, and produce speech. The intubated patient should be asked to write. The patient in coma (1a=3) will arbitrarily score 3 on this item. The examiner must choose a score in the patient with stupor or limited cooperation but a score of 3 should only be used if the patient is mute and follows no one step commands.	No aphasia, normal Mild to moderate aphasia; some obvious loss of fluency or facility of comprehension, without significant limitation of ideas or form of expression. Reduction of speech and/or comprehension, however, makes conversation about provided material difficult or impossible. For example in conversation about provided material examiner can identify picture or naming card from patients response Severe aphasia; all communication is through fragmentary expression; great need for inference, questioning and guessing by listener. Range of information that can be exchanged is limited; listener carries burden of communication. Examiner cannot identify materials provided from patients response. Mute, global aphasia; no usable speech or auditory comprehension	0 1 2 3	

CASE REPORT FORM **PAGE 20**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

DAY 3/DAY 4 DATA*

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) ITEMS 10 – 11

ITEM	DESCRIPTION	SCORE
10 Dysarthria: If patient is thought to be normal an adequate sample of speech must be obtained by asking patient to read or repeat words from the attached list. If the patient has severe aphasia, the clarity of articulation of spontaneous speech can be rated. Only if the patient is intubated or has other physical barrier to producing speech may the item be scored 9 and the examiner must clearly write an explanation for not scoring. Do not tell the patient why he/she is being tested.	Normal. Mild to moderate; patient slurs at least some words and, at worst, can be understood with some difficulty. Severe; patients speech is so slurred as to be unintelligible in the absence of, or out of proportion to any dysphasia, or is mute/aphasic. Intubated or other physical barrier, explain	0 1 2 9
11 Extinction and inattention (formerly neglect): Sufficient information to identify neglect may be obtained during the prior testing. If the patient has a severe visual loss preventing visual double simultaneous stimulation, and the cutaneous stimuli are normal, the score is normal. If the patient has aphasia but does appear to attend to both sides then the score is normal. The presence of visual spatial neglect or anosagnosia may also be taken as evidence of abnormality. Since the abnormality is scored only if present, the item is never untestable.	No abnormality. Visual, tactile, auditory, spatial or personal inattention or extinction to bilateral simultaneous stimulation in one of the sensory modalities. Profound hemi-inattention or hemi-inattention to more than one modality. Does not recognize own hand or orients to only one side of space.	0 1 2
		TOTAL

*To be done day 3 if patient randomised before 9am day 0, day 4 if patient randomised after 9am, day 0

CASE REPORT FORM **PAGE 21**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

DAY 4 DATA HE CS CL EB RD PB Date

PATIENT STATUS

Alive Dead Date of death Time

Specify primary cause of death

Withdrawn Date of withdrawal Time

Specify reason for withdrawal

Infusion stopped Times stopped / restarted

Specify reason

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

VITAL SIGNS

Pulse bpm Temperature °C

Blood Pressure / mmHg

HAEMATOLOGY, BIOCHEMISTRY and IL-1ra

Full blood count: Hb g/l; Wcc x10⁹; Neutrophils x10⁹; Lymphocytes x10⁹; Monocytes x10⁹; Eosinophils x10⁹; Basophils x10⁹; Platelets x10⁹

Liver function tests: Bilirubin µmol/l; Albumin g/l; Globulin g/l; ALP U/l; Total protein g/l; ALT g/l

Bone profile: Calcium mmol/l; Corr. Ca^{2+} mmol/l; Phosphate mmol/l; Glucose mmol/l

Urea and electrolytes: Na mmol/l; K mmol/l; Urea mmol/l; Creatinine µmol/l

IL-1ra pg/ml

CASE REPORT FORM **PAGE 22**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

DAY 5-7 DATA HE CS CL EB RD PB Date

PATIENT STATUS

Alive Dead Date of death Time

Specify primary cause of death

Withdrawn Date of withdrawal Time

Specify reason for withdrawal

Infusion stopped Times stopped / restarted

Specify reason

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

VITAL SIGNS

Pulse bpm Temperature °C

Blood Pressure / mmHg

HAEMATOLOGY AND BIOCHEMISTRY

Full blood count: Hb g/l; Wcc x10⁹; Neutrophils x10⁹; Lymphocytes x10⁹; Monocytes x10⁹; Eosinophils x10⁹; Basophils x10⁹; Platelets x10⁹; ESR mm/hr

Liver function tests: Bilirubin µmol/l; Albumin g/l; Globulin g/l; ALP U/l; Total protein g/l; ALT g/l

Bone profile: Calcium mmol/l; Corr. Ca^{2+} mmol/l; Phosphate mmol/l

Urea and electrolytes: Na mmol/l; K mmol/l; Urea mmol/l; Creatinine µmol/l

BIOLOGICAL MARKERS

CRP mg/l; Plasma cortisol nmol/l; IL-1β pg/ml; IL-1β production pg/ml; IL-1sRI pg/ml; IL-1sRII pg/ml; IL-1ra pg/ml; IL-6 pg/ml; IL-6 production pg/ml

TNFα pg/ml; TNF-RI pg/ml; TNF-RII pg/ml; IL-1β mRNA; IL-6 mRNA; Leptin pg/ml; Urinary neopterin: creatinine ratio

CASE REPORT FORM **PAGE 23**

Subject number

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

DAY 5-7 DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) ITEMS 1 – 4

ITEM	DESCRIPTION	SCORE
1a Level of consciousness: The investigator must choose a response, even if a full evaluation is prevented by such obstacles as an ET tube, language barrier, orotracheal trauma/bandages. A 3 is scored only if the patient makes no movement (other than reflexic posturing) in response to noxious stimulation	Alert, keenly responsive Not alert, but rousable by minor stimulation to obey, answer, or respond Not alert, requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped) Responds only with reflex motor or autonomic effects, or totally unresponsive flaccid, areflexic	0 1 2 3
1b LOC questions: The patient is asked the month and his/her age. The answer must be correct- there is no partial credit for being close. Aphasic and stuporous patients who do not comprehend the questions will score 2. Patients unable to speak because of endotracheal intubation, orotracheal trauma, severe dysarthria from any cause, language barrier or any other problem not secondary to aphasia are given a 1. It is important that only the initial answer be graded and the examiner not "help" the patient with verbal or non-verbal cues.	Answers both correctly Answers one correctly Answers neither correctly	0 1 2
1c LOC commands: The patient is asked to open and close the eyes, and then to grip and release the non-paretic hand. Substitute another one step command if the hands cannot be used. Credit is given if an unequivocal attempt is made but not completed due to weakness. If the patient does not respond to command, the task should be demonstrated to them and score the result (i.e. follows none, one or two commands). Patients with trauma, amputation or other physical impediments should be given suitable one-step commands. Only the first attempt is scored.	Performs both tasks correctly Performs one task correctly Performs neither task correctly	0 1 2
2 Best gaze: Only horizontal eye movements will be tested. Voluntary or reflexic (oculocephalic) eye movements will be scored but caloric testing is not done. If the patient has a conjugate deviation of the eyes that can be overcome by voluntary or reflexic activity the score will be 1. If a patient has an isolated peripheral nerve paresis (CN III, IV or VI) score a 1. Gaze is testable in all aphasic patients. Patients with ocular trauma, bandages and pre-existing blindness or other disorders of visual acuity or fields should be tested with reflexic movements and a choice made by the investigator. Establishing eye contact and then moving about the patient from side to side will occasionally clarify the presence of a partial gaze palsy.	Normal Partial gaze palsy; abnormal but not forced deviation Forced deviation/total gaze paresis not overcome by the oculocephalic manoeuvre	0 1 2
3 Visual: Visual fields (upper and lower quadrants) are tested by confrontation using finger counting or visual threat as appropriate. Patient must be encouraged but if they can look at the side of the moving fingers appropriately, this can be scored as normal. If there is unilateral blindness or enucleation, visual fields in the remaining eye are scored. Score 1 if only a clear cut asymmetry, including quadrantanopia is found. If the patient is blind from any cause, score 3. Double simultaneous stimulation is performed at this point. If there is extinction patient receives a 1 and the results are used to score question 11.	No visual loss Partial hemianopia Complete hemianopia Bilateral hemianopia (blind including cortical blindness)	0 1 2 3
4 Facial palsy: Ask, or use pantomime to encourage the patient to show teeth or raise eyebrows and close eyes. Score symmetry of grimace in response to noxious stimuli in the poorly responsive or non-responding patient. If facial trauma/bandages, orotracheal tube, tape or other physical barrier obscures the face, these should be removed to the extent possible.	Normal symmetrical movement Minor paralysis (flattened nasolabial fold, asymmetry on smiling) Partial paralysis (total or near total paralysis of lower face) Complete paralysis of on or both sides (absence of facial movements in the upper and lower face)	0 1 2 3

CASE REPORT FORM **PAGE 24**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

DAY 5-7 DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) **ITEMS 5 – 9**

ITEM	DESCRIPTION	SCORE
5 & 6 Motor arm and leg: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine) and the leg 30 degrees (always tested supine). Drift is scored if the arm falls before 10 seconds or the leg before 5 seconds. The aphasic patient is encouraged using urgency in the voice and pantomime but not noxious stimulation. Each limb is tested in turn, beginning with the non-paretic arm. Only in the case of amputation or joint fusion at the shoulder or the hip may the score be '9' and the examiner must clearly write the explanation for scoring as a '9'.	No drift, limb holds 90 (or 45) degrees for full 10 seconds. Drift, limb holds 90 (or 45) degrees, but drifts down before full 10 seconds; does not hit bed or other support. Some effort against gravity, limb cannot maintain (if cued) 90 (or 45), degrees, drifts down to bed, but has some effort against gravity. No effort against gravity, limb falls. No movement. Amputation, joint fusion explain:	0 R arm 1 L arm 2 R leg 3 L leg 4 9 Total
7 Limb ataxia: This test is aimed at finding evidence of a unilateral cerebellar lesion. Test with eyes open. In case of visual defect, ensure test is done in intact visual field. The finger-nose and heel-shin tests are done on both sides, and ataxia is only scored if present out of proportion to weakness. Ataxia is absent in the patient who cannot understand or who is paralysed. Only in case of amputation or joint fusion may the item be scored '9', and the examiner must clearly write the explanation for not scoring. In case of blindness, test by touching nose from extended arm position.	Absent. Present in one limb. Present in two limbs. If present, is ataxia in: Right arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Right leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain	0 1 2
8 Sensory: Sensation or grimace to pin prick when tested, or withdrawal from noxious stimuli in the obtunded or aphasic patient. Only sensory loss attributed to stroke is scored as abnormal and the examiner should test as many body areas as needed to accurately check for hemisensory loss. A score of 2 should only be given when a severe or total loss of sensation can be clearly demonstrated. Stuporous or aphasic patients will therefore probably score 1 or 0. The patient with brainstem stroke who has bilateral loss of sensation is scored 2. If the patient does not respond and is quadriplegic score 2. Patients in coma (item 1a = 3) are arbitrarily given a 2 on this item.	Normal, no sensory loss. Mild to moderate sensory loss; patients feels pinprick is less sharp or dull on the affected side; or there is a loss of superficial pain with pinprick but patient is aware he/she is being touched. Severe to total sensory loss; patient is not aware of being touched in the face, arm and leg.	0 1 2
9 Best language: The patient is asked to describe what is happening in the attached picture, to name the items on the attached naming sheet and to read from the attached list of sentences. Comprehension is judged from responses here as well as to all the commands in the preceding general neurological exam. If visual loss interferes with tests ask the patient to identify objects placed in the hand, repeat, and produce speech. The intubated patient should be asked to write. The patient in coma (1a=3) will arbitrarily score 3 on this item. The examiner must choose a score in the patient with stupor or limited cooperation but a score of 3 should only be used if the patient is mute and follows no one step commands.	No aphasia, normal. Mild to moderate aphasia; some obvious loss of fluency or facility of comprehension, without significant limitation of ideas or form of expression. Reduction of speech and/or comprehension, however, makes conversation about provided material difficult or impossible. For example in conversation about provided material examiner can identify picture or naming card from patients response. Severe aphasia; all communication is through fragmentary expression; great need for inference, questioning and guessing by listener. Range of information that can be exchanged is limited; listener carries burden of communication. Examiner cannot identify materials provided from patients response. Mute, global aphasia; no usable speech or auditory comprehension.	0 1 2 3

CASE REPORT FORM **PAGE 25**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

DAY 5-7 DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) **ITEMS 10 – 11**

ITEM	DESCRIPTION	SCORE
10 Dysarthria: If patient is thought to be normal an adequate sample of speech must be obtained by asking patient to read or repeat words from the attached list. If the patient has severe aphasia, the clarity of articulation of spontaneous speech can be rated. Only if the patient is intubated or has other physical barrier to producing speech may the item be scored '9' and the examiner must clearly write an explanation for not scoring. Do not tell the patient why he/she is being tested.	Normal. Mild to moderate; patient slurs at least some words and, at worst, can be understood with some difficulty. Severe; patients speech is so slurred as to be unintelligible in the absence of, or out of proportion to any dysphasia, or is mute/aphasic. Intubated or other physical barrier, explain:	0 1 2 9
11 Extinction and inattention (formerly neglect): Sufficient information to identify neglect may be obtained during the prior testing. If the patient has a severe visual loss preventing visual double simultaneous stimulation, and the cutaneous stimuli are normal, the score is normal. If the patient has aphasia but does appear to attend to both sides then the score is normal. The presence of visual spatial neglect or anosagnosia may also be taken as evidence of abnormality. Since the abnormality is scored only if present, the item is never untestable.	No abnormality. Visual, tactile, auditory, spatial or personal inattention or extinction to bilateral simultaneous stimulation in one of the sensory modalities. Profound hemi-inattention or hemi-inattention to more than one modality. Does not recognize own hand or orients to only one side of space.	0 1 2
		TOTAL

OCSP CLASSIFICATION

TACS Total Anterior Circulation Syndrome
All of
- Hemiplegia contralateral to the cerebral lesion
- Hemianopia contralateral to the cerebral lesion
- New disturbance of higher cerebral function (eg dysphasia, visuospatial disturbance)

PACS Partial Anterior Circulation Syndrome
Any of
- Motor/sensory deficit + hemianopia
- Motor/sensory deficit + new higher cerebral dysfunction
- New higher cerebral dysfunction + hemianopia
- Pure motor/sensory deficit less extensive than for LACS (eg monoparesis)
- New higher cerebral dysfunction alone

When more than one type of deficit is present, they must all reflect damage in the same cerebral hemisphere

POCS Posterior Circulation Syndrome
Any of
- Ipsilateral cranial nerve palsy (single or multiple) with contralateral motor and/or sensory deficit
- Bilateral motor and/or sensory deficit
- Disorder of conjugate eye movement (horizontal or vertical)
- Cerebellar dysfunction without ipsilateral long-tract deficit (as seen in ataxic hemiparesis)
- Isolated hemianopia or cortical blindness
- Cases where there is disturbance of higher cortical function alongside any of the above should be considered as POCS

LACS Lacunar Syndromes
Definition
Maximum deficit from a single vascular event
No visual field deficit
No new disturbance of higher cortical function
No sign of brainstem disturbance

Categories
Pure motor stroke (PMS), pure sensory stroke (PSS), Ataxic hemiparesis (AH) and sensorimotor stroke (SMS)
To be acceptable as a PMS, PSS or SMS, the relevant deficit must involve at least two out of three areas of the face, arm and leg, and, with particular reference to the arm, should involve the whole limb and not just the hand

CASE REPORT FORM **PAGE 26**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

3 MONTH / END OF STUDY HE C3 C4 ESR PCR PB Date

PATIENT STATUS

Alive Dead Date of death

Specify primary cause of death

Withdrawn Date of withdrawal

Specify reason for withdrawal

Place of residence Own home Residential home Nursing home Other (specify)

Adverse event(s) Refer to appendix

Serious adverse event(s) Refer to appendix

MEDICAL, SURGICAL HISTORY & CONCOMITANT DISEASES

Allergic/Immunologic	No	Yes	Specify
Dermatological	No	Yes	Specify
Ophthalmological	No	Yes	Specify
ENT	No	Yes	Specify
Endocrine	No	Yes	Specify
Cardiovascular	No	Yes	Specify
Respiratory	No	Yes	Specify
Gastrointestinal	No	Yes	Specify
Genitourinary	No	Yes	Specify
Neurological	No	Yes	Specify
Musculoskeletal	No	Yes	Specify
Haematological	No	Yes	Specify
Psychiatric	No	Yes	Specify
Other	No	Yes	Specify

PHYSICAL EXAMINATION

			Specify any abnormality
Cardiovascular			
JVP	Normal	Abnormal	
Carotid Bruits	Absent	Present	
Heart Sounds	Normal	Abnormal	
Murmurs	Absent	Present	
Peripheral Pulses	Present	Absent	
Respiratory system	Normal	Abnormal	
Abdomen	Normal	Abnormal	
Musculoskeletal	Normal	Abnormal	

(excluding abnormalities attributable to present stroke)

Other (please specify)

CASE REPORT FORM **PAGE 27**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

3 MONTH / END OF STUDY DATA

VITAL SIGNS

Pulse bpm Temperature °C

Blood Pressure mmHg

HAEMATOLOGY AND BIOCHEMISTRY

Full blood count	Hb	g/l	**Liver function tests**	Bilirubin	µmol/l	
	Wcc	x10⁹		Albumin	g/l	
	Neutrophils	x10⁹		Globulin	g/l	
	Lymphocytes	x10⁹		ALP	u/l	
	Monocytes	x10⁹		Total protein	g/l	
	Eosinophils	x10⁹		ALT	g/l	
	Basophils	x10⁹	**Bone profile**	Calcium	mmol/l	
	Platelets	x10⁹		Corr. Ca^{2+}	mmol/l	
	ESR	mm/hr		Phosphate	mmol/l	
Urea and electrolytes	Na	mmol/l		Glucose	mmol/l	
	K	mmol/l				
	Urea	mmol/l				
	Creatinine	µmol/l				

BIOLOGICAL MARKERS

CRP	mg/l	TNFa	pg/ml
Plasma cortisol	nmol/l	TNF-RI	pg/ml
IL-1β	pg/ml	TNF-RII	pg/ml
IL-1β production	pg/ml	IL-1β mRNA	
IL-1sRI	pg/ml	IL-6 mRNA	
IL-1sRII	pg/ml	Leptin	pg/ml
IL-1ra	pg/ml	Urinary neopterin: creatinine ratio	
IL-6	pg/ml		
IL-6 production	pg/ml		

CASE REPORT FORM **PAGE 28**

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

3 MONTH / END OF STUDY DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) ITEMS 1 – 4

ITEM	DESCRIPTION	SCORE
1a Level of consciousness: The investigator must choose a response, even if a full evaluation is prevented by such obstacles as an ET tube, language barrier, orotracheal trauma/bandages. A 3 is scored only if the patient makes no movement (other than reflexic posturing) in response to noxious stimulation	Alert, keenly responsive Not alert, but rousable by minor stimulation to obey, answer, or respond Not alert, requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped) Responds only with reflex motor or autonomic effects, or totally unresponsive, flaccid, areflexic	0 1 2 3
1b LOC questions: The patient is asked the month and his/her age. The answer must be correct- there is no partial credit for being close. Aphasic and stuporous patients who do not comprehend the questions will score 2. Patients unable to speak because of endotracheal intubation, orotracheal trauma, severe dysarthria from any cause, language barrier or any other problem not secondary to aphasia are given a 1. It is important that only the initial answer be graded and the examiner not "help" the patient with verbal or non-verbal cues.	Answers both correctly Answers one correctly Answers neither correctly	0 1 2
1c LOC commands: The patient is asked to open and close the eyes, and then to grip and release the non-paretic hand. Substitute another one step command if the hands cannot be used. Credit is given if an unequivocal attempt is made but not completed due to weakness. If the patient does not respond to command, the task should be demonstrated to them and score the result (i.e. follows none, one or two commands). Patients with trauma, amputation or other physical impediments should be given suitable one-step commands. Only the first attempt is scored.	Performs both tasks correctly Performs one task correctly Performs neither task correctly	0 1 2
2 Best gaze: Only horizontal eye movements will be tested. Voluntary or reflexic (oculocephalic) eye movements will be scored but caloric testing is not done. If the patient has a conjugate deviation of the eyes that can be overcome by voluntary or reflexic activity the score will be 1. If a patient has an isolated peripheral nerve paresis (CN III, IV or VI) score a 1. Gaze is testable in all aphasic patients. Patients with ocular trauma, bandages and pre-existing blindness or other disorders of visual acuity or fields should be tested with reflexic movements and a choice made by the investigator. Establishing eye contact and then moving about the patient from side to side will occasionally clarify the presence of a partial gaze palsy.	Normal Partial gaze palsy; abnormal but not forced deviation Forced deviation/total gaze paresis not overcome by the oculocephalic maneuvre	0 1 2
3 Visual: Visual fields (upper and lower quadrants) are tested by confrontation using finger counting or visual threat as appropriate. Patient must be encouraged but if they can look at the side of the moving fingers appropriately, this can be scored as normal. If there is unilateral blindness or enucleation, visual fields in the remaining eye are scored. Score 1 if only a clear cut asymmetry, including quadrantanopia is found. If the patient is blind from any cause, score 3. Double simultaneous stimulation is performed at this point. If there is extinction patient receives a 1 and the results are used to score question 11.	No visual loss Partial hemianopsia Complete hemianopsia Bilateral hemianopsia (blind including cortical blindness)	0 1 2 3
4 Facial palsy: Ask, or use pantomime to encourage the patient to show teeth or raise eyebrows and close eyes. Score symmetry of grimace in response to noxious stimuli in the poorly responsive or non-responding patient. If facial trauma/bandages, orotracheal tube, tape or other physical barrier obscures the face, these should be removed to the extent possible.	Normal symmetrical movements Minor paralysis (flattened nasolabial fold, asymmetry on smiling) Partial paralysis (total or near total paralysis of lower face) Complete paralysis of on or both sides (absence of facial movements in the upper and lower face)	0 1 2 3

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

3 MONTH / END OF STUDY DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) ITEMS 5 – 9

ITEM	DESCRIPTION	SCORE	
5 & 6 Motor arm and leg: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine) and the leg 30 degrees (always tested supine). Drift is scored if the arm falls before 10 seconds or the leg before 5 seconds. The aphasic patient is encouraged using urgency in the voice and pantomime but not noxious stimulation. Each limb is tested in turn, beginning with the non-paretic arm. Only in the case of amputation or joint fusion at the shoulder or the hip may the score be '9' and the examiner must clearly write the explanation for scoring as a '9'.	No drift, limb holds 90 (or 45) degrees for full 10 seconds. Drift. Limb holds 90 (or 45) degrees, but drifts down before full 10 seconds; does not hit bed or other support. Some effort against gravity, limb cannot maintain (if cued) 90 (or 45) degrees, drifts down to bed, but has some effort against gravity. No effort against gravity, limb falls. No movement Amputation, joint fusion explain:	0 1 2 3 4 9	R arm L arm R leg L leg Total
7 Limb ataxia: This test is aimed at finding evidence of a unilateral cerebellar lesion. Test with eyes open. In case of visual defect, ensure test is done in intact visual field. The finger-nose and heel-shin tests are done on both sides, and ataxia is only scored if present out of proportion to weakness. Ataxia is absent in the patient who cannot understand or who is paralysed. Only in case of amputation or joint fusion may the item be scored '9', and the examiner must clearly write the explanation for not scoring. In case of blindness, test by touching nose from extended arm position.	Absent. Present in one limb. Present in two limbs. If present, is ataxia in Right arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left arm 1 = Yes 2 = No 9 = amputation or joint fusion, explain Right leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain Left leg 1 = Yes 2 = No 9 = amputation or joint fusion, explain	0 1 2	
8 Sensory: Sensation or grimace to pin prick when tested, or withdrawal from noxious stimuli in the obtunded or aphasic patient. Only sensory loss attributed to stroke is scored as abnormal and the examiner should test as many body areas as needed to accurately check for hemisensory loss. A score of 2 should only be given when a severe or total loss of sensation can be clearly demonstrated. Stuporous or aphasic patients will therefore probably score 1 or 0. The patient with brainstem stroke who has bilateral loss of sensation is scored 2. If the patient does not respond and is quadriplegic score 2. Patients in coma (item 1a = 3) are arbitrarily given a 2 on this item.	Normal, no sensory loss Mild to moderate sensory loss; patients feels pinprick is less sharp or dull on the affected side; or there is a loss of superficial pain with pinprick but patient is aware he/she is being touched Severe to total sensory loss; patient is not aware of being touched in the face, arm and leg.	0 1 2	
9 Best language: The patient is asked to describe what is happening in the attached picture, to name the items on the attached naming sheet and to read from the attached list of sentences. Comprehension is judged from responses here as well as to all the commands in the preceding general neurological exam. If visual loss interferes with tests ask the patient to identify objects placed in the hand, repeat, and produce speech. The intubated patient should be asked to write. The patient in coma (1a=3) will arbitrarily score 3 on this item. The examiner must choose a score in the patient with stupor or limited cooperation but a score of 3 should only be used if the patient is mute and follows no one step commands.	No aphasia, normal Mild to moderate aphasia; some obvious loss of fluency or facility of comprehension, without significant limitation of ideas or form of expression. Reduction of speech and/or comprehension, however, makes conversation about provided material difficult or impossible. For example in conversation about provided material examiner can identify picture or naming card from patients response Severe aphasia; all communication is through fragmentary expression; great need for inference, questioning and guessing by listener. Range of information that can be exchanged is limited; listener carries burden of communication. Examiner cannot identify materials provided from patients response. Mute; global aphasia; no usable speech or auditory comprehension	0 1 2 3	

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

3 MONTH / END OF STUDY DATA

NATIONAL INSTITUTES OF HEALTH STROKE SCALE (NIHSS) ITEMS 10 – 11

ITEM	DESCRIPTION	SCORE
10 Dysarthria: If patient is thought to be normal an adequate sample of speech must be obtained by asking patient to read or repeat words from the attached list. If the patient has severe aphasia, the clarity of articulation of spontaneous speech can be rated. Only if the patient is intubated or has other physical barrier to producing speech may the item be scored '9' and the examiner must clearly write an explanation for not scoring. Do not tell the patient why he/she is being tested.	Normal. Mild to moderate; patient slurs at least some words and, at worst, can be understood with some difficulty. Severe; patients speech is so slurred as to be unintelligible in the absence of, or out of proportion to any dysphasia, or is mute/aphasic. Intubated or other physical barrier, explain	0 1 2 9
11 Extinction and inattention (formerly neglect): Sufficient information to identify neglect may be obtained during the prior testing. If the patient has a severe visual loss preventing visual double simultaneous stimulation, and the cutaneous stimuli are normal, the score is normal. If the patient has aphasia but does appear to attend to both sides then the score is normal. The presence of visual spatial neglect or anosognosia may also be taken as evidence of abnormality. Since the abnormality is scored only if present, the item is never untestable.	No abnormality. Visual, tactile, auditory, spatial or personal inattention or extinction to bilateral simultaneous stimulation in one of the sensory modalities. Profound hemi-inattention or hemi-inattention to more than one modality. Does not recognize own hand or orients to only one side of space.	0 1 2
	TOTAL	

Subject number

INFLAMMATION IN STROKE STUDY II **ANAKINRA IN ACUTE STROKE**

3 MONTH / END OF STUDY DATA

BARTHEL INDEX

Based on the last 4 weeks

INDEX ITEM	SCORE	DESCRIPTION	
BOWELS		Incontinent or needs enemas Occasional incontinence (+once per week) Continent	0 5 10
BLADDER		Incontinent/unable to manage catheter Occasional accident (+once per day) Continent	0 5 10
GROOMING		Needs help with shaving, washing, hair or teeth Independent	0 5
TOILET USE		Dependent Needs some help Independent on, off, dressing and cleaning	0 5 10
FEEDING		Dependent Needs some help (eg with cutting, spreading) Independent if food provided within reach	0 5 10
TRANSFER (eg bed to chair)		Unable and no sitting balance Needs major help Needs minor help	0 5 10
MOBILITY		Unable Wheelchair independent indoors Walks with help or supervision	0 5 10
DRESSING		Dependent Needs some help Independent including fasteners	0 5 10
STAIRS		Unable Needs some help or supervision Independent up and down	0 5 10
BATHING		Dependent Independent in bath or shower	0 5
TOTAL			

MODIFIED RANKIN SCALE

Based on the last 4 weeks

DESCRIPTION	GRADE	
No symptoms at all	0	
No significant disability despite symptoms; able to carry out all usual duties and activities	1	
Slight disability; unable to carry out all previous activities but able to look after own affairs	2	
Moderate disability; Requiring some help, but able to walk without assistance	3	
Moderately severe disability; unable to walk without assistance and unable to attend to own	4	
Severe disability; bedridden, incontinent and requiring constant nursing care and attention	5	

Subject number

AEn

ADVERSE EVENTS SUMMARY

Were there any AEs eg, during this period/interval? ☐ No ☐ Yes - If yes, specify below.

Line #	Adverse Event Diagnosis or Syndrome (if known) OR Sign(s) / Symptom(s) List one per line	Check if event continued from previous AE form	Date Started Day	Month	Year	Date Ended, Changed in Severity or Resulted in Death Day	Month	Year	Check if event continued on at End of Study	Duration If Less Than 24 hours Hours	Minutes	Intermittent	Severity (record one code) 1=Mild 2=Moderate 3=Severe 4=Life threatening 5=Fatal	Relationship Is there a reasonable possibility that the event may have been caused by test article? No / Yes	Relationship Is there a reasonable possibility that the event may have been caused by _____? No / Yes	Action Taken for This Event (record all that apply) (see code list)	Serious ? No / Yes
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10																	

***Criteria for Serious Adverse Event:**

Serious adverse event includes any event that (is):
- fatal
- life threatening (places subject at immediate risk of death)
- requires inpatient hospitalization or prolongation of existing hospitalization
- persistent or results in significant disability / incapacity
- a congenital anomaly / birth defect
- other significant medical hazard

If event is defined as serious, complete Serious Adverse Event Report form and phone / FAX immediately to Amgen International Clinical Safety.

Line #	Specify if "88 Other" Action Taken

Clinical Trial Serious Adverse Event Report Page1/2

1. Patient Details
Protocol No. | Site No. | Patient No.
Initials | Sex: ☐ Female ☐ Male | Date of Birth
Weight (kg) | Height (cm) | Race
Pregnant? ☐ Yes* ☐ No *Provide details in section13.

2. Reporter Details
Name
Address
Country
Phone/Fax
TITLE: ☐ Physician ☐ Nurse ☐ Pharmacist ☐ Other

3. Regulatory Serious Criteria
☐ Fatal
☐ Immediately life - threatening
☐ Required hospitalization — Date Admitted — Date Discharged
☐ Hospitalization prolonged
☐ Disabling or incapacitating
☐ Congenital anomaly
☐ Significant hazard. Medical and scientific judgement should be exercised in deciding whether expedited reporting is appropriate in other situations.

4a. Relevant Medical History (include primary diagnosis) — Date of Onset

4b. History of Allergy ☐ Yes ☐ No Details:

5. State Main Adverse Event (AE) — Date started — Has AE ended ☐ Yes ☐ No — If yes, when

Does the patient have prior history of this event type? ☐ Yes* ☐ No — *If yes, please specify date of onset

Drug associated? ☐ Yes ☐ No — Please specify

6. Severity: 1 ☐ Mild 2 ☐ Moderate 3 ☐ Severe 4 ☐ Life threatening 5 ☐ Fatal

7. Action Taken: ☐ None ☐ Dose reduced ☐ Drug withdrawn ☐ Drug restarted
Prescribed treatment for event? ☐ Yes* ☐ No
* If yes, please specify in section 10.

8. Outcome of Event:
☐ Recovered without treatment
☐ Recovered with treatment
☐ Symptoms recurred while on study drug
☐ Symptoms recurred while not on study drug
☐ Event continuing without treatment
☐ Event continuing and controlled with treatment
☐ Event continuing and not controlled with treatment
☐ Patient died

9. Suspect Study Drug Relationship
Is there a reasonable possibility that the event may have been caused by test article? ☐ Yes ☐ No

10. Describe Event(s) Chronological summary of symptoms, signs and vital signs; diagnosis, treatment, outcome and autopsy details if appropriate.
Please provide additional comments on separate sheet.

Date reported — Type of report ☐ New ☐ Follow up — Investigator Signature

Clinical Trial Serious Adverse Event Report Page2/2

11. Study Medication ☐ Active Drug, please specify _____ OR ☐ Blinded ☐ Control ☐ No treatment arm
Indication for use:

Start Date	Stop Date	Dose	Units	Route	Frequency

12. Concomitant Medications

Drug Name	Route	Dosage Dose	Units	Start Date	Stop Date	Ongoing? Yes / No	Indication

13. Additional comments including probable aetiology, relevant diagnostic tests and results:

14. Relevant Haematology Values

Date	WBC x10⁹/L	Platelet x10⁹/L	ANC x10⁹/L	Hb g/dL	HCT %	Retic %	Differentials (if available) % or Absolute (please specify units) SEG	BAND	META	BLAST	LYMP	MONO	EOS	BASO	MYEL

15. Relevant Biochemical Tests

Date	TEST / UNIT							

Date reported — Type of report ☐ New ☐ Follow up — Investigator Signature

SALFORD AND TRAFFORD RESEARCH CONSENT FORM

Project No: 00213
Title of Project: Safety of Anakinra in Patients with Acute Stroke

(The patient should complete the following part of this sheet himself/herself)

PLEASE DELETE AS NECESSARY

Have you read the Patient Information Sheet? YES/NO

Have you had an opportunity to ask questions and discuss this study? YES/NO

Have you received satisfactory answers to all your questions? YES/NO

Have you received enough information about the study? YES/NO

Are you willing for your G.P. to be informed? YES/NO

To whom have you spoken? Dr/Mr/Mrs .....

Do you understand that you do not need to take part in the study and if you do enter you are free to withdraw -

* at any time
* without having to give a reason for withdrawing
* and without affecting your future medical care? YES/NO

Do you agree to take part in this study? YES/NO

Signed: ..... Date.....

.....
(NAME IN BLOCK LETTERS)

SALFORD AND TRAFFORD RESEARCH ASSENT FORM

(Relative/carer/representative endorsement in circumstances where the patient is unable to consent formally for themselves)

Project No: 00213
Title of Project: Safety of Anakinra in Patients with Acute Stroke

Patient Name:

TO BE COMPLETED BY RESEARCH INVESTIGATOR/DOCTOR

I confirm that I have explained the study, and supplied the patient's relative/carer/representative with a full explanation sheet, in terms which in my judgement are suited to their understanding. I also consider the risks of the study to be negligible and justified.

Signature: ..... Date: .....

Name of investigator: .....

TO BE COMPLETED BY PATIENT'S RELATIVE/CARER/REPRESENTATIVE

- Please read this form and the information sheet very carefully.
- If there is anything that you don't understand about the explanation, or if you want more information, please ask the investigator/doctor.
- Please check that all the details on this form are correct. If they are and you understand the explanation, then sign the form.

I am the relative/carer/representative of the patient. (delete as necessary)

I confirm that I have received a full explanation sheet giving details of the study and outlining the potential risks, any distress or discomfort, and potential benefits to .....
(Name of Patient to be studied)

I have had sufficient time to consider the information in the explanation sheet. I understand that I am free to ask the investigator/doctor to withdraw the proposed patient from the study at any time without any effect on his/her future treatment. (delete as necessary).

Signature of relative/carer/representative: .....

MAMA

TIP – TOP

FIFTY – FIFTY

THANKS

HUCKLEBERRY

BASEBALL PLAYER

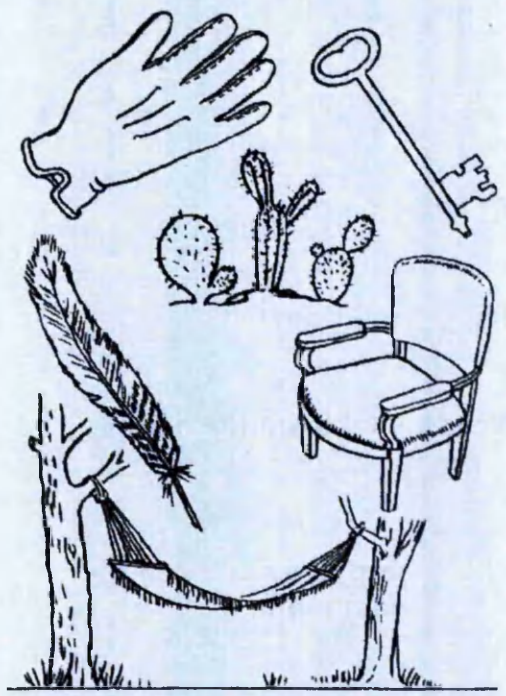
You know how.

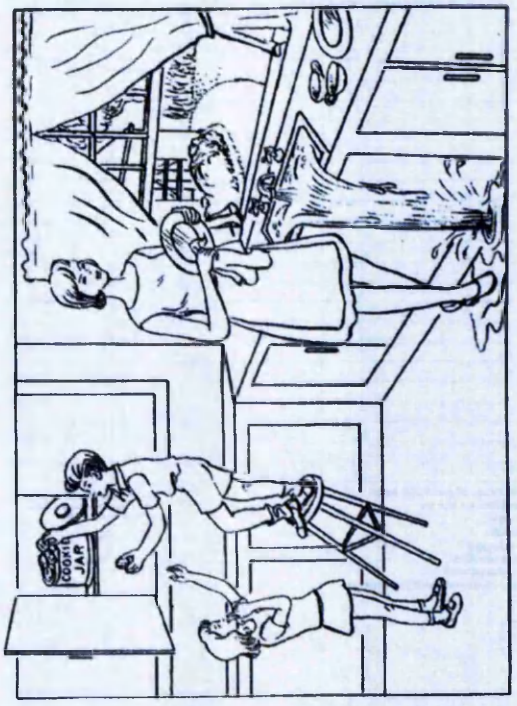
Down to earth.

I got home from work.

Near the table in the dining room

They heard him speak on the radio last night.





CONCOMITANT DRUG TREATMENT

Drug	Dose	Freq.	Indication	Pre-study?	If not, Start date	Stop date

NB Drugs of particular note:
Antiplatelet agents
Warfarin
NSAIDs
Analgesics
Beta blockers
Corticosteroids
Other immune modifying agents

If a drug is started and stopped more than once, please enter drug on multiple lines

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

INDEPENDENT SAFETY COMMITTEE – NOTIFICATION OF RANDOMISATION

PATIENT DETAILS

Patient Initials

Patient Number 1 Date of birth 2-Jan-01

RANDOMISATION DETAILS

Date of randomisation 2-Jan-01 Time 12:00

Age (yrs) 60
<70☐
≥70☐

NIHSS score at randomisation 24
4-9☐
10-20☐
≥21☐

Interval from onset to randomisation (h) .25
<4☐
≥4☐

STROKE DETAILS

CT Scan (if available)
Normal

Admission OCSP Classification
TACS

ADVERSE & SERIOUS ADVERSE EVENTS

Adverse events? Yes☐ No☐
Serious adverse events? Yes☐ No☐
} If yes, see AE/SAE forms

FORM COMPLETED BY

Name of investigator Dr Hedley Emsley Date 2-Jan-01

NOTIFICATION OF RANDOMISATION

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

INDEPENDENT SAFETY COMMITTEE – 7 DAY REPORT FORM

PATIENT DETAILS

Patient Initials

Patient Study Number 1 Date of birth 2-Jan-01

RANDOMISATION DETAILS

Date of randomisation 2-Jan-01 Time 12:00

Age (yrs) 0 NIHSS score at randomisation 0

Interval from onset to randomisation (h) .25

Number of vials administered

Test treatment completed? Yes☐ No☐

If no, specify reason for non-completion Withdrawal by investigator

Details

STROKE DETAILS

Admission CT Scan
Normal

5-7 Day CT Scan (if available)
Normal

Admission OCSP Classification TACS
72 hour NIHSS score 0 Deterioration by ≥4 from randomisation? ☐
5-7 Day NIHSS Score 0

ADVERSE & SERIOUS ADVERSE EVENTS

Adverse events? Yes☐ No☐
Serious adverse events? Yes☐ No☐
} If yes, see AE/SAE forms

FORM COMPLETED BY
Name of investigator Dr Hedley Emsley Date 2-Jan-01

7 DAY REPORT FORM

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

INDEPENDENT SAFETY COMMITTEE – 3 MONTH REPORT FORM

PATIENT DETAILS

Patient Initials

Patient Number Date of birth 1-Jan-03

Date of randomisation 1-Jan-03 Time 16:24

STROKE DETAILS

Final diagnosis Right LACUNAR infarction

NIHSS score at randomisation mRS at randomisation 0
5-7 Day NIHSS score 3 month mRS
3 month NIHSS score

BI at randomisation
3 month BI

ADVERSE & SERIOUS ADVERSE EVENTS

Adverse events? Yes☒ No☐
Serious adverse events? Yes☐ No☒
} If yes, see AE/SAE forms

FORM COMPLETED BY

Name of investigator Dr Hedley Emsley Date 12 July 2001

3 MONTH REPORT FORM

INFLAMMATION IN STROKE STUDY II ANAKINRA IN ACUTE STROKE

**INDEPENDENT SAFETY COMMITTEE –
NOTIFICATION OF ADVERSE/SERIOUS ADVERSE EVENTS**

PATIENT DETAILS

Patient Initials

Patient Study Number 1 Date of birth 2-Jan-01

RANDOMISATION DETAILS

Date of randomisation 2-Jan-01 Time 12:00

ADVERSE & SERIOUS ADVERSE EVENTS

Adverse events? Yes☐ No☐
Serious adverse events? Yes☐ No☐
} If yes, see AE/SAE forms

Date of occurrence 2-Jan-01
Date of identification 2-Jan-01

Has patient died? Yes☐ No☐

Date of death 2-Jan-01 Time of death 12:00

Cause of death

Reported to coroner? Yes☐ No☐ Post-mortem to be held? Yes☐ No☐

Details of Post-mortem if known

FORM COMPLETED BY

Name of investigator Dr Hedley Emsley Date 2-Jan-01

NOTIFICATION OF ADVERSE/SERIOUS ADVERSE EVENTS